ENHANCED BIOREMEDIATION OF A SOIL CONTAMINATED WITH BOTH PETROLEUM HYDROCARBONS AND HEAVY METALS WITH IN-SOIL BIOSURFACTANT PRODUCTION.

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ABSTRACT

"Enhanced Bioremediation of a Soil Contaminated with both Petroleum Hydrocarbons and Heavy Metals with In-Soil Biosurfactant Production."

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Soils that are co-contaminated with petroleum hydrocarbons and metals account for 55% of all hazardous waste sites and are hard to decontaminate because of the different nature of the remedial treatment required. Biosurfactant-producing microorganisms are present in many environments and can be stimulated to produce them under favorable conditions. This dissertation investigates the effect of biosurfactant production by indigenous microorganisms of a co-contaminated soil.

A soil that was heavily contaminated with petroleum hydrocarbons and metals was acquired from the Toronto Harbour area. Phase one of the study evaluated the extent of the biodegradation of organic pollutants as well as the feasibility of biosurfactant production by the produced microorganisms. Results show that by the end of the experiment (50 days) batches amended with nutrients produced biosurfactants up to 3 times their critical micelle concentration (CMC). The produced surfactants caused the concentration of TPH and metals in the filtrate to increase from 2 to 8% and from 2 to 4%, respectively. In the phase two of the experiments the production of biosurfactants was enhanced by 40% by limiting the inorganic source of nitrogen in the batches, following a short growth phase. The produced biosurfactants were able to wash 10% of TPH and 6% of the metal content of the soil. The results indicate that biosurfactants can

be produced by the indigenous soil microorganisms using organic contaminants as the sole carbon source. Furthermore, the produced biosurfactants showed potential to enhance biodegradation of petroleum hydrocarbons as well as to improve flushing of the remaining soil pollutant from soil.

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At the end of my thesis I would like to thank all those people who made this thesis possible and a challenging experience for me.

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Finally, I would like to express my deepest gratitude for the constant support, understanding, and encouragement that I received from the faculty and staff of the Department of Building, Civil, and Environmental Engineering at Concordia University during my undergraduate and graduate studies.

DEDICATION

I dedicate this thesis to my family, for their continuous support and encouragement not just throughout my academic career, but my entire life;

to my father who taught me the value of patience and hard work;

to my mother who encouraged me to be self-reliant and independent;

my gratitude to you is impossible to fully express.

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LIST OF ABBREVIATIONS

ACS = American Chemical Society

ANOVA = Analysis of Variance

ASTM = American Society of Testing and Standards

ATSDR = Agency for Toxic Substances and Disease Registry

Cd = Cadmium

CEC = Cation Exchange Capacity

CFU = Colony Forming Units

CMC = Critical Micelle Concentration

CMD = Critical Micelle Dilution

Cr = Chromium

Cu = Copper

EDTA = Ethylenediaminetetraacetic Acid

EPA = Environmental Protection Agency

FID = Flame Ionization Detector

FLAA = Flame Atomic Absorption Spectroscopy

GC = Gas Chromatography

HPC = Heterotrophic Plate Count

IARC = International Agency for Research on Cancer

NA = Nutrient Amended

NAPL = Non-Aqueous Phase Liquids

Ni = Nickel

NPL = National Priority List

p = Probability

PAH = Polycyclic Aromatic Hydrocarbons

Pb = Lead

PCB = Poly-Chlorinated Benzenes

PH = Petroleum Hydrocarbons

ppm = Parts Per Million

ROD = Records of Decisions

RPM = Revolutions per Minutes

S/S = Solidification/Stabilization

SARA = Superfund Amendments and Reauthorization Act

THC = Toronto Harbour Commission

TPH = Total Petroleum Hydrocarbons

USDA = United States Department of Agriculture

UST = Underground Storage Tanks

Zn = Zinc

α = Acceptable Error (level of significance)

1 INTRODUCTION

1.1 Background

Contamination of soil and groundwater by hazardous waste is a global problem affecting both industrialized and developing countries. Annually, an estimated 250 million metric tons of various hazardous wastes are generated from agricultural enterprises, industrial factories and other anthropogenic activities in the United States alone (Getis, 1991). Soils are contaminated when these hazardous wastes are either spilled or buried directly in the soil or migrate to a soil from a spill that has occurred elsewhere. Soils that are co-contaminated with hydrocarbon compounds and metals, present a complex challenge for remediation because of the different nature of the remedial treatment required; yet, such contaminated soils account for 55% of all hazardous waste sites (Roane et al., 2001).

Petroleum hydrocarbons are used extensively as fuel and chemical compounds. The uncontrolled release of petroleum hydrocarbons from leaking underground storage tanks (UST), broken oil pipelines, petroleum refineries and storage facilities, spills of petroleum products in chemical plants and transportation processes (Sherman and Stroo, 1989) causes serious environmental problems such as fire/explosion hazard, human and environmental toxicity, and movement through soil to air or water. Hutchins et al. (1991) reported that approximately 6 million tons of petroleum products spill and leak into soil each year in the US alone.

Metals are natural constituents in soils. Usually their concentration is low and the types and amounts of metals present reflect the parent soil material (Forstner, 1995). Interestingly, some metals such as Cu, Zn, and Fe, are essential micronutrients for microbial, plant, and animal species, as well as humans. Essential micronutrients are elements that are required in very low amounts for metabolic functioning or as constituents of essential enzymes. Their presence in soil at low concentrations is thus desirable (Barman and Bhargava, 1997). However, in many areas the concentration of metals in soil and groundwater has increased as a result of industrial activities. In such sites, metals can accumulate to toxic levels and disrupt ecological processes. Contamination by metals is found at a majority of Superfund sites, where soil and groundwater problems associated with metals are present at approximately 65% of the Superfund sites with signed records of decisions (ROD) (EPA, 1997a).

Forty percent of hazardous waste sites on the U. S. Environmental Protection Agency's National Priority List (NPL) are co-contaminated with organic and metal pollutants. The remediation of co-contaminated soils is typically difficult, as cited above, because of the mixed nature of the contaminants; however, remediation can be further complicated by such factors as physical heterogeneities of the subsurface, sorption of the contaminant onto soil particle surfaces, diffusion of the contaminant into inaccessible portions of the soil matrix, and inadequate site characterization (NRC, 1994).

In-situ bioremediation is a technique that was first developed in the 1970s to deal with contaminated soils. This method essentially involves a two-stage process where water with added oxygen and nutrients is first applied on and injected into the contaminated

area. This will stimulate the soil's indigenous microbial populations to utilize any organic pollutants as their carbon source, concomitantly increasing the soil's biomass. In addition, microorganisms and microbial by-products can facilitate the removal of metals from the soil matrix as well as attenuate the toxicity of certain metals. Further downstream, the water is subsequently removed via extraction wells and treated on the surface to eliminate any mobilized contaminants (Mulligan, 1998).

Bioremediation has a number of advantages over conventional remediation approaches (Alexander, 1999; Romantschuk et al., 2000). First, bioremediation can be used in-situ. This eliminates the cost associated with excavation and ex-situ treatment of contaminants. Bioremediation also involves less pumping than pump and treat processes and even though less pumping is involved in bioremediation, the rate of cleanup is usually much faster. Another significant advantage of bioremediation over more conventional approaches is that bioremediation typically produces only carbon dioxide and water as byproducts as opposed to the toxic byproducts often associated with some physical/chemical treatment methods (NRC, 1994).

1.2 Statement of the Problem

Conventional approaches to remediation of co-contaminated sites, which comprise excavation of contaminated soil and transportation for incineration or disposal in hazardous landfills, have all but been abandoned due to high costs and lack of available landfill sites (Clarke et al., 1991). In addition, with the implementation of the Superfund Amendments and Reauthorization Act of 1986 (SARA) that requires pretreatment prior to landfilling of contaminates soils, the focus of the technologies in the remediation of

Superfund sites shifted from containment or disposal to permanent remedies (treatment) (Peters and Shem, 1992). This trend continues today, and by 2002 more than 69% of the sites on NPL with a Record of Decision (ROD) contained provisions for treatment (EPA, 2004).

Furthermore, the percentage of the in-situ treatment technologies used in the remediation of Superfund sites increased from 15% to 39% of all treatment technology projects employed between 1985 and 2002. Bioremediation is the most common innovative approach to remediating soils, accounting for approximately 57% of all innovative technologies used on U. S. Superfund sites (EPA, 2004). This has led to increasing interest in the application of microorganisms and microbial products for in-situ remediation of co-contaminated subsurface soils (EPA, 2004, 1993).

Most of the pollutants in the soil environments are sorbed onto soil particle surfaces and organic matter (Mulligan, 1998; Maier et al., 2000). Because organic pollutants require solubilization before being degraded by microorganisms and metals need desorption from soil in order to be removed, bioremediation rates are governed by desorption of contaminants from soil (Alloway, 1995; Cookson, 1995; Miller, 1995a). Therefore, the rates of remediation at many sites are still so low that decades would be required to reach current maximum contaminant levels required by law (Winegarden, 1996). So in many instances additives are added to the flush water to increase its removal efficiency.

One of the groups of additives that have been extensively used is the surfactant (Mulligan, 1998). Surface-active molecules (surfactants) are amphiphilic compounds that can assist in the solubilization, dispersal and desorption of hydrophobic compounds and

heavy metals (Mulligan, 2005). Biosurfactants are naturally produced by microorganisms and are biodegradable. They have also proven to be more effective than synthetic surfactants. Biosurfactants also have low toxicity, high specificity, and the potential to be used in extreme environmental conditions (Kosaric, 2001; Mulligan and Gibbs, 2004).

In-situ production of biosurfactants has tremendous environmental significance and will eliminate the cost of purchasing biosurfactants, further reducing bioremediation costs. In addition, by taking advantage of biosurfactant specificity, the produced biosurfactant can be more effective in removal of organic and metals contaminants than currently available commercial biosurfactants.

In-soil biosurfactant production has been observed during laboratory experiments conducted by Cassidy and Irvine (1997, 2001), Straube et al. (1999), Cassidy et al. (2000, 2002a), Cassidy and Hudak (2001), Hudak and Cassidy (2004), Menezes Bento et al. (2005), and Jennings (2006) on soils contaminated with organic contaminants. Furthermore, in-situ biosurfactant production at contaminated sites has been reported in the research literature (Cassidy et al., 2002b; Youssef et al., 2006). However, the feasibility of in-soil biosurfactant production by indigenous microorganisms in co-contaminated environments has not yet been investigated.

1.3 Objectives

The goal of this study was to advance current knowledge in the area of soil remediation by investigating in-soil production of biosurfactant by indigenous microbial populations in co-contaminated soils to facilitate bioremediation. The principal objectives of the research are delineated below.

To test the potential of bioremediation in a co-contaminated soil.

To determine the feasibility of biosurfactant production by the native microbial population of a co-contaminated soil.

To investigate the effect of biosurfactant production on bioavailability and biodegradation of petroleum hydrocarbon soil contaminants.

To study the effects of biosurfactant production on desorption and removal of heavy metals (chromium, copper, lead and zinc) from the soil matrix.

To enhance biosurfactant production by limiting nitrogen and to evaluate its affect on bioremediation of co-contaminated soil.

1.4 Thesis Outline

This dissertation consists of five chapters followed by an appendix. The contents are as follows:

Chapter 1 introduces the nature and description of the problem and the objectives and organization the thesis.

Chapter 2 presents some background information as well as a literature review of related past and current research.

Chapter 3 includes the materials, equipment, and methodology used in this investigation.

Chapter 4 presents the results of this study as well as a discussion of their implications and significance.

Chapter 5 summarizes the conclusions.

Chapter 6 presents some suggestions for future work.

Appendix includes the calculation for nutrient and sodium azide solutions.

2 LITERATURE REVIEW

2.1 Petroleum Hydrocarbons in the Soil

Crude oil is refined to provide liquid fuels, solvents, lubricants, and many other marketable products. These products provide energy to heat homes and workplaces, fuel the transportation systems, power manufacturing processes and tools, as well as a source for numerous synthetic materials used in daily life. Used as intended, hydrocarbons provide great benefits to society; however, when released to the soil environment as raw feedstocks, refined fuels, or lubricants, a number of problems can result.

First, if volatile petroleum hydrocarbons enter confined spaces they pose a fire/explosion hazard. Second, most petroleum hydrocarbon components are toxic to some degree but assessing their toxicity and environmental impact is made difficult by the complexity of the component mixtures, variability of the sources, and other site specific circumstances. Third, low molecular weight hydrocarbons are mobile and are transported in ground, water or air, contaminating large areas from their point of release. Fourth, large molecular weight and branched hydrocarbons, as well as polycyclic aromatic hydrocarbons (PAH) are resistant to degradation and will persist in the environment. Finally, under some conditions, petroleum hydrocarbons can interfere with the soil's water retention and transmission ability (ATSDR, 1999; Canadian Council of Ministers of the Environment, 2001).

Hydrocarbons can be released from burning of fossil fuels, leaking underground storage tanks (UST), broken oil pipelines, wood-treatment facilities, petroleum refineries and storage facilities, spills of petroleum products in chemical plants and transportation processes (Sherman and Stroo, 1989). According to the Canadian Council of Ministers of the Environment (2001) about 60% of Canada's contaminated sites involve petroleum hydrocarbon contamination.

Groundwater contamination is one of the most significant environmental impacts from the leakage of organic pollutants. More importantly, one gallon of the leakage can render one million gallons of water unsuitable for drinking (Noonan and Curtis, 1990). In addition, release of hydrocarbon products is also a major component of soil contamination. Moreover, volatile chemicals leaked could eventually reach the air and result in air pollution.

2.1.1 Composition of Crude Oils

Crude oil (petroleum) is essentially a mixture of gaseous, liquid, and solid hydrocarbons with varying degrees of water solubility, chemical structure, volatility, and toxicity that occur in sedimentary rock deposits. It has been estimated that there are between 20,000 and 5,000,000 compounds present in crude oils (Braun and Burnham, 1988). On the molecular level, petroleum is a complex mixture of hydrocarbons (83 to 87% carbon and 10 to 14% hydrogen), organic compounds of sulfur, nitrogen, and oxygen (0.05 to 6% sulfur, 0.1 to 2% nitrogen, and 0.05 to 1.5% oxygen), and compounds containing metallic constituents, particularly vanadium, nickel, iron, and copper (up to 0.1% metals) (Speight, 1991).

Petroleum in the crude state (crude oil) is a natural resource, which is refined to liquid fuels, solvents, lubricants, and many other products. Crude petroleum usually contains hydrocarbons classified as alkanes or paraffins (with structure of C_nH_{2n+2}), cycloalkanes or naphthenes (with structure of C_nH_{2n}), aromatics and polycyclic aromatics or PAHs (with one or more fused aromatic rings), resins (polar compounds containing nitrogen, sulfur, and oxygen), and asphaltenes (high molecular weight compounds containing metals elements). In addition, in refined petroleum products there are small amounts of alkenes that are generated by the refining processes (Speight, 1991).

Crude oil is a complex mixture of thousands of individual hydrocarbons from light, volatile, short-chained compounds to heavy, long-chained, branched compounds. These compounds arise from variations in chain length, branching, or interclass combinations, as well as the presence of elements, such as oxygen, nitrogen, sulfur, or metals (Speight, 1991; Eweis, 1998). The physical and chemical properties of individual hydrocarbon compounds depend on the size and structure of the molecule. Generally, larger and heavier molecules have higher boiling points, lower volatility, and less solubility in water. Solubity is also directly influenced by the polarity of a molecule (Speight, 1991; Potter et al., 1998).

When crude oil is subjected to fractional distillation and other processes, such as catalytic cracking in a refinery various petroleum products such as, petroleum gas, gasoline, kerosene, and fuel oils are formed. Except for gasoline, most of these products are made primarily by collecting a particular range of boiling point fractions of crude oil from a distillation column. Because distillation columns separate these petroleum products

primarily based on their boiling point and not by chemical composition, the composition of these products vary according to the type of crude oil and refining processes used (Speight, 1991; Gary and Handwerk, 1993; ATSDR, 1999).

2.1.2 Definition of Petroleum Hydrocarbons

The Canadian Council of Ministers of the Environment, (2001) defined Petroleum hydrocarbons (PH) as: "A general term used to describe mixtures of organic compounds found in or derived from geological substances such as oil, bitumen, and coal". The most common petroleum hydrocarbons are gasoline, diesel, fuel oils, and lubricating oils. The properties of hydrocarbons depend on the petroleum source and type, the chemical composition, degree of processing, and the extent of exposure to the environmental factors also known as weathering (Speight, 1991; The Canadian Council of Ministers of the Environment, 2001).

The carbon atom content of most petroleum hydrocarbons is between C_6 and C_{25}. Gasoline is mostly light fractions (C_6 to C_{10}) with a boiling temperature ranging from 23°C to 204°C. Diesel fuel contains the middle distillate compounds (C_6 to C_{22}) with boiling temperature between 202°C and 320°C. Lubricants contain compounds in the range of C_{20} and C_{50} with a boiling range of 300°C to 370°C. Fuel oil is heavy hydrocarbons between C_{20} and C_{70} with boiling temperature between 370°C to 600°C (Parr et al., 1994; Potter et al., 1998; ATSDR, 1999).

2.1.3 Toxic Impacts of Petroleum Hydrocarbons

Health effects from exposure to petroleum hydrocarbons depend on many factors. These include the type of hydrocarbon compounds exposed to, the route of exposure, the

duration of exposure, and the total amount of hydrocarbon compounds exposed to (ATSDR, 1999). The different hydrocarbon fractions affect the body in different ways. Some volatile compounds, such as benzene, toluene, and xylene, can affect the central nervous system. For example, breathing toluene at high concentrations (greater than 100 ppm) for more than several hours can cause fatigue, headache, nausea, and drowsiness. If exposures are high enough, even death can occur (ATSDR, 1999).

Compounds such as, n-hexane can cause a nerve disorder called "peripheral neuropathy" consisting of numbness in the feet and legs and, in severe cases, paralysis. This has occurred in workers exposed to 500-2,500 ppm of n-hexane in the air. Other compounds can cause adverse effects on the lungs, liver, kidney, blood, immune system, reproduction, fetus development, skin, and eyes from exposure to petroleum hydrocarbon compounds. Usually with low exposure levels the symptoms will subside when exposure is stopped. However, if someone is exposed to very high concentration or for a long time, damage can be permanent (ATSDR, 1999). According to the International Agency for Research on Cancer (IARC) at least some hydrocarbon compounds or petroleum products, such as benzene, benzo(a)pyrene and gasoline, are carcinogenic to humans, but most petroleum hydrocarbons are not considered classifiable by IARC (ATSDR, 1999).

2.1.4 Fate of Petroleum Contaminants

The fate of hydrocarbons in the subsurface environment depends on the characteristics of the spill site, as well as, the composition and properties of the released petroleum hydrocarbons (Alexander, 1999; Xueqing et al., 2001). The most important soil properties effecting fate of hydrocarbons are grain size distribution, porosity,

permeability, and organic matter content. Other contributing factors concern the biological properties of the media including presence of substrate, nutrients, and microorganism genera. The chemical composition and physical characteristics of individual components of petroleum hydrocarbons, such as non-hydrocarbon fractions, water solubility, soil adsorbtivity, vapor pressure, and bioavailability are also significant factors (Williamson et al., 1998; Alexander, 1999; Loehr et al., 2000; Xueqing et al., 2001).

Therefore, determining the fate of all individual compounds released to the environment is unrealistic due to the complexity of these mixtures. This is further complicated by hydrodynamic, abiotic, and biotic changes that occur upon the release of the petroleum hydrocarbons in the environment. The longer the release is exposed to the environment, the greater the change in chemical character and the harder it is to obtain accurate analytical results reflecting the identity and fate of the release (Maier et al., 2000; Xueqing et al., 2001).

With respect to water content, the soil environment can be unsaturated or saturated. In unsaturated zones, hydrocarbons may exist as vapor in the pore spaces, adsorbed to soil solids, dissolved in water, or as nonaqueous phase liquids (NAPLs); whereas, in saturated zones, they could exist in adsorbed, dissolved and nonaqueous phases (Bekins et al., 2001).

According to Alexander (1999), the pathways that govern the fate of petroleum hydrocarbons in the subsurface soils can be given as follows:

- Hydrolysis in aqueous phase: hydrolysis is a chemical process in which an organic chemical is split into two parts by reacting with a molecule of water.

- Oxidation/reduction or redox reactions: a redox reaction is the loss or gain of an electron or reaction of the organic compound with oxygen or hydrogen atoms, respectively.

- Volatilization: volatilization is the removal of light-weight components of oil from the liquid phase to the gas phase in the unsaturated zone; depending on the oil spilled, volatilization/evaporation can account for up to 40% of the weight of the oil (Liu and Liptak, 2000).

- Adsorption: adsorption is a physical process that decreases the mobility of organic contaminants by a temporary adhesion of contaminant molecules to soil particles, such as soil organic matter and to a leaser extent, soil minerals.

- Biodegradation: biodegradation is the breakdown of organic compounds through microbial metabolism and co-metabolism pathways. Metabolism is when microorganisms use organics as substrate for growth or energy, whereas, cometabolism is the transformation of organic compounds without them being used for growth or energy.

2.1.5 Growth of Microorganisms

Microorganisms are ubiquitous and can be found almost in every habitat, even as deep as 7 km beneath the earth's surface (Szewzyk et al., 1994; Eweis et al., 1998).

Microorganisms in soils are also diverse in types (bacteria, fungi, etc.) and species (*Pseudomonas*, *Bacillus*, etc.) as well as numbers (Prescott et al., 1999).

A microbial growth curve is a graph that illustrates biomass increase over time (Figure 2.1). The most common is the bacterial growth curve, where bacteria cell number or mass, expressed in a logarithmic scale, is plotted as a function of time. The growth cycle of the organisms reproducing by binary fission can be divided into four principal phases: lag, exponential, stationary, and death. In the lag phase bacteria are adapting to the new environment and do not multiply. During the second phase, bacteria utilize substrates and nutrients to form new cells. The rate of growth in this phase is the maximum growth rate possible under those specific conditions. Next phase is the stationary phase during which the total number of bacteria will remain constant. This can occur either as growth and death of bacteria come to a balance or as cells cease to grow but are still active. The last phase (death phase) is when the growth has stopped and the bacteria are dying and thus the number of cells is in decline (Prescott et al., 1999; Maier et al., 2000).

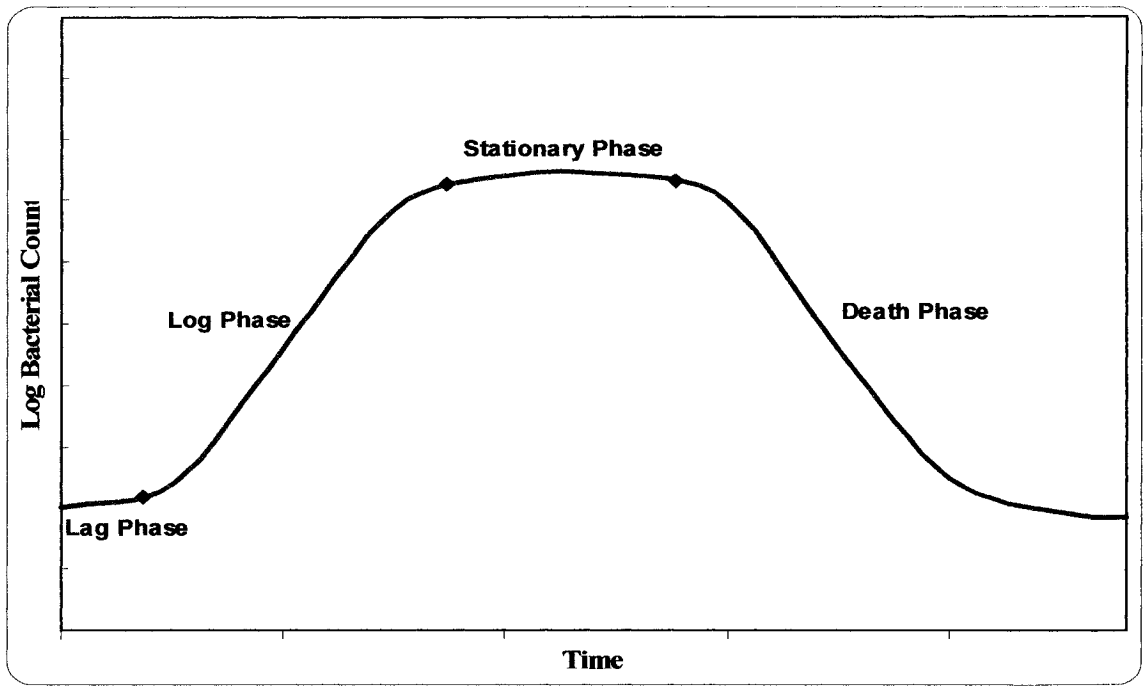

Figure 2.1: A typical growth curve for a bacterial population.

2.1.6 Factors Effecting Microbial Growth

The factors that affect microbial growth and activity can be divided into two main categories: abiotic (physical, chemical) and biotic (biological).

A. Abiotic Factors

Abiotic factors affect the rate and extent of biodegradation of petroleum hydrocarbons by influencing both the growth of microbial populations and the physical characteristics of hydrocarbons is soil media. Some of these factors are: availability of electron acceptors (oxygen, nitrate, etc.), soil structure, nutrients, temperature, soil pH, soil moisture, and presence of inhibitors (Atlas 1984; Cookson, 1995).

Availability of Electron Accepters

The type and quantity of electron acceptors greatly influence the rate and extent of biodegradation in any media. Based on the type of the electron acceptor, biodegradation can be divided into two main categories: aerobic and anaerobic. Aerobic processes use oxygen, which is the most efficient electron acceptor, to oxidize the organic compound into energy, cell mass, and carbon dioxide (Barker and Patrick, 1985; Cookson, 1995). When oxygen is not available (anaerobic biodegradation), ferric iron, nitrate, sulfate, and carbon dioxide serve as electron acceptors. In general, anaerobic processes are slower that aerobic processes (Atlas, 1984).

Nutrients

Nutrients are substances that are required for growth and activity of microorganisms. Normally, the amounts of the substances that are required are approximated from their composition within the biomass. The composition of macronutrients in the biomass is approximately $C_5H_7O_2NP_{0.03}$ (Cookson, 1995). Other elements that are required in smaller amounts (sulfur, potassium, sodium, calcium, iron, etc.) are also present in the biomass and are called micronutrients or trace elements. For microorganisms to grow and optimally metabolize petroleum compounds, all of the macro and micro-nutrients have to be present in sufficient concentrations. The source of carbon in the contaminated soil is the organic pollutant; hydrogen and oxygen are supplied from water; and nitrogen, phosphorus, and sulfur are acquired from soil organic matter and inorganic sources. The micronutrients are present in most soils in sufficient concentrations and are provided by inorganic salts (Cookson, 1995; Alexander, 1999; Atlas and Unterman, 1999).

However, the increase in the concentration of carbon as a result of the contamination with hydrocarbon compounds disrupts the balance in the carbon, nitrogen, phosphorous ratio and creates a demand for nitrogen and phosphorous (Maier et al., 2000). As a result, biodegradation can become nutrient limited, causing the rate of degradation to slow down. To prevent this, nitrogen and phosphorus amendments are normally added to the soil in a process known as biostimulation. The goal of biostimulation is to create and maintain a carbon, nitrogen, and phosphorus ratio (C:N:P) of 100:10:1 (Miller, 1990; Cookson, 1995; Maier et al., 2000). Other ratios have been proposed for example: 100:15:3 (Zitrides, 1983) and 120:10:1 (Alexander, 1977), but the 100:10:1 is most widely used ratio. Also due to toxicity concerns nutrients are preferably added in small frequent applications (Dibble and Bartha, 1979; McMillen et al., 1993).

Temperature

Microbial growth and activity are particularly affected by soil temperature, because they are poikilothermic, meaning they have the same temperature as their surroundings (Sims and Bass, 1984; Cookson, 1995). For a microbial species there is a minimum temperature below which growth does not occur, an optimal temperature at which growth is optimum, and a maximum temperature above which growth is implausible (Prescott et al., 1999). Moran and Hickey (1997) reported that the optimal temperature for common petroleum-degrading microorganisms is usually about the room temperature (22°C).

Soil Moisture

On average 90% of a microorganisms weight is water and water plays an important role in the transfer of substrate, nutrients, oxygen, and organisms in the soil media (McGill et al., 1981; Cookson, 1995). In addition, water also diffuses microbial by-products,

reducing their toxicity. Generally, the optimum activity of aerobic microorganisms occurs when the soil moisture is 38 to 81% of the soil pore space (Maier et al., 2000). When, the soil is too dry (moisture content is lower than 10% of the holding capacity), the bioactivity becomes marginal and if water content of the soil is too high, oxygen replenishment is limited; thereby, bioactivity will be reduced (Eweis, 1998; Maier et al., 2000).

Soil pH

Natural soil pH can be highly variable and it can affect soil microorganism growth and activity. While some microorganisms can survive in a wide range of pH, others are sensitive to small variations (Atlas and Bartha, 1993). Most heterotrophic bacteria prefer neutral to alkaline environments with pH values between 6.5 and 8.5 (Dibble and Bartha, 1979; Cookson, 1995). In acidic conditions, fungi are more predominant which usually results in lower rates of hydrocarbon degradation (Maier et al., 2000).

Soil Structure

The structure of the soil affects microbial activity because it controls the transport of water, oxygen, and nutrients to the microorganisms in the media. Generally, permeable soils, such as sands and gravels, transport these substances fast, but drain fast as well. On the other hand, finer particles such as, clay and silt, have high water and nutrient holding capacities, but they transfer them slower. Other soil characteristics that can influence remediation process are particle size distribution, cation exchange capacity, moisture content, and organic matter content (Cookson, 1995; Sparks, 1995).

Presence of Inhibitors

Presence of inhibitors, such as toxins (hydrocarbon compounds, pesticides, etc.) and heavy metals affect microbial growth or activity in soil. Although all toxic contaminates inhibit microbial activity to some extent, the degree that they can affect different microbial populations varies and depends on the microorganism, compound types, concentrations, and environmental conditions of the soil (Kropp and Fedorak, 1998; Wang et al., 2002). Other microbes are even stimulated by presence toxic hydrocarbons as well as heavy metals (Baath, 1989; Lin, 1993; Ward et al., 1997; Cassidy et al., 2002b).

B. Biotic Factors

Biotic factors define the relationship between organisms and their environments. The main biotic factors include microbial factors, biodegradability, bioavailability, bioaugmentation, and adaptation and previous exposure.

Microbial Factors

Many microorganisms, including bacteria, fungi, yeasts as well as some algae are capable of degrading petroleum hydrocarbons (Cookson 1995). These microorganisms are widely distributed in freshwater, marine, and soil environments, even when the environment is pristine (Atlas, 1981; Pfaender and Buckley 1984; Bartha and Atlas 1987; Leahy and Colwell, 1990). Different species and genera have different biodegradation rates for hydrocarbon compounds (Bartha and Atlas, 1987). For degradation of a mixture of hydrocarbons a consortium of organisms with a broad catabolic potential is required, because individual microbial species are able to metabolize only a limited range of

hydrocarbons as substrates (Cookson, 1995). Examples of the hydrocarbon-degrading bacteria are *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Nocardia,* and *Pseudomonas* subspecies (Leahy and Colwell, 1990).

Biodegradability

Biodegradable compounds are the compounds that can be naturally broken down by microorganisms (Cookson, 1995). On the other hand, hydrocarbons that are not biodegradable or degrade at an extremely slow rate are considered recalcitrant (Huesemann, 1997). Molecular structure and size are the most important factors in determining a biodegradability of hydrocarbon compounds.

Presence of branching and functional groups often reduce biodegradability of compounds. Based on their structure characteristics, the biodegradability of hydrocarbons have generally been ranked in the following order of decreasing susceptibility by Hinchee et al., (1994) and Leahy and Colwell (1990):

n-alkanes > branched alkanes > low-molecular weight aromatics > cyclic alkanes >

high-molecular weight alkanes > polar compounds

In general the biodegradability of organic compounds decreases when molecule size increases (Alexander, 1999; Kanaly and Harayama, 2000). Petroleum hydrocarbons occupy an intermediate position between highly biodegradable and recalcitrant organic mixtures. Lighter mixtures of petroleum hydrocarbons, such as gasoline can be readily

biodegraded, while heavier products, such as diesel or fuel oils degrade much slower (Bartha, 1986; Baker and Herson, 1994).

Bioavailability

Bioavailability is the accessibility of a substrate, such as hydrocarbon compounds, to the microorganisms. When non-limiting condition exist the extent and rate of biodegradation, depend on the bioavailability of the degraded compounds (Alexander, 1999). Before metabolism can occur, microorganisms have to uptake hydrocarbon compounds into the microbial cells. The modes of hydrocarbon transport to microbial cells are (Goswami and Singh, 1991):

1. Interaction of cells with hydrocarbon dissolved in the aqueous phase.
2. Direct contact of cells with large hydrocarbon drops.
3. Interaction of cells with fine or emulsified hydrocarbon droplets dispersed into solution.

Therefore bioavailability is directly affected by solubility of hydrocarbon compounds (Cooksan, 1995; Eweis, 1998). Bioavailability is also affected by sorption to soil organic and inorganic particles (Winegardner, 1996). The portion of the organic pollutants that are physically adsorbed to soil can be desorbed and become available for microbial utilization (Maier et al., 2000).

Bioaugmentation

Although, natural soil microorganisms are the preferred source of microorganisms for biodegradation, in some places they may not have the metabolic capability or necessary

population to perform the remediation process (Leahy and Colwell, 1990; Roane et al., 2001). In those instances, a group of cultured natural microbial strains or genetically engineered variants are added to the soil to enhance the rate and extent of bioremediation. The added organisms should not have pathogenic characteristics to plants and animals or produce toxic byproducts (Errampalli et al., 1997; Maier et al., 2000).

Adaptation and Previous Exposure

Often, microorganisms do not have the capability to degrade hydrocarbon compounds upon initial exposure, but they may develop it after prolonged exposure. Therefore, prior exposure is important as far as the subsequent extent and speed at which degradation occurs (Leahy and Colwell 1990). Three mechanisms have been proposed for metabolic adaptation (Pinholt et al. 1979; Leahy and Colwell 1990; NRC, 1993):

1. Induced enzyme production: Microorganisms can induce the production of specific enzymes required for break down of a specific hydrocarbon.

2. Genetic exchange: Genetic exchange enables microorganisms to degrade a compound by developing a new metabolic pathway.

3. Microbial community change: Selective enrichment results in an increase in the number and genera of hydrocarbon degrading microorganisms.

2.2 Heavy Metals in the Soil

Traces of metals are naturally present in soil, water, air, and biota. Some metals, such as Cu, Zn and Mn, when present in low concentrations, are beneficial for living organisms

while others, including Cd, Pb, and Hg have no beneficial effect at any concentration (Cameron, 1992; Barman and Bhargava, 1997).

The main sources of metals in natural soils are the primary and secondary minerals, which gradually release them as they are weathered (Forstner, 1995). Except for ore deposits, which are the main source of commercial metal production, the concentration of individual metals in soil normally does not exceed 100 mg/kg (Sposito, 1989; Alloway, 1995).

The anthropogenic release of metals into the environment, on the other hand, has caused the concentration of metals found in some soils to exceed the naturally expected level by several orders of magnitude. The main sources of metals include mining and smelting operations, municipal solid wastes, sewage sludge, fuel combustion aerosols, and agricultural fertilizers and pesticides (Alloway, 1995; Forstner, 1995; Mulligan 1998). The most commonly found metals in soil and groundwater are Pb, Cd, Cu, Cr, Hg, Ni, Zn, As, Ba, Mn, Fe, Co, and Sn (Cameron, 1992; Forstner, 1995; Kafka and Kuras, 1997). Metal contaminated soils usually have more than one metal element and often contain other contaminants, such as organic compounds.

2.2.1 Definition of Heavy Metals

By definition, a heavy metal is a metal that has a specific gravity of about 5.0 or greater, especially if it is poisonous. The term heavy metal, however, is broadly applied to other potentially hazardous elements (metals and metalloids), even if they do not meet the above chemical definition (Hawkes, 1997). The most common heavy metals are cadmium, chromium, cobalt, copper, iron, mercury, manganese, molybdenum, nickel,

lead, tin, and zinc (Cameron, 1992). According to ATSDR (2005a), there were four heavy metals (arsenic first, lead second, mercury third, and cadmium eighth) on the list of top 20 hazardous substances in 2005.

2.2.2 Toxic Impacts of Heavy Metals

Biota may be exposed to heavy metals through food, water, air, or absorption through the skin. Heavy metals become toxic when they accumulate in the tissues of plants, animals, and humans to critical levels (International Occupational Safety and Health Information Centre, 1999).

Heavy metals have been shown to damage or reduce mental and central nervous functions, lower energy levels, and damage blood composition, lungs, kidneys, liver, and other vital organs in humans (International Occupational Safety and Health Information Centre, 1999).

A. Chromium

Chromium is a block VI transition metal on the periodic table and is the 21st most abundant element in the earth's crust at 100 parts per million (ppm) (Lide, 2007). Since, chromium is a naturally occurring element; it is widely distributed in the environment, being found in air, water, and biota. The most common forms or species of chromium are chromium (0), chromium (III) (trivalent chromium), and chromium (VI) (hexavalent chromium). Chromium metal is a hard and brittle steel-gray solid with a high melting point. No known taste or odor is associated with chromium compounds. Chromium (VI) is extremely toxic and carcinogenic (Enterline, 1974; Mertz, 1974), while Cr (III) on the

other hand is less toxic and mobile, and adsorbs on most soils (Cameron, 1992; Fendorf and Sparks, 1994; Barnhart, 1997).

Chromium is used in chrome plating, dyes and pigments manufacturing, steel and alloy production, leather tanning, and wood preservatives. Smaller amounts are used in drilling muds, rust, and corrosion inhibitors, textiles, and toner for copying machines (Reddy and Parupudi, 1997; Orcino, 1998a).

The level of chromium in air and water is generally low. The concentration of total chromium in air generally ranges between 0.01 and 0.03 μg per cubic meter of air ($\mu g/m^3$). Chromium concentrations in drinking water are generally very low, less than 2 parts of chromium in a billion parts of water (ppb) (Health Canada, 1986). The maximum allowable for chromium in drinking water has been established by Health Canada (2006) at 0.05 mg/L total chromium.

Chromium (III) is an essential nutrient that helps the body use sugar and fat. An intake of 50 to 200 μg of chromium (III) per day is recommended for adults (Supply and Services Canada, 1990). However, breathing in high levels (greater than 2 $\mu g/m^3$) of chromium can cause irritation to the nose, sneezing, itching, nosebleeds, ulcers, and holes in the nasal septum. Swallowing of large amounts of chromium may cause stomach upsets and ulcers, convulsions, kidney and liver damage, and even death. Long-term exposure to chromium has also been associated with lung cancer (James, 1996; Lytle et al., 1998; ATSDR, 2000).

B. Copper

Copper (Cu) is a pliable, malleable metal, with a bright reddish metallic luster and is an excellent conductor of both electricity and heat. It is used in making textiles, marine paints, electrical conductors and wires, plumbing fixtures and pipes as well as coins and cooking utensils.

Copper occurs naturally in rock, soil, water, sediment, and air. Because copper is an essential micronutrient it is naturally found in both plants and animals (Supply and Services Canada, 1990). Adult humans require 0.03 mg/kg of copper per day (World Health Organization, 1973).

The concentration of copper in air ranges from a few nanograms in a cubic meter to about 200 ng/m^3. The average copper concentration in the earth's crust is about 50 ppm, although concentrations close to 7000 ppm have been found near copper smelters. The average concentration of copper in tap water ranges from 20 to 75 ppb. However, due to copper pipes and brass faucets, higher concentrations (more than 1 mg/L) can also be found (Health Canada, 1992a). According to the guidelines for Canadian drinking water quality (2006), the amount of total copper in drinking water should be limited to 1.0 mg/L.

Exposure to copper can occur by breathing air, drinking water, eating food, and by skin contact with soil, water, and other copper containing substances. Long-term exposure to copper dust can irritate the nose, mouth, throat, and eyes, and cause headaches, dizziness, nausea, and diarrhea. Drinking waters containing high levels of copper may cause vomiting, diarrhea, stomach cramps, and nausea. Intakes of copper can damage brain,

kidney, cornea, eyes, and other organs and in some cases even cause death. Copper is not known to cause cancer (ATSDR, 2004).

C. Lead

Lead (Pb) is a soft, heavy, toxic, and low-melting metal that occurs naturally in small amounts in the earth's crust. It is most commonly found in the galena (PbS) form and has a bluish white color when freshly cut, but tarnishes to dull gray when exposed to air. Lead is used in lead-zinc smelters, ammunition, solder, glass, piping, insecticides, paints and batteries (Jaworsky, 1978). Lead is released into the air from burning of wastes and fossil fuels and subsequently lands onto the soil. It may also directly reach the soil from landfills and paints (Cameron, 1992; ATSDR, 2005b).

The average concentration of lead in natural soil is less than 10 mg/kg. The concentration of lead in the drinking waters is normally less than 0.005 ppm (Health Canada, 1992b; ATSDR, 2005b). In Canada, the maximum acceptable concentration in the drinking water is set to 0.01 mg/L (Health Canada, 2006).

Exposure to lead can happen from breathing workplace air or dust, eating contaminated foods, or drinking contaminated water. Lead is a potent neurotoxin, which accumulates in soft tissues and bone over time. It some cases lead exposure has shown to cause weakness in fingers, wrists, or ankles. Exposure to lead affects almost every organ, especially in high levels it can severely damage the brain and kidneys in humans and even cause death (ATSDR, 2005). In pregnant women, high levels of exposure to lead may cause miscarriage and in men it could lead to impotence (Wilson, 1966; ATSDR, 2005a).

D. Zinc

Zinc is a transition metal and one of the most common elements in the earth's crust. It has a bluish-white color that tarnishes in moist air. From 100 to 210°C zinc metal is malleable, whereas above 210°C, it becomes brittle and will be pulverized by beating. Zinc is found in the air, soil, and water and is present in all foods. Trace amounts of zinc is necessary for all living organisms, since it is an essential nutrient; however, too much zinc can be harmful to health (Health Canada, 1979; Cameron, 1992).

Zinc is used extensively in the world. Its major uses are for galvanizing steel, producing alloys, batteries, wood preservatives, ceramics, and paints. Zinc compounds are used by the pharmaceutical and cosmetic industries as ingredients in sun blocks, diaper rash ointments, deodorants, and shampoos (Cameron, 1992). Zinc is released into air through coal burning and burning of wastes. Water and soil contamination may result from the mining and metal purifying operations as well as release of discharge from galvanizing plants and municipal waste treatment plants (ATSDR, 2005c).

Zinc in air is mostly present as fine dust particles with an average concentration of less than 1 $\mu g/m^3$. Most of the zinc in soil is insoluble and bound to the soil mineral particles and organic matter. Natural levels of zinc in soils range from 30 to 150 mg/kg, while concentrations above 400 mg/kg are considered toxic to plants. Low levels of zinc are also present in most drinking water (Health Canada, 1979; ATSDR, 2005c). The Canadian drinking water quality guidelines limit the amount of total zinc in drinking water to less than 5 mg/L (Health Canada, 2006).

Zinc can enter the body through inhalation as well as the ingestion of zinc-containing food or water. The recommended average daily zinc intake through the diet is 10 milligrams (USDA, 2001). Ingestion of excess amounts of zinc (10-15 times the recommended daily doze) is harmful and can result in stomach cramps, nausea, and vomiting. Chronic exposure to high doses can lead to anemia, pancreas damage, and decreased levels of so-called good cholesterol. Drinking water with 600 to 2,000 mg/L of zinc can lead to the symptoms of zinc poisoning, which include vomiting, dehydration, fever, and anemia (Smith et al., 1976; ATSDR, 2005c). High exposure to zinc dust can cause skin irritation and coughs with phlegm. Zinc has not been classified as a human carcinogen (ATSDR, 2005c).

2.2.3 Fate of Metals

Metals are not thermally decomposable or microbially degradable in the environment, thus, the key processes that affect their fate and behavior in soils are retention and transport. Retention is the capacity of the soil mineral and organic matter surfaces to sorb or retain material; in this case metals. Transport is the movement of metals in the soil system by groundwater flow and convection. Obviously the transport of metals in soil is influenced by their retention. The mobility and retention of metal are affected by their adsorption-desorption and precipitation-dissolution reactions with soil particles (Alloway, 1995; EPA, 1997b).

A. Metal Dissolution/Precipitation

Usually only the soluble fraction of metals is able to readily move through the soil system while metal-containing microorganisms and large colloidal-metal complexes are to some

extent filtered by soil pores. Thus, metal mobility is a function of the dissolution-precipitation equilibria of soil minerals (Alloway, 1995). When metal concentrations in the soil solution are lower than equilibrium levels, metals will dissolve in into the aqueous phase and become mobile. On the contrary, as the dissolved metal concentrations start to approach levels that are higher than equilibrium values, metals will precipitate as insoluble forms. The dissolution-precipitation equilibrium is affected by the soil physico-chemical properties, such as pH, redox potential, and water content (Sposito, 1989). Therefore, metal solubility and bioavailability will depend to a great extent on the dissolution-precipitation reaction (Alloway, 1995).

B. Metal Adsorption

Adsorption is a physical process that involves the binding of molecules or particles to a soil surface. The binding is usually weak and reversible and is dependent on the metal-soil surface affinity (Petruzzelli, 1997). The sorption/desorption process, depends on the chemical characteristics of the metal as well as the characteristics of the surface. For metals the fraction of free ions, ion pairs, and complex metals are important. In terms of the surface, the most important constituents with respect to metal adsorption are the types and amounts of clay minerals, oxides/hydrated metal oxides, and organic matter (Bohn et al., 1985; Sposito, 1989).

According to Alloway, (1995) the mechanisms for metal sorption are:

1. Ion Exchange Adsorption: Ion exchange or non-specific adsorption is the adsorption of metal cations on the surface of negatively charges soil particles. This is a reversible process arising form the electrostatic attraction of oppositely

charged particles and depends on the density of negative charges on the soil solid surfaces (Petruzzelli, 1997). Selectivity sequences are mainly related to the metal valence, but for metals of the same valence, the degree of hydration also affects the sequence (Sparks, 1995). The adsorption process may be affected by soil characteristics, such as pH, cation exchange capacity (CEC), ionic strength, competing cations, and the presence of organic and inorganic ligands (Petruzzelli, 1997).

2. Specific Adsorption of Metals: In a specific adsorption, also called chemisorption, metals adhere to surface ligands to share an electron and form partial covalent bonds. Chemisorption is stronger than sorption through ion exchange, and is affected by the pH, and the ionic strength of the aqueous phase (Sparks, 1995; Petruzzelli, 1997). The main metals involved in chemisorption are Al, Fe, and Mn (Petruzzelli, 1997).

3. Organic Complexation of Metals: A complex is formed when an organic ligand coordinates by providing a pair of electrons and forms ionic or covalent bonds with a metal ion. High molecular weight humic substances with acidic functional groups account for most of the metal immobilization by soil organic matter because they have high charge densities.

2.3 Surfactants

Liquid surfaces tend to contract to the minimum area possible because the attraction from the underlying molecules is greater than that of the vapor molecules above the surface. The net inward attraction force per unit length of the surface is called the surface tension

of a liquid (Christofi and Ivshina, 2002). Surface tension has also been defined as the energy required to convey a molecule from the bulk phase to the surface (Rosen, 1978).

In general, surfactants have amphiphilic molecules consisting of two distinct structural parts: a non-polar (hydrophobic) tail and a polar (hydrophilic) head (Edwards et al., 1992; Mulligan and Gibbs, 2004). Hydrophilic head groups, have sufficient affinity for the polar solvent to bring the entire molecule into solution, while the hydrophobic portion has less affinity for the solvent than the solvent molecules have for each other. If forces affecting this hydrophobic portion are sufficient, the solute will tend to concentrate at an interface.

Two possible mechanisms exist for surfactant treatment of contaminated soils. At low concentrations, surfactants are present as individual molecules and gather at interfaces. The interface can be at the free solution surface (liquid/vapor interface), at the container wall (solid/liquid interface), or at the solution and an immiscible fluid boundary (liquid/liquid interface) (Zajic and Seffens, 1984). By replacing the bulk molecules at the interfaces, surfactants reduce surface and interfacial tensions, and facilitate the formation of emulsions between liquids of different polarities (Mulligan, 1998; Christofi and Ivshina, 2002; Jennings, 2006). An emulsion is the dispersion of a liquid in another immiscible liquid in the form of droplets with diameters of at least 0.1 μm (Mulligan and Gibbs, 2004). The reduction in interfacial tension also lowers the capillary forces, reduces the contact angle, and increases the mobility of the contaminant (Lake, 1989; Pennell et al., 1994; Mulligan et al., 2001b; Urum and Pekdemir, 2004).

The reduction in the surface tension is proportionate to the increase in the concentration of the surfactant up to a level known as the critical micelle concentration (CMC) (Zajic and Panchel, 1976). Increasing the concentration of surfactants above the CMC has no effect on the surface tension of the solution (Figure 2.2), instead surfactants monomers start to form aggregates know as micelles (Mitchell and Ninhman, 1981; Hiemenz, 1997 Mulligan, 2005). Thus, the CMC is also referred to as the maximum concentration of surfactant monomers (Mulligan and Gibbs, 2004).

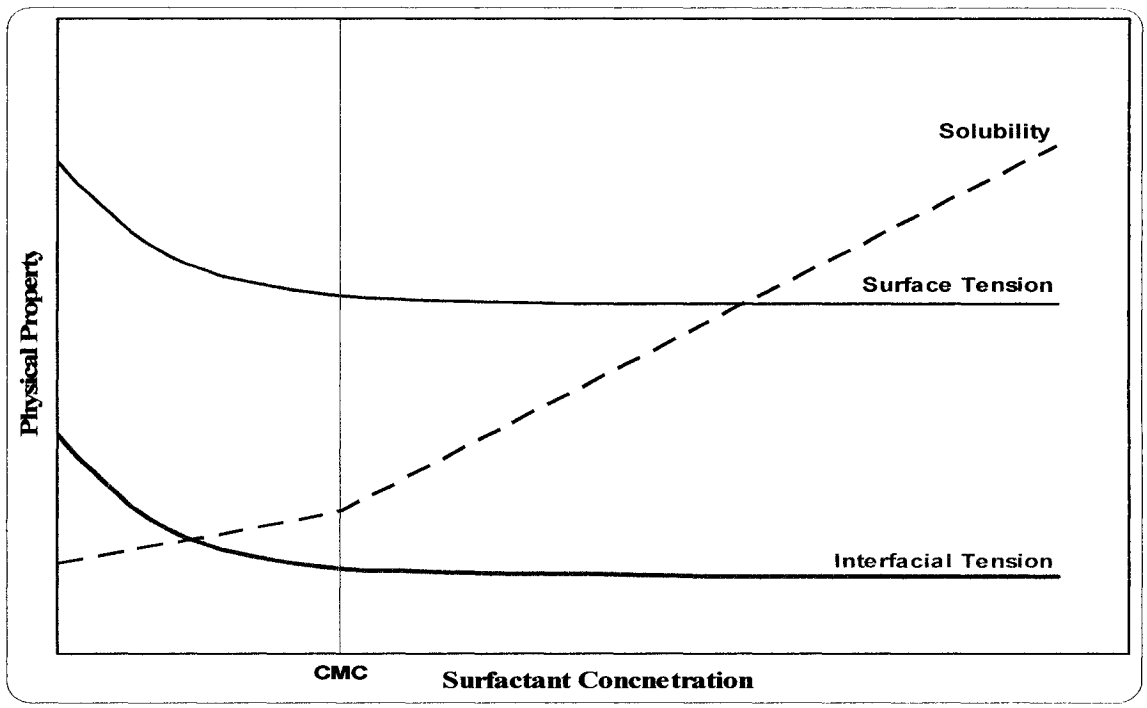

Figure 2.2: Correlation of surface tension, interfacial tension, and solubility with concentration of surfactants in the solution.

Aggregated surfactants can form structures, such as spheres, bilayers, and vesicles. A sphere is the simplest micellar structural form, and generally has a diameter of less than 5 nm (Mitchell and Ninhman, 1981). In spherical micelles the polar heads of the surfactant monomers are oriented toward the surrounding aqueous solution and the tails

than 5 nm (Mitchell and Ninhman, 1981). In spherical micelles the polar heads of the surfactant monomers are oriented toward the surrounding aqueous solution and the tails are oriented toward the oleophilic center of the micelle. Surfactant bilayers have their polar heads facing the outside while the nonpolar surfactant tails are confined together inside making both sides of the bilayer hydrophilic and the portion within hydrophobic. A vesicle is a bilayer that has a small enclosed compartment structure similar to a biological membrane. Vesicle size ranges from 10 nm to more than 500 nm in diameter, whereas, a bilayer with flexible sheet structures can be unlimited in size (Mitchell and Ninhman, 1981; Hiemenz, 1997). The type and size of the aggregate formed depends on pH, temperature, structure of surfactant monomer, and ionic strength of the solution (Bai et al., 1998; Mulligan and Gibbs, 2004).

Formation of micelles (concentration above CMC) results in the partitioning of the hydrophobic compounds into the hydrophobic core of the micelle. Hydrophobic contaminants are thus dispersed in the aqueous phase above their solubility limits (Falatko and Novak, 1992; Pennell et al., 1993), increasing their bioavailability (Miller, 1996; Deshpande et al., 1999), and subsequently in the case of organics their biodegradation (Rouse et al., 1994; Deshpande et al., 1999). Surfactants also enhance the mobility and removal of heavy metals from soil by complexation (Beveridge and Pickering, 1983; Herman et al., 1995; Mulligan, 1998).

Surfactants are used in industries that deal with multiphasic systems specifically in petroleum and pharmaceutical industries, as well as in soil decontamination, because of their desirable properties, such as surface and interfacial tension reduction, solubility

enhancement, wettability, and foaming capacity (Mulligan and Gibbs, 2004). Examples of their use in soil decontamination include the use of Triton X-100 and Tween 80 to increase the concentration of polycyclic aromatic hydrocarbons (PAH) in the aqueous phase (Tiehm, 1994; Grasso et al., 2001; Cuypers et al., 2002; Prak and Pritchard, 2002). Other studies indicate that surfactants can increase the biodegradation rates of soil xenobiotics including alkanes, PAHs, and a range of other hydrocarbons (Aronstein et al., 1991; Bury and Miller, 1993; Bruheim et al., 1999; Margesin and Schinner, 1999). Despite this, the use of chemical surfactants in the area of soil remediation has been hampered due to their adverse effects, such as toxicity, persistence to degradation, and reduced availability of compounds incorporated into micelles (Tiehm, 1994; Mulligan et al., 2001b; Cort et al., 2002).

2.3.1 Biosurfactants

By definition biosurfactants are surfactants produced by animals, plants, and microorganisms (Lin, 1996; Mulligan et al., 2001b). Biosurfactants are used as an alternative to synthetic surfactants in a variety of applications, because of their many advantages (Kosaric, 2001; Hudak and Cassidy, 2004; Mulligan and Gibbs, 2004), including:

- Biodegradability
- Low toxicity
- Potential for in-situ production
- Greater ability to complex heavy metals

- Specificity: Biosurfactants have complex organic structures with specific capabilities

- Effectiveness: Biosurfactants can be employed under extreme pH and salinity

Biosurfactants are evaluated by their effectiveness as well as their efficiency. Biosurfactant effectiveness is a measure of their capacity to lower the surface tension of water (Mulligan, 1998). The lower the surface tension of water that is achievable the more effective the biosurfactant is. A biosurfactant's efficiency is determined by its CMC, and a biosurfactant with lower CMC is considered to be more efficient (Mulligan, 2005). Typically, biosurfactants are capable of lowering the surface tension of water from 72 mN/m to as low as 27 mN/m and have CMCs from 1 to 200 mg/L (Rosen, 1978; Javaheri et al., 1985; Lang and Wagner, 1987; Persson et al., 1988).

Biosurfactants are generally grouped by their charge type, molecular weight, or chemical composition. A biosurfactant's charge (positive or negative), like most of its other chemical properties, is usually dictated by the hydrophilic head group (Bai et al., 1997). An anionic biosurfactant has a negative charge at its head group, while a cationic biosurfactant carries a positive charge. When the head group has both charges the surfactant is defined as zwitterionic. If there is no charge in the head group, the biosurfactant is termed a nonionic biosurfactant (West and Harwell, 1992). Most of the biosurfactants identified thus far are either anionic or neutral, and only a few have cationic properties (Mulligan et al., 2001b).

Based on their molecular weight, biosurfactants are categorized as low molecular weight and high molecular weight biosurfactants. Low molecular weight biosurfactants are

effective surface and interfacial tension reducers, while high molecular weight biosurfactants are generally long chain polymers capable of wetting, coating, and emulsifying immiscible liquids; but normally do not lower the surface tension of the solvent appreciably (Cooper, 1986; Ron and Rosenberg, 2001).

Unlike chemical surfactants that are classified by the nature of their polar groups (alcohol sulphates, alkyl phenol ethoxylates, etc.) biosurfactants are structurally diverse molecules. Based on their molecular structure, biosurfactants can be classified into several broad groups: glycolipids, lipopeptides, phospholipids, fatty acids, and neutral lipids (Biermann et al., 1987).

Glycolipids are the most common types of biosurfactants. The constituents are carbohydrate (sugar) head groups and fatty acid tails. Examples are rhamnolipids and sophorolipids (Mulligan and Gibbs, 2004). Lipopeptides contain several amino acids as the head group and one or two fatty acids as the tail. The best known lipopeptide is surfactin, which is produced by *Bacillus subtilis*. Phospholipids possess a negatively charged phosphate head group and are produced when certain bacteria (*Acinetobacter, Thiobacillus thiooxidans*) or yeasts are grown on alkanes (Cirigliano, et al., 1985; Mulligan and Gibbs, 2004). Fatty acids and natural lipids are produced by the oxidation of alkane substrates by microorganisms, and have shown surface activity properties (Zajic and Seffens, 1984).

Studies have shown that biosurfactants can be employed in enhanced oil recovery (Zhang and Miller, 1995; Al-Tahhan et al., 2000; Mulligan et al., 2001b), metal detoxifying (Sandrin et al., 2000), metal removal (Holden et al., 1989; Tan et al., 1994; Miller, 1995a;

Mulligan et al., 1999; Ochoa-Loza et al., 2001; Hong et al., 2002; Neilson et al., 2003), enhanced soil washing and flushing (Bai et al., 1997; Page et al., 1999), and in biodegradation of contaminated soils (Zhnag and Miller, 1992, 1995; Burd and Ward, 1996; Sandrin et al., 2000; Gallego, 2001; Cassidy et al., 2002a; Noordman and Janssen, 2002).

2.3.2 Mechanisms of Removal

Like their synthetic counterparts, a few mechanisms are believed to be involved in the removal of xenobiotic organic and metallic compounds by biosurfactants. When an immiscible compound like a non-polar hydrocarbon is placed in aqueous solution containing biosurfactant at concentrations below its CMC, the hydrophilic portion of the biosurfactant will orient itself toward the water molecules, while the hydrophobic portion will orient itself toward the hydrophobic compounds (hydrocarbons, surface air, etc.). Accumulation of biosurfactants at the interfaces reduces the surface and interfacial tensions between immiscible phases allowing them to become miscible by releasing the entrapped hydrocarbon compounds and creating additional surfaces (Bai et al., 1998; Christofi and Ivshina, 2002).

In the case of metals, accumulated biosurfactants at the solid-liquid interface come into direct contact with metals that are bonded or precipitated on soil particles (Christofi and Ivshina, 2002). Therefore, anionic biosurfactants attract and form bonds with the positively charged metals. Since these bonds are stronger than the metals bond with the soil, the metals will enter the liquid phase (Miller, 1995a; Torrens et al., 1998). Cationic

biosurfactants can also limit sorption of metals to soils by competing for the positively charged solid surfaces of soil (Christofi and Ivshina, 2002).

Above their critical micelle concentrations, biosurfactants partition the hydrophobic hydrocarbons into the interior of the formed micelles (Bai et al., 1998; Urum and Pekdemir, 2004). This can enhance the solubility and subsequently the bioavailability of hydrophobic compounds dramatically (Rouse et al, 1994; Miller, 1996; Deshpande et al., 1999; Urum and Pekdemir, 2004).

With concentrations of biosurfactants above the CMC, the free metals in solution form complexes with biosurfactants and are removed from the solution. This reduces the amount of metals in the solution and promotes desorption of metals from soil (Herman et al., 1995; Miller, 1995a; Mulligan, 1998). Furthermore, metal-organic matter complexes can be incorporated into the micelles as well, further increasing the concentration of metal in solution (Mulligan, 1998).

2.3.3 Production of Biosurfactants

According to Mulligan and Gibbs (2004), there are two main modes of biosurfactant production (Figure 2.3) by microorganisms:

A. Growth-Associated Production

Under growth-associated conditions biosurfactants production has a direct relationship with substrate utilization and microbial growth (Desai and Ibrahim, 1997). Biosurfactants produced on hydrocarbons are usually growth-associated (Syldatk et al., 1985; Robert et al., 1989; Deziel et al., 1996; Lepo and Cripe, 1999; Mulligan and Gibbs, 2004), but

growth on other substrates have also been observed (Robert et al., 1989; Nitschke et al., 2004; Maneerat, 2005; Nitschke and Pastore, 2006).

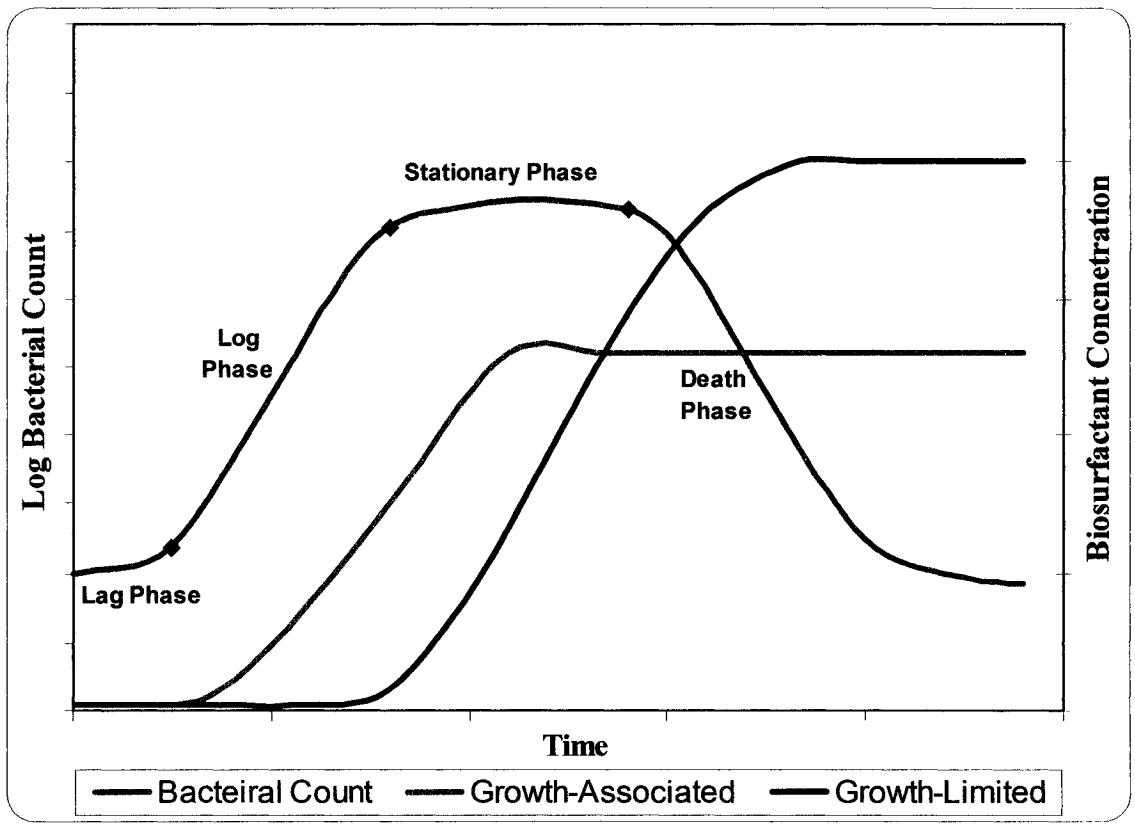

Figure 2.3: Biosurfactant production modes.

B. Growth-Limited Production

In contrast to growth-associated production, production under growth-limiting conditions is not a function of the growth of microorganisms. Here, biosurfactants are produced at the end of the logarithmic and/or stationary phases of growth of microorganisms, when one of the essential growth factors, such as substrate or nutrients becomes limited (Desai and Ibrahim, 1997; Mulligan and Gibbs, 2004).

The best example of growth-limited productions of biosurfactants is production of rhamnolipid under nitrogen limitation (Mulligan and Gibbs, 1989; Yateem et al., 2002;

Hudak and Cassidy, 2004). Other examples include production under limiting conditions of iron (Guerra-Santos, 1984), phosphorous (Mulligan et al., 1989; Chayabutra, 2001), oxygen (Lin, 1994), sulfur, magnesium, and calcium (Chayabutra, 2001).

An advantage of production under nutrient-limited conditions is that the produced biosurfactants are less subjected to biodegradation because of reduced population of microorganisms and unavailability of nutrients (Hudak and Cassidy, 2004).

2.3.4 Factors Effecting Biosurfactant Production

Composition and yields of biosurfactants produced by microorganisms depend on producing species, carbon source, nutrient composition, and environmental factors (Mulligan and Gibbs, 1993; Desai and Ibrahim, 1997).

A. Producing Species:

The types and compositions of biosurfactants generated are dictated by the producing microorganism. Most microorganisms have the capacity to produce biosurfactants, especially among bacteria, they are widely distributed in both pristine and contaminated soils (Bouchez-Nairali et al., 1999; Bodour et al., 2003; Menezes Bento et al., 2005;). Different species or genera of microorganisms produce biosurfactants with different charge types (anionic, cationic, etc.) and chemical structures (glycolipids, phospholipids, etc.). A single isolate usually produces a mixture of different chemical variations of the same biosurfactant (Bodouret et al., 2003). For example, various *Pseudomonas* species produce rhamnolipids that have different number of rhamnose molecules and tail lengths (Arinoet et al., 1996; Deziel et al., 1999).

B. Carbon Source:

The type, quality, and quantity of biosurfactant produced are influenced by the nature and concentration of the carbon source in the medium (Cassidy and Irvine, 1997; Desai and Ibrahim, 1997; Hudak, 2004). Some organisms produce biosurfactants when they are growing on water immiscible hydrocarbons, such as n-alkanes and olive oil, and will not produce or have very small production rates when grown on water soluble substrates, such as glucose and vise versa (Copper et al., 1981; Syldatk, 1985; Duvnjak and Kosaric, 1985; Robert, 1989; Matsuyama et al., 1990).

The concentration of the hydrocarbon source is also important in production of biosurfactants and their characteristics (Li et al., 1984; Cassidy and Irvine, 1997). Studies show that increasing the concentration of the carbon source not only increases biosurfactant production rates but also changes their type and performance (Desai and Ibrahim, 1997; Cassidy et al., 2002a; Hudak, 2004).

C. Nutrient Composition:

The sources and concentrations of other medium constituents, such as nitrogen, phosphorus, and trace metals also affect the production rate and performance of biosurfactants (Syldatk, 1985; Desai and Ibrahim, 1997). Concentrations of the nitrogen and phosphorous, as well as carbon-to-nitrogen ratios, and the carbon-to-phosphorous ratios affect the production and performance of biosurfactants by growth-associated microorganisms (Guerra-Santos et al., 1984). Furthermore, as explained earlier, nitrogen or phosphorous limitation can initiate or induce growth-limiting microorganisms to

overproduce biosurfactants (Mulligan and Gibbs, 1989; Mulligan et al., 1989; Kim et al., 1990; Desai and Ibrahim, 1997).

The presence and amount of various metal cations (Fe, Mn, Ca, etc.) also effect biosurfactant production (Guerra-Santos et al., 1986; Chayabutra, 2001). In addition, metal cations can also affect biosurfactants properties, such as the solubilization capacity, solubility, and sorption to soil (Bai et al., 1998).

D. Environmental Factors:

Since biosurfactants are produced by microorganisms, their production rate and characteristics are influenced by microbial growth factors such as pH, temperature, agitation speed, and salinity when cultivated in the laboratory (Guerra-Santos et al., 1986; Desai and Ibrahim, 1997; Bai et al., 1998). Ambient pH plays an important role in the growth of microbes and the subsequent production and behavior of biosurfactants (Guerra-Santos et al., 1986; Mulligan and Gibbs, 1993). For example the optimum pH for rhamnolipid production by *Pseudomonas aeruginosa* is 7 (Cassidy et al., 2002a). Some microbes produce biosurfactants only at specific temperature ranges, while others are not temperature sensitive (Matsuyama et al., 1986; Matsuyama et at, 1990). Increasing the agitation speed has been shown to decrease the production of biosurfactants in fermentation reactors as well as increase their aerobic production by yeast (Spencer et al., 1979; Kosaric, 1993). High concentrations of salt prohibit growth of microorganisms and decrease the production of biosurfactants in most cases (Abu-Ruwaida et al., 1991).

2.4 Remediation Strategies

The most important factors in selecting a remediation technology are the type and concentration of contaminants in the soil. Other important factors include their volatility, solubility, and biodegradability (Mulligan, 1998). Contaminants are divided into two main groups of organics and inorganics (EPA, 2004). Organics are the carbon-containing compounds, including non-volatile hydrocarbons, volatile hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), organic pesticides, and polychlorinated benzenes (PCBs). Inorganics contain heavy metals, cyanides, and radionuclides.

Remediation methods are categorized as either containment or treatment methods (EPA, 2004). Containment include the isolation and control of the source or groundwater with the use of caps, liners, covers, barriers, and landfilling to prevent movement of contaminants and/or reduce the permeability and increase bearing capacity of the soil (EPA, 1985, 1992a; 2004; Mulligan, 1998). Barriers can be made of steel, cement, and grout or hydraulically created by pumping (EPA, 2004). Another containment method is solidification/stabilization that restricts the movement of contaminants by binding soil particles with physical and chemicals means (EPA, 2000b, 2004).

Remediation technologies are also grouped as ex-situ or in-situ. In ex-situ methods, contaminated media are usually excavated, dredged, and removed, before being subjected to an aggressive treatment technology in a controlled environment. In-situ technologies remove the contaminants without excavating, pumping, or moving the contaminated media to the surface. Ex-situ methods are more expensive because they require

excavation of the medium, whereas in-situ methods are conducted in a less controlled environment, therefore typically they require longer treatment times (EPA, 1997a, 2004).

EPA (2004) has listed 28 methods for cleanup of contaminated sites. These include source control treatments (primarily soil), in-situ groundwater methods, pump and treat technologies, monitored natural attenuation, and groundwater containment techniques. Below the main remediation methods that are applicable to soils contaminated with both organics and inorganic pollutants are briefly explained.

Chemical Treatment

Chemical treatments include application of chemical amendments to induce specific chemical reactions within the soil to oxidize or reduce contaminants into nonhazardous, less toxic, more stable, less mobile, and/or inert compounds (Mulligan, 1998; EPA, 2004; Martin and Ruby, 2004). For the purposes of this dissertation chemical treatments do not include encapsulation of the contaminants (solidification/stabilization). This method can be applied to solids or groundwater in-situ or ex-situ (EPA, 2004).

Chemical treatment also includes redox reactions, which involve the transfer of electrons between two chemical species; where one compound is oxidized (loses electrons) and the other is reduced (gains electrons). The oxidizing agents used for treatment of hazardous contaminants in soil include ozone, hydrogen peroxide, potassium permanganate, phosphate, manganese, iron, and chlorine. Examples include phosphate amendment stabilization of lead, cadmium, and zinc (Zhang et al., 1998; Mosby, 2000; Hamon et al., 2002), sorption of metals to iron and/or manganese (Chen et al., 2000), and oxidation of organic pollutants by hydrogen peroxide (Kanel, 2003).

Electrokinetics

Electrokinetic processes involve inserting electrodes into soil matrix and passing a low intensity direct electric current through them (EPA, 1995; Rodsand and Acar, 1995; USAEC, 2006). This will result in electrolysis, which is chemical reaction due to electric current, and electrokinetic phenomenon described as movement of ions, fluids, and charged particles due to the electric field (FRTR, 2001; Mulligan, 1998). Because, the electric currents can't remove the contaminants from the polluted media, electrokinetic methods are used to concentrate the contaminants, in order to be removed via excavation, precipitation, or pumping of water near the electrode, (EPA, 1997b, 2000c; FRTR, 2001). This process can be used in-situ or ex-situ.

Phytoremediation

Phytoremediation or vegetative remediation is the direct use of plants, such as *Thlaspi,* Alfalfa, Indian mustard, *Chenopodium*, and *Urticao* to stabilize or remove contaminants from shallow soils (EPA, 1998; 1999b; Chen, 2000). Stabilization of contaminants involves sorption, precipitation, and complexation of contaminates to reduce their mobility and bioavailability, whereas the removal is the result of their uptake and accumulation in the tissue of the plants (Brown et al., 1994a, b; Mulligan, 1998; EPA, 1999b; Pierzynski et al., 2000). The contaminated plant biomass has to be collected and disposed of using appropriate techniques, such as drying, incineration, acid extractions, and anaerobic digestion (EPA, 1998; 2000c; Bolenz et al., 1990).

The factors that influence the effectiveness of phytoremediation include the concentration and form of the contaminants, the type and strength of additives (acid, base, chelation

agent, etc.), and the growing conditions of the plants (EPA, 1998; 1999b). Examples of phytoremediation include removal of cadmium by vetiver grass (Chen et al., 2000), remediation of zinc and cadmium contaminated soils by *Thlaspi* (Brown et al., 1994b, 1995), and lead removal by Indian mustard (Blaylock et al., 1997; Elless and Blaylock, 2000).

Solidification/Stabilization

Solidification/stabilization (S/S) is the most common method for containment of polluted soils both ex-situ and in-situ (Evanko and Dzombak, 1997). In this process, binding agents are mixed or injected into the soil media to alter the contaminants chemically and physically, making them less soluble, mobile, and bioavailable (ITRC, 1997; EPA, 2000b, 2004). According to the EPA (2000), cement, phosphate, fly ash, lime, and sulfur are the most common binders and reagents used in remediation of superfund sites. These products will react chemically, encapsulate, and reduce the permeability and surface area of the contaminants (EPA, 1989, 1997b). S/S can be applied to sites contaminated with a wide range of contaminant types, concentrations, and depths (EPA, 1989, 2004; Frankenberger and Losi, 1995). Examples include, stabilization of lead with phosphorous and manganese oxides (Hettiarachi et al., 2002) and immobilizing hexavalent chromium contaminated soils by cement (Wang and Vipulanandan, 2006).

Vitrification

In vitrification an electric current capable of melting the soil at high temperatures (1600°C to 2000°C) is applied to the contaminated soils to convert them into a glass-like

amorphous solid, free of any crystalline structure (EPA, 2004; 1985). The organic contaminants of the soil are either volatilized or pyrolyzed by the generated heat, while metals are immobilized within the end product resulting in an inert, impermeable, chemically stable, and leach-resistant material (EPA, 1997a). Because vitrification is expensive it is usually applied to sites contaminated with high levels of both organic and metal contaminants. The contaminated soils must also contain sufficient glass-forming materials and be free of any volatile metal (EPA, 1997b; Martin and Ruby, 2004).

Soil Washing and Flushing

Soil washing and soil flushing are technologies that separate and extract contaminants from soil matrix via water or other suitable aqueous solutions (EPA, 2004, 1985). Soil washing refers to ex-situ technologies performed in reactors or tanks, where the environmental conditions can be manipulated and controlled without restrictions (EPA, 1992a; Martin and Ruby, 2004). Soil flushing on the other hand, is an in-situ method implemented by applying water to soil or injecting it into subsurface, followed by its recovery and treatment above ground to remove residual contaminants (EPA, 1997b).

If contaminant solubility is low, removal efficiency can usually be improved with additives (EPA, 1997a). Inorganic and organic acids (hydrochloric acid, citric acid, etc.) bases (sodium hydroxide, etc.), chelating agents (ethylenediaminetetraacetic acid (EDTA), etc.), solvents (methanol, etc.), biosurfactants (rhamnolipid, etc.), and oxidizing or reducing agents (hydrogen peroxide, etc.) have been used as additives in soil washing and soil flushing (EPA, 1991, 1997b, 2000c, 2004).

In addition to the solubility coefficient of the contaminant, the removal efficiency also depends on soil type, soil pH, cation exchange capacity (CEC), particle size distribution, contaminants type, and permeability and hydrogeology of the soil (EPA, 1992a, 2004; West, 1992; NRC, 1994).

The recovered water together with the flushed contaminants are treated, and then recycled if necessary. According to the EPA (2004), the recovered water can be treated by adsorption, air stripping, bioremediation, chemical treatment, filtration, ion exchange, metal precipitation, or membrane filtration.

Bioremediation

Unlike other fate pathways that disperse contaminants in the environment, bioremediation can eliminate the adverse health and environmental effects of the pollutants (Cookson, 1995; Alexander, 1999). Bioremediation is the in-situ or ex-situ use of microorganisms and their products to break down, remove, and/or neutralize contaminants in soil, sludge, solids, and groundwater (EPA, 2004). During bioremediation of organic pollutants, the natural ability of microorganisms to degrade is stimulated by providing favorable conditions (pH, temperature, etc.) and improving availability of materials (electron acceptors, nutrients, etc.) and microorganisms (specific genera or species, etc.) (EPA, 1993; 2000c; Eweis, 1998). Under favorable conditions microorganisms catabolize (destructive metabolism) complex substrates to provide the cell with carbon, energy, and other nutrients required for growth (Atlas and Bartha, 1993; Bourquin and Pedersen, 1995; White and Bertola, 2005). Microbes can accomplish degradation of organic compounds through cometabolism as well. Cometabolism is the

degradation of recalcitrant material in the presence of other organic compounds that serve as the primary substrate (Leahy and Colwell, 1990; Cookson, 1995). The degradation of the recalcitrant compound dose not provide energy, carbon, or nutrients, therefore, the compound is only partially degraded (Horvarth, 1972; Atlas and Bartha, 1993).

In biological treatment of inorganics, microorganisms can oxidize, reduce, precipitate, and complex metals to immobilize them by binding them tightly to the soil matrix or transform them into less toxic and less bioavailable species (Tichy et al., 1992; EPA, 1993, 2004; Karavaiko et al, 1988). Other methods promote migration and removal of metals by desorbing and solubilizing them in the aqueous media (Mulligan, 1998; Miller, 1995a).

In-situ bioremediation techniques involve creating a favorable environment for microorganisms to grow in soil (Mulligan 1998; Alvarez and Illman, 2006). These methods may be hindered by low permeability of soils, low bioavailability of contaminants, or presence of inhibitors, such as toxins and metals (EPA, 1993). Ex-situ bioremediation include treatment of excavated soils or extracted waters in bioreactors, piles, etc. Another type of ex-situ bioremediation is landfarming, which involves the spreading of the excavated soils in a thin layer on the ground surface, mixing them with native topsoil, and stimulating their degradation by providing essential growth factors (Mulligan 1998; EPA, 2004; Alvarez and Illman, 2006).

2.5 Rationale

As explained earlier, selected technologies for remediation of Superfund sites show an increasing trend toward in-situ treatment methods to reduce costs and to satisfy legal

requirements (EPA, 2004). Furthermore, among the in-situ treatment methods, bioremediation is by far the most widely used technology because it is a natural process and is less intrusive and disruptive to the ecosystem undergoing treatment, and produces environmental friendly by-products (NRC, 1994).

Recently, biodegradable surfactants have been studied extensively as an alternative to chemical additives employed to improve the rate of bioremediation (Holden et al., 1989; Burd and Ward, 1996; Mulligan, 1998; Al-Tahhan et al., 2000; Sandrin et al., 2000). Furthermore; reports of in-situ biosurfactant production at contaminated sites using contaminants as substrate (Cassidy et al., 2002b; Youssef et al., 2006) prompted researchers to evaluate the potential for in-soil biosurfactant production by indigenous soil microorganisms under laboratory conditions (Cassidy and Irvine, 1997, 2001; Straube et al.; 1999;).

Cassidy et al. (2000, 2002a) conducted a series of experiments in which biosurfactants were produced in diesel fuel, PAHs, and lubricating oil contaminated soils using a slurry reactor. Biosurfactants were produced in sufficient quantities so as to promote biodegradation and facilitate washing of remaining petroleum hydrocarbons, including polychlorinated biphenyls (PCBs), which are essentially recalcitrant with respect to biodegradation. The success of those studies prompted questions regarding the feasibility of enhanced bioremediation with biosurfactant production in co-contaminated soils to increased mobilization and removal of metallic soil contaminants in addition to petroleum hydrocarbon biodegradation. This dissertation addresses those questions.

3 MATERIALS AND METHODS

This chapter outlines the material, equipment, and methodology employed in this study. Common laboratory chemicals were purchased from Fisher Scientific. Chemicals and reagents including any test kit used are cited in the pertinent method section. All distilled water was prepared in the laboratory using a Barnstead Classic Still A1011-B distiller. Table 3.1 lists all the major equipment used.

3.1 Origin of the Soil

The soil was obtained from the Toronto Harbour area (Toronto, Ont.) run by the Toronto Harbour Commission (THC). The site had been used for metal finishing, refineries and petroleum storage, resulting in co-contamination of the soil with hydrocarbons and metals (Richardson and Ehrenreich, 1994; NCDENR, 1997). The soil was collected in a 5 L plastic pail and stored at room temperature. Prior to initial characterization of the samples, homogenization and removal of oversize material was performed to create uniform samples. The material remaining on sieve No. 5 (4 mm) was removed and the remaining soil was homogenized by provisions cited in ASTM D422-63 (ASTM, 2006). To minimize loss of petroleum hydrocarbons by aerobic degradation and evaporation, homogenized soil were placed into six 250 mL clean sterilized amber glass jars with minimal headspace.

Table 3.1: List of major equipment used.

Instrument	Brand and Model
Atomic Absorption Spectrometer	Perkin Elmer Atomic Absorption Analyst 100 Spectrometer
Autoclave	Yamato Sterilizer SM300
Balance	Mettler Toledo PB1502-S
Biological Hood	Forma Scientific Class II A/B3 Biological Safety Cabinet
Centrifuge	International Equipment Company, HN-SII
Desiccator	Sanplatec SANPLA Dry Keeper
Furnace	Fisher Scientific Programmable Isotemp® Basic Muffle Furnace, 550 Series Model No. 125
Gas Chromatography	Varian Model 3800 equipped with a Varian CP-8400 AutoSampler and a Flame Ionization Detector (FID)
Hot plate	Fisher Stirring Hot Plate
Incubator (Experimental Tests)	Fisher Scientific Isotemp® Incubator Model 304
Incubator (Microbial Enumeration)	Hotpack Environmental Chamber
Orbital Shaker	Canadawide Scientific Digital Orbital Shaker
Oven	Lindberg/Blue Mechanical Gravity Oven Model GO1330A
pH Meter	Fisher Scientific AR25, Dual Channel pH/Ion Meter
Rotary Evaporator	Heidolph Laborota 4000 Equipped with a Heidolph ROTOVAC Pump
Spectrophotometer	Hach DR/2800
Tensiometer	Fisher Scientific Semiautomatic Model 21 Tensiomat®
Ultrasonic Bath	Ney Ultrasonik 300
Vortex Shaker	Caframo™ Reax 2000 Vortex
Water Distiller	Barnstead Classic Still A1011-B

3.2 Soil Characteristics

3.2.1 Amorphous Content

The amorphous material content (Fe_2O_3, Al_2O_3, and SiO_2) of the soil was measured according to Segalen (1968). In this method soil samples are washed by alternate solutions of acid and base, and centrifuged to separate supernatant from soil. Using an atomic absorption spectrometer the base and acid washes were analyzed for Fe, Si, and Al. Individual contents were summed to determine the total concentration of each element. The amorphous content was quantified by converting the concentration of the elements to their equivalent oxide forms.

3.2.2 Cation Exchange Capacity

The procedure of Chapman (1965) was used to measure the cation exchange capacity (CEC) of the soil sample. This is a pH-buffered CEC measurement.

The procedure is as follows:

1. 5 g of soil was added to a 50 mL polypropylene centrifuge tube.

2. 20 mL of 1 M potassium acetate was added to the sample and placed on a mechanical orbital shaker for 5 minutes. This procedure will replace soil bound cations with potassium ions.

3. To quantitatively transfer all soil to the liquid the inside of the tube was washed with distilled water.

4. The tube was then centrifuged for 15 minutes at 1000 rpm and the supernatants removed.

5. Steps 2, 3, and 4 were repeated for several times to replace all soil cations with potassium ions.

6. The soil particles were brought into suspension by adding 20 mL of methyl alcohol to the tube and shaking it.

7. The tube was again centrifuged for 15 minutes at 1000 rpm and the supernatants removed.

8. Steps 6 and 7 were repeated for several times to remove all soluble potassium ions.

9. The soil particles were brought into suspension again by adding 25 mL of 1 M ammonium acetate to the tube and shaking it.

10. To quantitatively transfer all soil particles to the liquid the inside of the tube was washed with ammonium acetate solution.

11. The tube was again centrifuged for 15 minutes at 1000 rpm and the supernatants removed.

12. Steps 9, 10, and 11 were repeated for several times to ensure that all adsorbed potassium ions were replaced by ammonium.

The potassium ions desorbed into solution is measured to determine the number of the exchange sites that were occupied by potassium. The CEC can thus be estimated based on milliequivalents of potassium exchanged per unit weight of soil.

3.2.3 Heavy Metal Content

To determine the heavy metal content the soil was prepared by digestion using an Environment Canada method (1990). The procedure is as follows:

1. 1 g sample of the soil was weighed and placed into a 1 L beaker.
2. Slowly, 100 mL 16 N nitric acid was added to the beaker.
3. 40 mL of a 30% hydrogen peroxide (H_2O_2) solution was carefully added and the mixture was left to react.
4. The beaker was heated on a hot plate until boiling.
5. The beaker was removed and placed in a fume hood to cool to room temperature.
6. 200 mL of aqua regia was added to the beaker. The aqua regia was prepared by mixing 200 mL of concentrated nitric acid, 500 mL of distilled water, 50 mL of concentrated hydrochloric acid, and finally 350 mL of distilled water to bring the total volume to 1000 mL.
7. Finally the total volume in the beaker was brought to 500 mL by adding distilled water.

The prepared solution was analyzed by flame atomic absorption spectroscopy (FLAA). The standards, blanks, and samples were prepared according to EPA standard methods and complied with manufacturer recommendations. SCP Science certified stock standards were used. Preparation and analysis were repeated four times and the average values reported as the heavy metal content.

3.2.4 Soil Moisture

The soil moisture was measured using both an air-drying method and oven-drying method. Soil was weighed in a loosely covered, pre-weighted aluminum dish and placed in fume hood for 48 hours. The change in total weight, divided by the soil's initial weight, was multiplied by 100 to give the percent soil moisture.

The oven-drying method was a modification of the method described in ASTM method D-2216 (ASTM, 2006). The following is a summary of the procedure used. Approximately 10 g sample of soil was placed in a pre-weighed, clean and dry porcelain dish and dried overnight in an oven set at 110°C. The dish was then placed in a desiccator for two hours to reach the room temperature and weighed. The percent moisture content is calculated as the difference in the weight before and after drying divided by the original soil weight multiplied by 100.

3.2.5 Organic Matter Content

The organic matter content of the soil was determined by the weight loss on ignition method, as well as the digestion by hydrogen peroxide (H_2O_2) method (APHA/AWWA/WPCF, 1998). The ignition method (dry method) is as follows:

1. A porcelain dish was washed, dried and weighed.
2. 3 g of air-dried soil was weighed into the pre-weighed porcelain dish.
3. The dish was placed in a furnace set to 550°C for 1.5 h.
4. The porcelain dish was left overnight in the desiccator.

5. The dish was then weighed and the difference in weight was divided by the initial soil weight and multiplied by 100 to give the percentage organic matter.

The other method (wet method) involved digestion with hydrogen peroxide (H_2O_2). This method involved:

1. A 250 mL beaker was washed, dried, and weighed.
2. 3 g of air-dried soil was added to the beaker.
3. 10 mL aliquots of a 30% hydrogen peroxide (H_2O_2) solution were added to the beaker until the reaction (bubbling) stopped.
4. The solution was washed into pre-weighted centrifuge tubes and centrifuged at 3000 rpm for 30 minutes.
5. The supernatant was collected and disposed of.
6. The beaker and centrifuge tubes were air-dried and weighed.

The difference in weight was divided on the initial weight of the soil and multiplied by 100 to give the percentage organic matter.

3.2.6 Particle Size Distribution

The grain size distribution of the soil was performed according to ASTM D422 method (ASTM, 2006). Approximately, 300 g of soil were dried in an oven at 100°C for 24 h and cooled to room temperature in a desiccator. Standard brass sieves were arranged from sieve No. 5 to 200, corresponding to mesh numbers from 1 to 200. The sieve column was

placed on a sieve shaker for 15 minutes and the dry weight retained by each sieve was determined. The same procedure was again repeated for a soil that had its organic matter removed by the loss on ignition method as explained in section 3.2.5.

3.2.7 Soil pH

Soil pH was measured using a soil to water ratio of 1:10. In this procedure 20 g of soil was placed in a 400 mL beaker and 200 mL of distilled water added. The solution was placed on an orbital shaker for 30 minutes and left for one hour to ensure equilibrium was reached prior to measurement.

3.2.8 Total Petroleum Hydrocarbon Content

Total petroleum hydrocarbon content was determined according to modified method of Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WPCF, 1998). This method uses 30 mL of n-hexane (Optima®, Fisher Scientific, meets ACS specifications) to wash the petroleum hydrocarbons from 0.3 g of air-dried soil into a Pyrex glass centrifuge tube. The tube is placed in an ultrasonic bath for 30 min, while being agitated by hand every 5 min. After the ultrasonic treatment the soil was centrifuged at 3000 rpm for 10 minutes, the hexane was pipetted off, and the soil was dried in a fume hood. The difference in the weight of the soil sample was divided on the initial soil weight and multiplied by 100 to give the percentage of total petroleum hydrocarbon.

3.3 Experimental Approach

As explained before, this study has two phases: The first phase concerns the feasibility of biosurfactant production by the indigenous microorganisms of a co-contaminated soil and

its effect on contaminant removal; whereas, the second phase examines the effect of nitrogen limitation on the production of biosurfactant and the associated effect on contaminant removal. Although the experimental design and analyses for both parts are the same, some aspects of the sampling and procedures are not. Therefore, methods and procedures for the first phase will be explained in detail with procedural differences for the second phase outlined.

3.3.1 Batch Preparation

A. Phase One

Soil batch studies were preformed to evaluate the feasibility of biosurfactant production in the co-contaminated sample soil. Mixing soil in water enhances bioremediation by maximizing the contact between microorganisms, hydrocarbons, nutrients, and oxygen (Cookson, 1995). All tests were performed in duplicate.

There are three types of batches involved: blank batches, control batches, and nutrient amended batches. The batches were designed to contain a soil to water ratio of 1:50 (dry-wt/v) based on the air-dried moisture content described in section 3.2.4. Each batch has approximately 2 g of soil and 100 mL of distilled water placed in a 500 mL Erlenmeyer flask. Flasks were fitted with foam stoppers to prevent the entry of dust and microorganisms into the system, while allowing aeration. Aeration was achieved by rotating the flasks at 200 rpm on an orbital shaker. In order to minimize the effect of photodegradation and temperature variation the shaker was placed inside an incubator set to 20°C.

The blank batches are designed to account for the loss of petroleum hydrocarbons due to abiotic sources, such as adsorption, volatilization, and photodegradation (Shannon and Unterman, 1993). In order to nullify contaminant loss or removals through biotic sources sodium azide was added to the blank batches in concentration exceeding 30 times their effective concentrations (1 mmol/L) (Pramer and Bartha, 1972). A detail description of the calculation and preparation for the sodium azide can be found in the Appendix.

Control batches are simply soil and distilled water, and are intended to evaluate the natural ability of the soil to remediate through intrinsic bioremediation and abiotic processes. In addition, batch pH, a parameter not within the scope of this study, but which may have an effect on the bioremediation of the soil, was monitored in the control batches.

Finally, in nutrient-amended batches the aim was to improve the biodegradation rates obtained by intrinsic bioremediation. Therefore, the soil was supplemented with mineral salts of nitrogen and phosphorous to attain a C:N:P ratio of 100:10:1 as recommended by Cookson (1995). Sodium nitrate was used as the nitrogen source. Monobasic and dibasic potassium phosphates were added as the phosphorous source as well as a buffer solution to maintain a constant pH in accordance with that of the control batches. The concentration of added phosphorus was doubled to account for its precipitation (Walworth and Reynolds, 1995). Furthermore, since adding the total amount of nutrients estimated as necessary for complete biodegradation in a single application may have an inhibitory effect on the microorganism's growth; nutrients were administered

incrementally over time (Walworth and Reynolds, 1995). A detailed description of the calculation and preparation for nutrient solutions can be found in the Appendix.

Before proceeding with experimental tests, a trial run was performed with the same parameters as explained above, for a period of three weeks, to determine the amount of water loss due to evaporation and to monitor the variations in the solution pH. To determine water loss the weight of the flasks, including soil, distilled water, and foam stopper were monitored during this period.

B. Phase Two

Phase two experiments were restricted to nutrient amended batches. All other aspects and parameters were identical to the phase one study.

3.3.2 Analysis

A. Phase One

For each batch type there were a total of six sampling time intervals at 0 (30 minutes), 5, 15, 30, 45, and 50 days. At each time interval, the flasks were completely sacrificed. The batches were analyzed as follows:

1. 1 mL of the supernatant was taken for microbial enumeration (section 3.3.3).
2. The flasks were allowed to settle for around 10 minutes.
3. 60 mL of the supernatant was pipetted off from the middle of the flask and passed through a Whatman No. 40 ashless filter (8 μm).
4. The pH of the filtrate supernatant was measured.

5. 40 mL of the filtered supernatant was poured into a 100 mL beaker to determine its surface tension (section 3.3.4).

6. If the surface tension of the supernatant was equal to the surface tension at the critical micelle concentration then the critical micelle dilution (CMD) was determined (section 3.3.6).

7. The 40 mL filtrate was then used for analysis of total mobilized metals in the supernatant (section 3.3.9).

8. The remaining 20 mL of the filtrate was used to determine total petroleum hydrocarbons in the supernatant (section 3.3.7.A).

9. The remaining soil and supernatants were extracted inside the flask to determine the total petroleum hydrocarbons removal (section 3.3.7.B).

B. Phase Two

For phase two experiments, the following six sampling time intervals were selected: 0 (30 minutes), 5, 15, 20, 25, and 30 days. Nitrate concentration in the liquid phases was monitored by withdrawing 2 mL aliquots and determining the nitrate-nitrogen content (section 3.3.10); prior to microbial enumeration (step 1 of section 3.3.2.A). All other measurements and analytical procedures follow phase one testing procedures.

3.3.3 Enumeration of Microorganisms

The heterotrophic plate count (HPC), formerly known as the standard plate count was used to monitor the number of viable heterotrophic bacteria during batch experiments

using the spread plate method (method 9215C) from Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WPCF, 1998).

First, an agar media suspension was prepared by thoroughly mixing 18.2 g of a Difco™ R2A Agar powder in 1 L of distilled water and boiling it for 1 minute while stirring. The mixture was then autoclaved for 15 minutes at 121°C and 20 mL aliquots were transferred using disposable sterile plastic pipettes to 100x15 mm pre-sterilized, disposable plastic petri dishes inside a laminar flow biological hood. The plates were then turned upside down and allowed to cool overnight in the biological hood.

In order to dilute the 1 mL supernatant aliquots prior to plating, 9 mL of phosphate buffer solutions (pH 7.0) were added to disposable glass sample vials and autoclaved for 15 minutes at 121°C. Using a sterile plastic pipette 1 mL of the batch liquid sample was transferred to the vial and vortexed for 10 seconds, resulting in a 10^{-1} dilution. Serial dilution using the sterilized buffer solutions was used to produce dilutions up to 10^{-8}.

Two replicate plates were prepared by evenly spreading 0.1 mL of each dilution sample onto prepared petri dishes using a flame-sterilized, bent stainless steel rod. The plates were labeled with batch type, batch number, and dilution factor and placed in an incubator set to 28°C for 7 days. All colonies on plates containing 30 to 300 colonies were counted and averaged. The colony forming units (CFU) per mL of each batch liquid phase was determined by multiplying the average plate count by the dilution factor x 10 (since 0.1 mL of sample aliquots was used in each plate).

3.3.4 Surface Tension Measurements

The reduction in surface tension, which accompanies surfactant production at levels below their critical micelle concentrations, was used as an indicator for the relative concentration of biosurfactants produced. The surface tension of the filtered supernatant was measured using a Fisher Scientific Tensiometer. This device is based on the ASTM method D1331-89 (ASTM, 2006) that employs the du Noüy ring method for direct results with no calculations. The du Noüy ring method measures the force necessary to separate a platinum-iridium ring from liquid, and has an accuracy of ± 0.25 mN/m. The device was calibrated as instructed by the manufacturer and its accuracy checked by measuring the surface tension of distilled water at room temperature and comparing it to the reported value in the literature (72 mN/m) (Mulligan and Gibbs, 2004).

3.3.5 Biosurfactant Screening

In order to verify that the reduction in surface tension is not due to anything other than biosurfactants the filtered supernatants were screened according to the method described by Cassidy et al. (2002b). In this screening method, instead of direct measurement of biosurfactants, which is lengthy and expensive, the surface tension of filtered supernatants concentrated in a rotary evaporator was monitored.

For this experiment additional control batches were prepared for 0 and 45 days durations. The sample supernatant was filtered through a 0.45 μm filter (Fisherbrand 25 mm diameter sterilized mixed cellulose ester syringe filters) and its surface tension monitored while being increasingly concentrated in a rotary evaporator set at full vacuum and 90°C.

By concentrating the samples with a rotary evaporator other naturally occurring organic compounds, such as volatile organic acids and alcohols, that may have surfactant properties, are removed by means of thermal degradation and evaporation. Biosurfactants on the other hand, have high molecular weights (Desai and Ibrahim, 1997) and are stable up to 100°C (Miller, 1995b; Cassidy et al., 2002b).

3.3.6 Critical Micelle Dilution

The concentration of the biosurfactants above their CMC levels was measured using critical micelle dilution (CMD) according to the method described by Hudak (2004). As explained earlier, at concentrations above the CMC, surface tension remains constant, and increases only when the surfactant concentration drops below the CMC by means such as, dilution, precipitation, or biodegradation. The CMD method quantifies the dilution required to bring the surface tension of the filtered supernatant above its CMC level. Distilled water was added in 5 mL increments to filtrate until the surface tension increased above its CMC levels. The CMD was the representative dilution factor.

3.3.7 Extraction Procedure

A. Petroleum Hydrocarbons in the Supernatants

The amount of petroleum hydrocarbons in the supernatant was measured using the EPA standard method 1664A (n-hexane extractable material) with slight modifications (EPA, 1999a). This method uses n-hexane and a separatory funnel to extract relatively non-volatile hydrocarbons from water, wastewater, and aqueous wastes.

The following is a summary of the procedure used. For extraction, 20 mL of the filtrate (see section 3.3.2.A.8) and 10 mL of Optima® n-hexane (Fisher Scientific, meets ACS

specifications) were transferred to a 60 mL Erlenmeyer separatory funnel. The funnel was vigorously shaken for two minutes, with frequently venting to release vapor pressure. The mixture was then allowed to settle for 10 minutes until the two phases were completely separated. The water was then eluted through the stopcock into a 100 mL Erlenmeyer flask and the process repeated three times. The collected hexane was dried over 10 g of sodium sulfate (granular anhydrous ACS grade) to remove residual water, and filtered thorough a Whatman No. 40 filter. The extracts were then brought to a total volume of 30 mL, transferred to a 40 mL amber vial, and stored at 4°C until the time of analysis by gas chromatography (GC). Liquid/liquid extraction blanks (only distilled water with hexane in a separatory funnel) were prepared according to the standard method and extracted using the same procedure as above.

B. Petroleum Hydrocarbons Remaining in the Soil

Because of the attachment of petroleum hydrocarbons to soil and the adherence of soil particles and petroleum hydrocarbons to the interior of the Erlenmeyer flask walls, a method was developed to measure the oil remaining in the soil. This method involved solvent extraction (n-hexane) of the remaining soil and supernatant (approximately 39 mL of supernatant and 2 g of soil) inside the Erlenmeyer flasks. The procedure involved:

1. The filter that was used to in section 3.3.2.A.3 to separate supernatant and soil particles was opened and placed in a 400 mL beaker.

2. The funnel and pipette that was used to in section 3.3.2.A.3 were washed with 30 mL of n-hexane (Optima®) and the hexane used was collected in the beaker containing the filter.

3. The beaker was swirled for 30 seconds and the hexane was poured into the Erlenmeyer flask.

4. The flask was sealed with a Teflon stopper and vigorously shaken for 5 minutes with frequently venting to allow the fumes to escape.

5. The solution was allowed to settle for 10 minutes to allow hexane and supernatant to separate.

6. The hexane was pipetted off as much as possible without removing any of the supernatant and collected in 50 mL Pyrex glass centrifuge tubes.

7. This was repeated twice more and the three hexane extractions were collected into centrifuge tubes.

8. The tubes were centrifuged at 3000 rpm for 10 minutes to break all of the emulsions formed during the extraction and separate hexane and water phases.

9. The hexane/petroleum hydrocarbon extracts were dried over 10 g of sodium sulfate (granular anhydrous ACS grade) to remove residual water and filtered thorough a Whatman No. 40 filter.

10. The extracts were then brought to a total volume of 90 mL in a graduated cylinder, transferred to 40 mL amber vials and stored at 4°C until the time of analysis by gas chromatography (GC).

Extraction blanks were prepared according to the EPA standard method 1664a (EPA, 1999a) and extracted as explained above. Furthermore, prior to extraction of experiment batches the extraction efficiency and repeatability of the developed method was evaluated. To determine the extraction efficiency, soil and supernatant were washed with 30 mL of hexane a fourth time in the centrifuge tubes and the extract was analyzed with GC. The fourth extract gave the same chromatograph reading as a pure n-hexane therefore it can be assumed that all of the hexane extractable material was removed during the first three extractions. Repeatability was determined by preparing, extracting, and then comparing two separate trial batches, and was calculated to be within ± 4%.

3.3.8 Gas Chromatography Analysis

Hexane extracts of the samples, blanks, and controls were analyzed based on EPA standard method 8015C for nonhalogenated organics using GC/FID with modification (EPA, 2000a). Chromatographic separation was achieved using a DB-1 (J & W Scientific) high resolution capillary column coated with 100% dimethylpolysiloxane. The column was 30 meters long with an inner diameter of 0.25 mm and a 0.25 μm film thickness.

A. Chromatography Data Acquisition

After preliminary studies the GC operational parameters that resulted in the best peak resolutions were selected for analysis of the batch hexane extracts, as follows:

1. Helium was used as the carrier gas at a constant flow rate of 2 mL/min and makeup gas flow rate of 30 mL/min.

2. Hydrogen gas and air flow rates were 30 mL/min and 300 mL/min, respectively in the FID detector.

3. Injector and detector temperatures were kept constant at 250°C.

4. 1 µL of the sample was injected to the column in the split/splitless mode.

5. The oven temperature program was set at 40°C for 1.00 minute, increased to 250°C at a rate of 8.0°C/min, and held at 250°C for 6.00 minutes (total run time of 33.25 minutes).

B. Data Handling

Quantitative analysis of acquired chromatograms was done using the Varian Star Chromatography Workstation Version 5.5 software. The cumulative area under the chromatogram, excluding the solvent peak, was selected as the total area for that sample. The TPH of each batch was calculated by subtracting the total area of the extraction blank from the total area of each sample. The percentage TPH in the supernatant was calculated using equation (1):

$$TPH_x \text{ superantant (\%)} = \frac{5 \times TPH_{wx}}{TPH_{sx} + 3.05 \times TPH_{wx}} \times 100\% \qquad (1)$$

where TPH_{sx} and TPH_{wx} are the TPH remaining in the batch and mobilized in the supernatant after x days and extracted from them as explained in the sections 3.3.7.B and

3.3.7.A, respectively. The numerator was multiplied by 5 since only 20 mL of supernatant was extracted from a total batch liquid phase of 100 mL. The factor 3.05 arises from the fact that 61 mL of supernatant were removed from the batches, but only 20 mL were used for TPH extraction.

The percentage TPH remaining in the batch was calculated using equation (2):

$$TPH_x \text{ Remaining } (\%) = \frac{TPH_{sx} + 3.05 \times TPH_{wx}}{TPH_{si} + 3.05 \times TPH_{wi}} \times 100\% \qquad (2)$$

where TPH_{si} and TPH_{wi} are the initial (day 0) TPH remaining in the batch and mobilized in the supernatant , receptively for the same batch type. The TPH values were expressed as a percentage of the initial (day 0) TPH value because the chromatogram contained numerous peaks and their individual identification and quantification were not feasible and beyond the scope of this study.

For phase two of the experiment every detail of the procedure explained above was repeated except that the percentage TPH in the supernatant and remaining in the batch was calculated using equations (3) and (4), respectively:

$$TPH_x \text{ Supernatant } (\%) = \frac{5 \times TPH_{wx}}{TPH_{sx} + 3.15 \times TPH_{wx}} \times 100\% \qquad (3)$$

$$TPH_x \text{ Remaining } (\%) = \frac{TPH_{sx} + 3.15 \times TPH_{wx}}{TPH_{si} + 3.15 \times TPH_{wi}} \times 100\% \qquad (4)$$

where the factor 3.15 represents extraction of 20 mL of supernatant when a total of 63 mL was removed from the batch liquid phase.

3.3.9 Analysis of Mobilized Metals

The filtered supernatant was prepared by acid digestion using EPA standard method 3005A (EPA, 1992c). For this 40 mL of the aliquot were filtered (section 3.3.2.A.7), acidified with nitric acid (5 mL/L), and transferred to a 100 mL beaker. Digestion was carried out by adding 2 mL of concentrated nitric acid and 5 mL of concentrated hydrochloric acid for each analyzed metal to the beaker. The beaker was covered with a watch glass and placed on a hot plate until it boiled. When the volume had been reduced to about 30 mL, the beaker was removed and allowed to cool. The beaker walls and the watch glass were washed with distilled water and the sample was transferred and brought to a final volume of 40 mL using a graduated cylinder.

The concentrations of metals in the digest were determined by flame atomic absorption spectroscopy (FLAA). The standards, blanks, and samples were prepared according to EPA standard methods and complied with manufacturer recommendations. Certified SCP Science stock standards were used. The mass of metals in the supernatant (mass metal $_{Supernatant}$) was calculated by multiplying the FLAA reading by the volume of sample (40 mL) and 2.5 (40 mL of 100 mL supernatant was digested for analysis of mobilized metals). The total mass of metals in the batch (mass metal $_{Batch}$) was determined by multiplying the concentration of metals in the soil by the amount of soil in each batch. The determined concentrations of heavy metals in the supernatants were then expressed as a percentage of initial soil heavy metal, defined by the following formula (5):

$$Mobilized\ Metals\ (\%) = \frac{mass\ metal_{\ Supernatant}}{mass\ metal_{\ Batch}} \times 100\% \qquad (5)$$

3.3.10 Nitrate Measurement

For the second phase of the study the concentration of nitrate (NO_3^-) was measured in the sample supernatants using Hach test kits (TNTplus 835) and a Hach DR/2800 Spectrophotometer according to method 10206 (Hach, 2005). This method is capable of measuring nitrate-nitrogen (NO_3^--N) in the range of 0.23 to 13.50 mg/L. For nitrogen concentration above the detection limit, sample supernatants were diluted using de-ionized water. Reagent and sample blanks were prepared as described by the method and the values were subtracted from the original sample readings to give the corrected sample concentration. The accuracy of the results was checked by measuring the nitrate-nitrogen concentration of a 10 mg/L NO_3^--N standard, as recommended by the manufacturer. Since nitrate-nitrogen was the sole added nitrogen to the nutrient amended batches, and the original or control batch levels of nitrate-nitrogen were insignificant, monitoring the nitrate levels was the only method for nitrogen used in this study.

3.4 Statistical Analysis

A single factor analysis of variance (ANOVA) was conducted on the initial and final concentrations of mobilized metals and TPH. Single factor ANOVA estimates the possibility (p- value) that the variations in a data series are by chance and compares it to a given acceptable error or critical value "α". If the p-value found is less than the critical value, then the effect is said to be significant at that probability level and implies that the means differ more than would be expected by chance alone. When the p-value is bigger than α, the effects are not considered significant. The critical value was selected as 0.05, or at the 95% confidence level.

4 EXPERIMENTAL RESULTS AND DISCUSSION

4.1 Soil Characterization Results

This section describes the soil type as well as some physical and chemical characteristics of the soil used in this study. The nature and extent of the contaminants will be examined as well.

4.1.1 Amorphous Content

The total amorphous content of the soil was 12.9%. The individual contents of amorphous iron (Fe_2O_3), silicon (SiO_2), and aluminum (AI_2O_3) were 84.3 ± 1.8, 26.1 ± 2.2, and 18.9 ± 1.4 g/kg, respectively.

4.1.2 CEC, pH, and Moisture Content

The cation exchange capacity (CEC) of the soil was determined as 8.2 meq/100g. The soil thus exhibits a moderate to low capacity to retain exchangeable cations. Soils with high cation exchange capacity can potentially have significant exchangeable metal cation content; especially for more soluble species, such as copper. However, since the majority of metals are generally quite insoluble within usual soil pH ranges, the exchangeable metal component may not be significant in soils heavily contaminated with metal unless the CEC is extremely high. Soil pH should also be considered in this respect since metal compounds tend to be more soluble at lower soil pH.

The average soil pH was found to be 7.4 ± 0.3. This soil has a neutral pH, and enhanced solubility of metallic compounds and complexes in this soil would not be an important consideration. Soils that are heavily contaminated with metals and that have a low CEC and neutral pH could be potential candidates for remediation involving biosurfactant, since biosurfactant-mediated enhanced metal mobility under controlled flush and pump conditions. Soil pH is also an important consideration in bioremediation projects since different microbial populations prefer different soil pH (Cookson, 1995). As mentioned earlier in this text, fungi prefer acidic soil whereas bacteria prefer neutral soil. In this case the soil pH is ideal for the maintenance of bacterial growth. Since bacteria are the most productive microorganisms with respect to petroleum hydrocarbon biodegradation, the pH of this soil is suitable for bacterial remediation (Maier et al., 2000).

The air-dried and oven-dried moisture content of the samples was 2.4 and 6.1%, respectively. Air-dried soil moisture levels are always lower than oven dried values since some moisture is usually tightly bound within soil pores. The specific moisture content of this soil is not important since this sample may bear no similarity to its current in-situ soil condition. However, moisture content in general is an important consideration in any bioremediation project (Eweis, 1998). Sufficient soil moisture must be maintained so as to promote microbial growth; however, saturated soil conditions limit oxygen transfer within the soil substrata and are to be avoided during periods of active microbial biodegradation during the remediation procedure (Maier et al., 2000). Although soil particles were completely saturated in batch tests in this study, air transfer and the continuous shaking of flask contents ensured sufficient oxygen levels.

4.1.3 Organic Matter Content

The total amount of organic matter in the soil by the weight loss on ignition method (dry method) was determined to be 22.3%. Using the wet method (digestion by hydrogen peroxide) the organic content of the soil was 14.3%. These two values contain the natural organic matter content plus the petroleum hydrocarbon contaminants of the soil because during both methods hydrocarbon compounds will be lost as well. The value obtained for each method differs greatly. The furnace method will completely remove all traces of organic matter and even possibly tightly bound water that was not removed during drying and generally gives higher values for organic matter than the peroxidation method (EPA, 2002). If we estimate the total organic matter by averaging these two values, there is about 18.3%. The organic matter content alone is not very meaningful without knowing the level of TPH contamination for this soil.

4.1.4 Heavy Metal Content

In terms of metal content the soil has considerable amounts of Cr (682 mg/kg), Cu (378 mg/kg), Pb (298 mg/kg), and Zn (751 mg/kg), while the concentration of Ni (59 mg/kg) and Cd (23 mg/kg) were quite low. Therefore, Cr, Cu, Pb, and Zn were selected for examination during this study. High levels of heavy metals in a soil with a fairly low CEC indicate that most of the cationic metals are non-exchangeable, since most exchange sites would be occupied with the more soluble naturally occurring soil cations, such as sodium, potassium, magnesium, and calcium. The insoluble metal contaminants, especially at this soil's pH and CEC, would tend to be fairly immobile.

4.1.5 Total Petroleum Hydrocarbon

The initial total petroleum hydrocarbon (TPH) content of the soil was 111 g/kg. Due to evaporation of the hydrocarbons during the homogenization step as well as occasional aeration as a result of handling, and potential consequent aerobic biodegradation the total petroleum hydrocarbon content was reduced to 110.4 g/kg by the end of the experiments. The difference accounts only for 0.5% of the initial TPH and will not affect the results because comparing to treatment TPH reductions it is negligible. The level of petroleum contamination is extremely high and accounts for about 11% of the total soil weight. This level of contamination along with the high level of metal contamination certainly qualifies this soil to be a good candidate for a remedial treatment study involving a co-contaminated soil.

4.1.6 Particle Size Distribution

Figure 4.2 shows the grain size distribution of the contaminated source soil. In addition, the grain size distribution for the same soil but without organic matter is shown. The removal was achieved by igniting the soil at 550 °C (section 3.2.5). For the contaminated soil about 79% of the particles were finer than 2 mm and only 9% passed through a 0.075 mm sieve (91% sand and 9% silt). However, this distribution does not reflect the soil particle distribution when the hydrocarbon contaminants are removed. The ignited soil contains 15% sand, 65% silt, and 20% clay; therefore, based on United States Department of Agriculture (USDA) texture classification, it is a silt loam when organic matter is removed (Soil Survey Division Staff, 1993).

Although the ignited soil is not in fact a natural, pre-contaminated, or pristine soil surrogate, since even naturally occurring humus was removed, it serves to illustrate how organic matter can cause soil particles to agglomerate into effectively larger grain size, thus dramatically affecting the grain size distribution differences and consequently properties of the soil. The TPH content of the soil was about 11%, while the average total organic content was about 18%. Thus the contribution to total organic matter by petroleum contaminants is considerable.

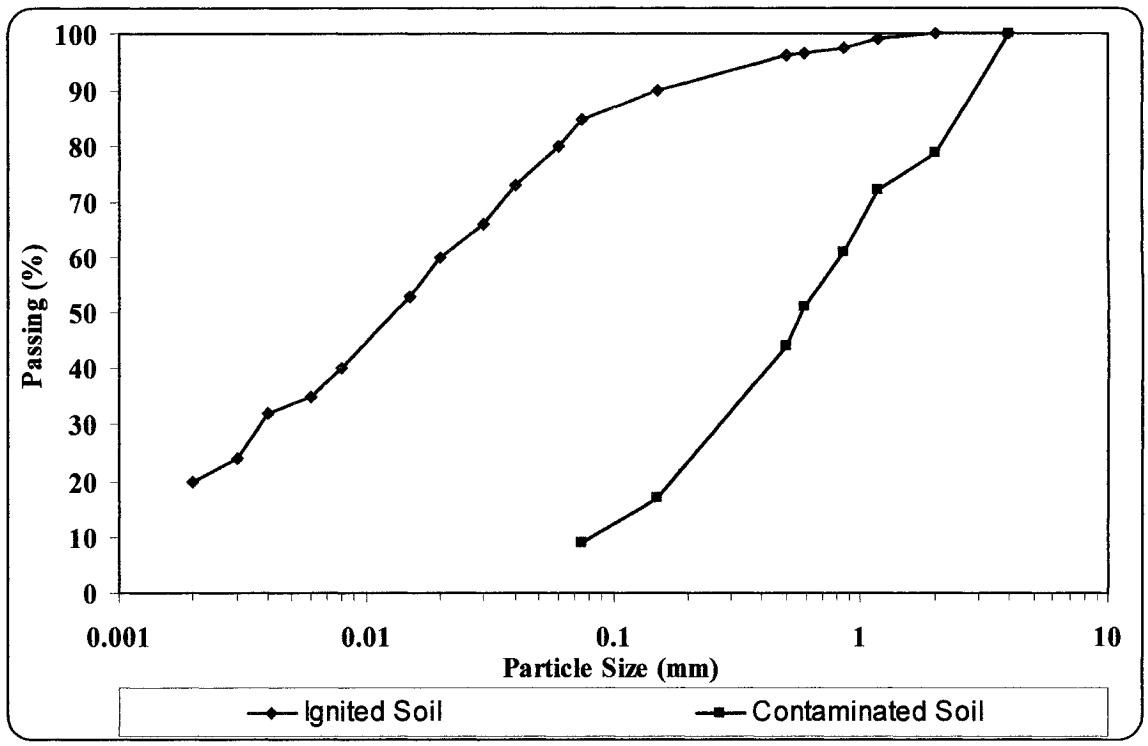

Figure 4.1: Grain size distribution of contaminated and treated soil sample.

Table 4.1 summarizes all of the above results for the contaminated soil's characteristics.

Table 4.1: Summary of the sample soil characterization.

Parameter	Value
pH	7.4
Moisture content, oven dried (%)	6.1
Moisture content, air dried (%)	2.4
Total organic matter, ignition (%)	23.3
Total organic matter, hydrogen peroxide (%)	14.3
CEC (meq/100 g)	8.2
Total petroleum hydrocarbons (%)	11.1
Cadmium (mg/kg)	23
Chromium (mg/kg)	682
Copper (mg/kg)	378
Lead (mg/kg)	298
Nickel (mg/kg)	59
Zinc (mg/kg)	751
Clay particles, source soil (%)	0
Silt particles, source soil (%)	9
Sand particles, source soil (%)	91
Clay particles, ignited soil (%)	20
Silt particles, ignited soil (%)	65
Sand particles, ignited soil (%)	15
Al_2O_3 (%)	1.9
Fe_2O_3 (%)	8.4
SiO_2 (%)	2.6

4.2 Trial Run Results

During the trial run it was found that the average water loss due to evaporation was 3 mL per week per batch reactor. Therefore, this amount was added to each flask to compensate for evaporation and maintain a soil to water ratio of 1:50. During the trial run it was also observed that the pH of the trail batches remained almost constant at about 7.5. Thus, in preparing the nutrient amended batches, solutions of monobasic and dibasic potassium phosphate were prepared with a K_2HPO_4: KH_2PO_4 ratio of 84:16. The pH of the solution was adjusted to prepare a buffer with a pH of 7.5. Detailed calculation for nutrients stock solution preparation and addition to the batches can be found in Appendix.

4.3 Phase One Results: Feasibility of Biosurfactant Production

As explained before, phase one tests investigated the feasibility of biosurfactant production by indigenous microorganisms of a co-contaminated soil in batch tests. During batch tests abiotic loss was measured in batch 'blanks' by suppressing microbial activity. In 'control' batches the natural growth of aerobic microorganisms and their ability to degrade hydrocarbon compounds and produce biosurfactants was evaluated. Finally, the potential of indigenous microorganisms to biodegrade and produce biosurfactant in a non-limiting environment was assessed by nutrient addition in 'nutrient amended' batches.

4.3.1 Microbial Enumeration

Figure 4.2 show the total counts of heterotrophic bacteria for the three batch types over time. The extremely low numbers of bacteria in the blank flasks confirm that addition of sodium azide not only inhibited metabolism but actually poisoned bacterial populations.

The total number of heterotrophic bacteria in these batches were less than 20 colony forming units per mL, which is similar to the method procedural blanks (distilled water added to petri dish) readings. On the other hand, control and nutrient amended batches showed similar, significantly greater initial microbial densities. Both control and nutrient amended batch bacterial populations exhibit an exponential growth phase within about the first 20 days after which both approach a stationary growth phase. However, the exponential phase is much more evident in the nutrient amended batch. Comparing the microbial enumeration data for control and nutrient amended batches shows that nutrients were limiting in the control batches, since microbial density spiked with the addition of nutrients. Microbial population densities in the nutrient amended batches were about 100 times more than control densities from about day 30 to the end of the experiment. Limiting environments are typical of hydrocarbon contaminated soils, because of the lack of basic nutrients in the PHs (Alexander, 1999; Atlas and Unterman, 1999; Lepo and Cripe, 1999; Gallego et al., 2001).

The onset of an exponential growth phase is characteristic of a situation where substrate is not limiting. Once substrate does become limiting, in relation to the increased demand, the population growth curve tends to become stationary, indicating no net growth. Subsequent to this phase microbial populations tend to decrease as an endogenous metabolic period ensues (Eweis, 1998; Maier et al., 2000). Without additional substrate input, the net growth is negative as the population dies off. The population density curve below would also indicate that the substrate had not yet been completely exhausted by the end of the testing period.

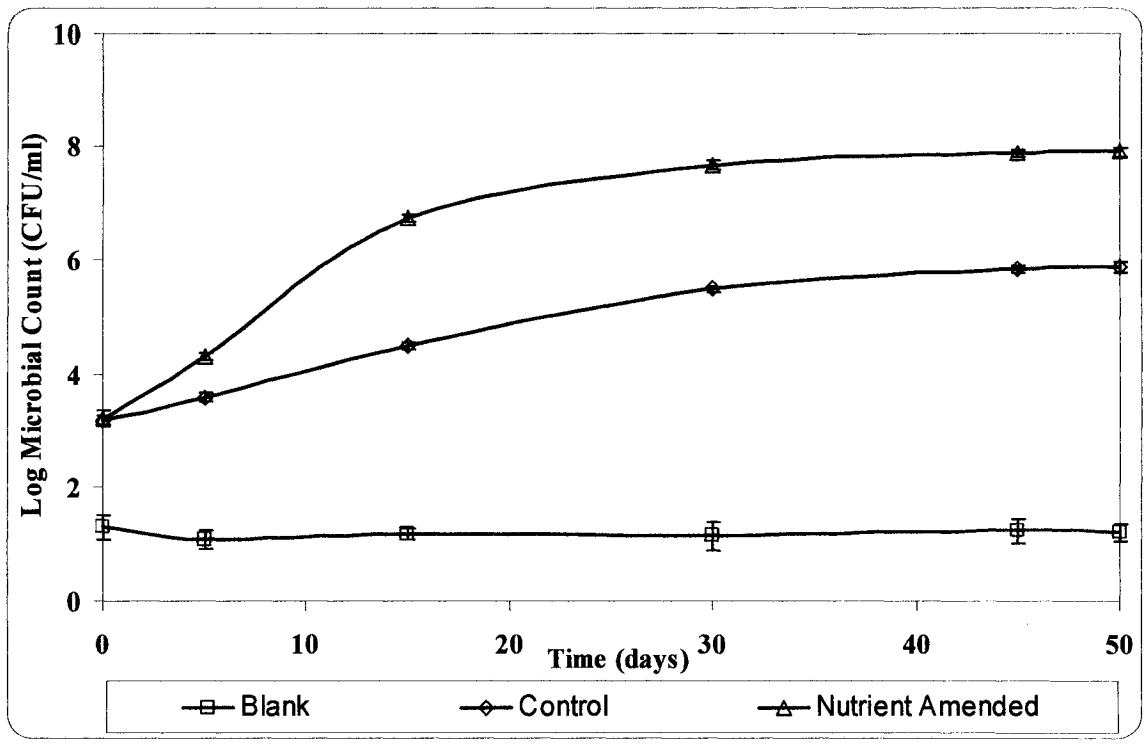

Figure 4.2: Heterotrophic microbial count for the three batch types over time.

4.3.2 Biosurfactant Screening

In order to demonstrate biosurfactant production, filtered supernatant from the control batches were concentrated in the rotary evaporator. Figure 4.3 shows the surface tension versus concentration factor of control batch filtered supernatants at two different times, 0 and 45 days. Surface tension of the filtered supernatants continually decreased with increasing concentration factor to a single minimum value. Initial sample surface tensions at time 0 and 45 days were at 65 and 61 mN/m, receptively, and decreased with almost identical slopes to a final constant value of 34 mN/m. Further concentration did not decrease the surface tension of the samples below this value.

This behavior is characteristic of surfactants in solution and indicates that the CMC for the surfactant was reached at about a 12-fold concentration of the original surfactant

levels. These results also indicate that there was already active biosurfactant production in the original soil sample (Cassidy et al., 2002a). In addition, it appears that biosurfactant production did not occur to any great extent in the control batch, since the surface tension at day 45 was not much less than at day 0. This is further discussed in the section on biosurfactant production below.

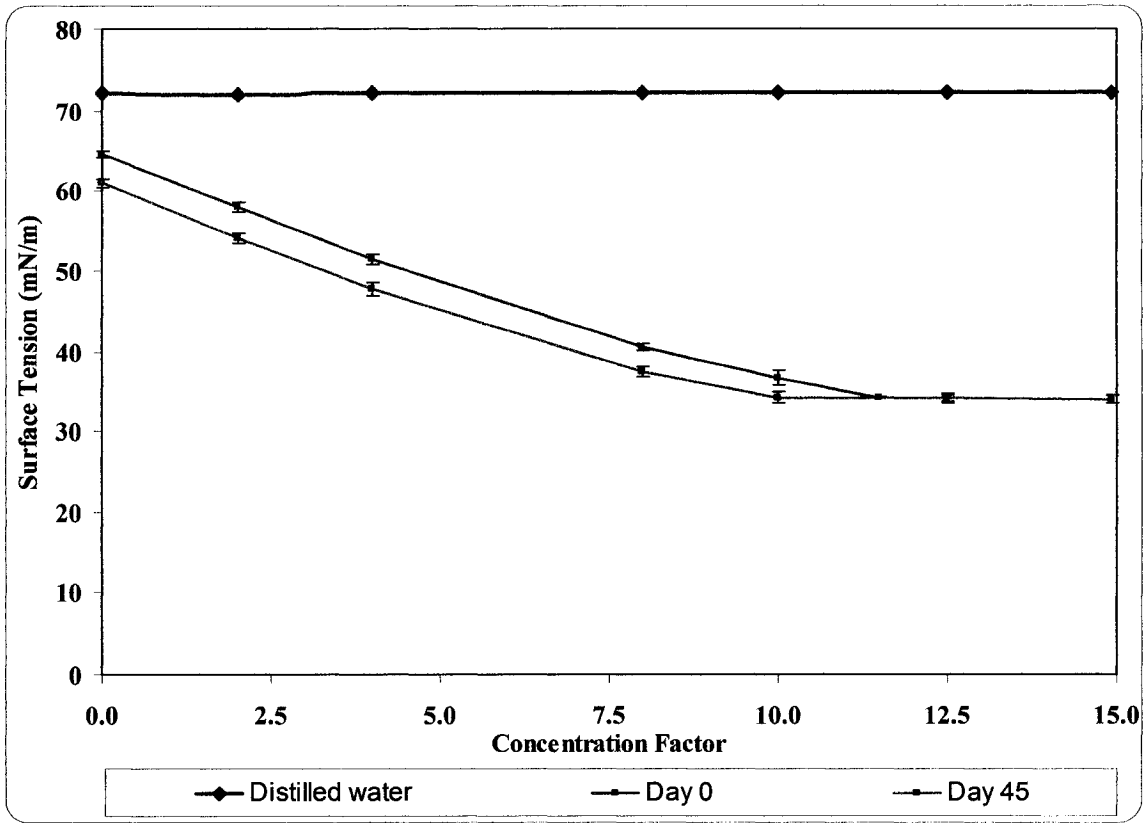

Figure 4.3: Surface tension vs. concentration factor for control batch filtered supernatants.

4.3.3 TPH Removal

Figure 4.4 shows the remaining TPH in the nutrient amended (NA), control, and blank treatment batches throughout the entire study period. Based on the initial (time 0) TPH value for the blank, the loss of TPH through abiotic processes was estimated at 7% after

50 days. The percent TPH removal in the control batches were significantly higher than the blank batches indicating the presence of hydrocarbon degrading microorganisms. Removal in the control batches increased to a final value of 22%. In NA batches the initial TPH removal rate was greater than that for the control batches. The maximum removal in the NA batches reached almost 43%, which is approximately twice the removal of the control treatment value.

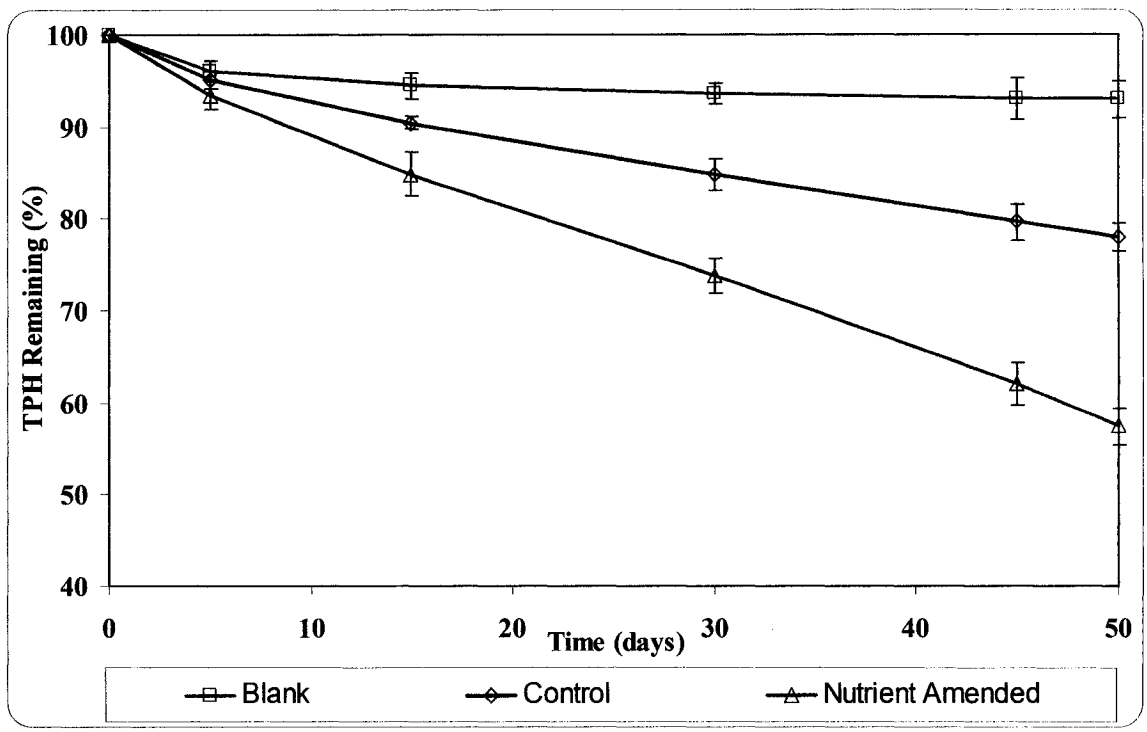

Figure 4.4: TPH remaining over time in blank, control, and nutrient amended batches.

The removal by biodegradation can be estimated by subtracting the abiotic loss from the total loss for each time period. This gives a final total loss through biodegradation for the control batch and the NA batch of about 15% and 36%, respectively. Figure 4.5 illustrates the percent loss through biodegradation for the control and NA treatment batches throughout the experiment.

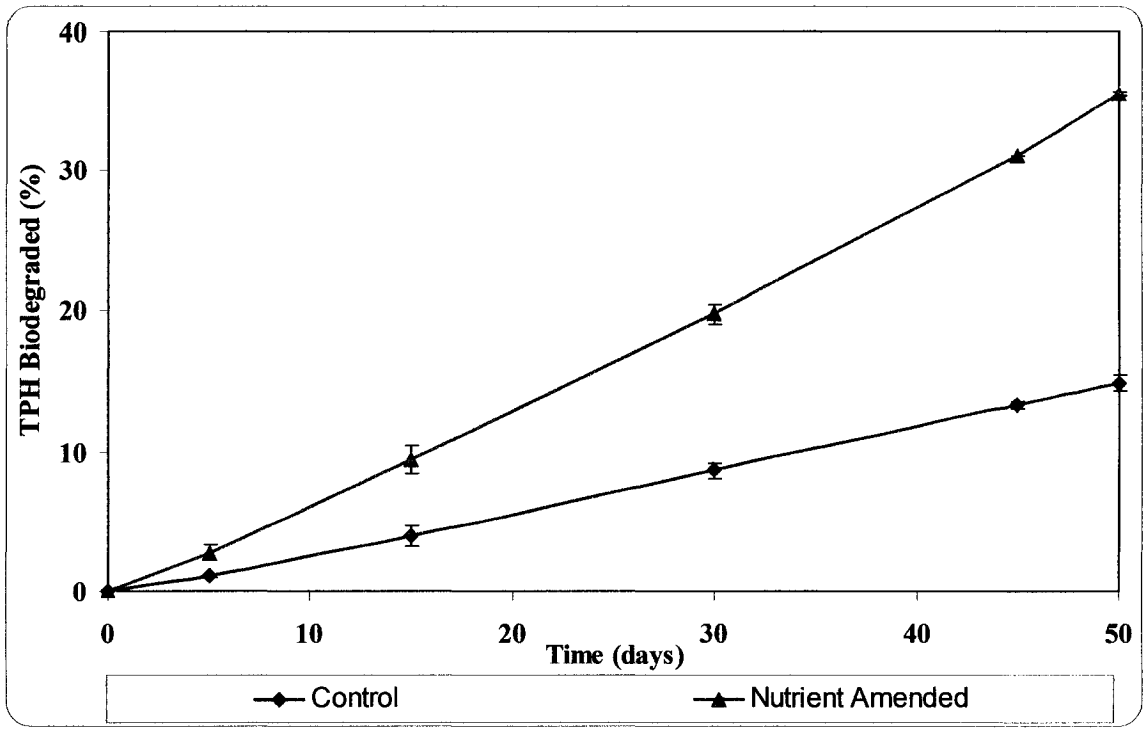

Figure 4.5: TPH removal by biodegradation in control and nutrient amended batches.

As mentioned in the trial run results, the pH values for the control batches remained between 7.4 and 7.6 throughout the experiment (Figure 4.6). The pH of the NA batches was buffered at 7.5 and did not change substantially during the test period. The pH value for the blank flasks ranged between 7.4 and 7.7. Thus, pH conditions were comparable between treatments and should not have differentially affected bacterial growth. The increase in TPH removal in the NA batch would seem to be result of increased biodegradation. Comparison of the microbial density for NA and control batches would validate this claim.

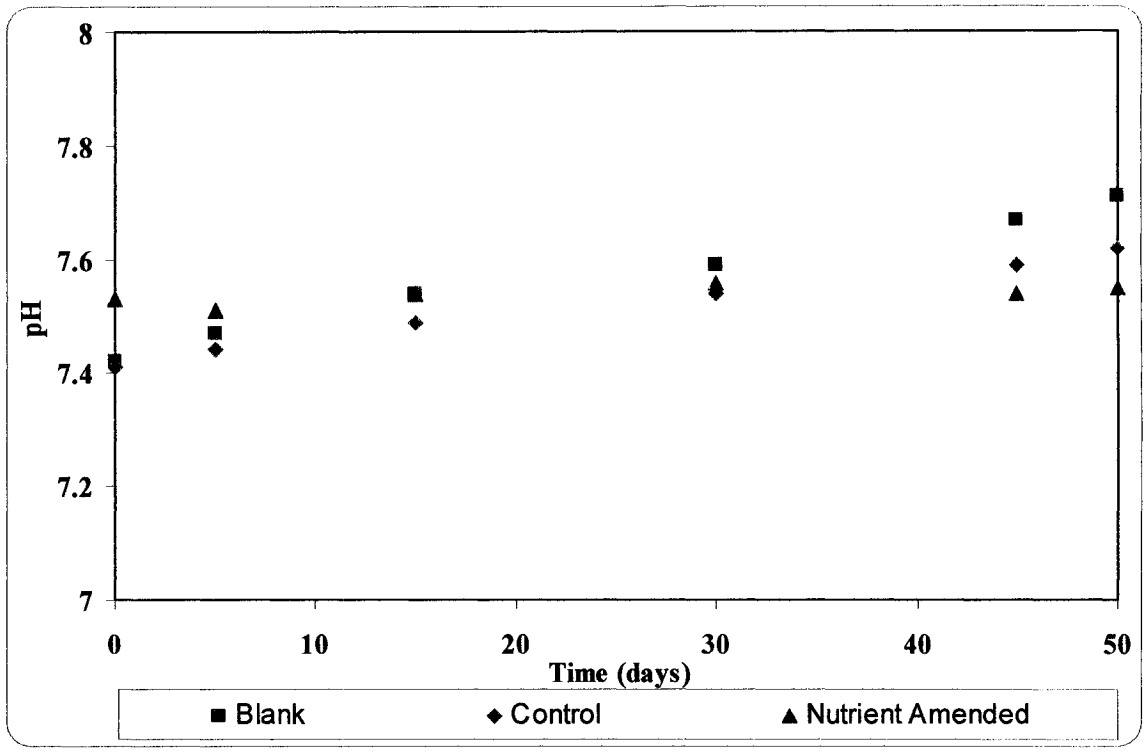

Figure 4.6: Variations in the pH of blank, control, and nutrient amended batches.

4.3.4 Biosurfactant Production

As mentioned earlier, concentration of biosurfactants in solution will be indirectly measured with changes in the surface tension (concentrations below CMC) and CMD (concentration above CMC) of sample filtered supernatant. Figure 4.7 shows the results for surface tension and CMD for each batch type over time. The initial surface tension of the sample solutions for all three batch types was about 64 mN/m, indicating that biosurfactant producing microorganisms are present and active in the source soil, as observed by other researchers (Miller, 1995b; Cassidy et al., 2002a; Bodour et al., 2003).

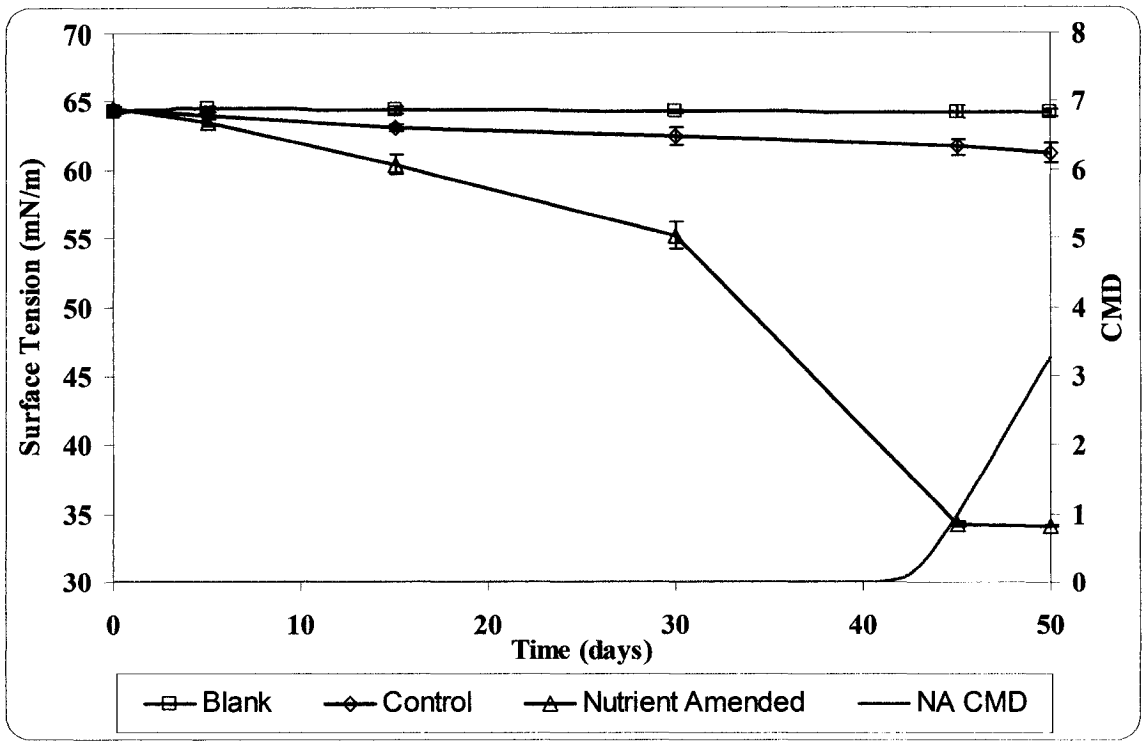

Figure 4.7: Surface tension and CMD values of filtered supernatant over time for all three batch types.

In blank batches the surface tension of the filtered supernatant did not change substantially during the experimental period, maintaining a value of about 64 mN/m. Surface tension for control batches gradually decreased to 61 mN/m from its initial value of 64 mN/m. NA batch supernatants decreased steadily from 65 mN/m to 55 mN/m within the first 30 days. Surface tension values dropped more abruptly after that to approximately 34 mN/m, over the next 15 days, and remained there for the last 5 days of the experiment. Since neither blank nor control batches saw substantial decrease in their surface tension, CMD determination was not applicable to them. In nutrient amended batches the CMD for the first 30 days was zero, increased to 1 by day 45, and reached 3.25 by the end of the experiment. The surface tension of filtered NA liquid phase dropped to a minimum value close to the one observed in the Biosurfactant Screening

(section 4.3.2). In addition, remaining of surface tension readings at its minimum value for the last 5 days indicated a non-zero CMD value; and is evidence that biosurfactants were present at above CMC levels.

The results clearly demonstrate that in nutrient amended batches biosurfactants were produced in concentrations greater than the CMC. An increase in the net biosurfactant production rate for the NA batch, which occurred at about day 30 and continued to the end of the experiment, coincided with the stationary phase for microbial growth (Figure 4.2). This would seem to indicate that as the microbial density increased, over that of the control batch, due to the effect of added nutrients, the substrate started to become more limiting, resulting in the growth-limited production of more biosurfactant in order to solubilize more petroleum hydrocarbons (Cassidy and Hudak, 2002; Cassidy et al., 2002a). It should be recalled that the microbial density for the NA batches was about 100 times that for the control. Little net biosurfactant production occurred in the control batches. It is important at this point to mention the distinction between production and net production when discussing biosurfactant levels in the aqueous phase, since they are subject to uptake and biodegradation themselves. Thus, when discussing surfactant levels plus surfactant-solubilized petroleum hydrocarbons substrate levels, the dynamic process of production and biodegradation must always be kept in mind.

Figure 4.8 shows the results for aqueous phase TPH levels during the course of the experiment in the filtrate batch supernatants. Initial aqueous TPH values for all treatments was very low, because the source soil was highly weathered, which means that the more soluble hydrocarbon compounds have been removed by biodegradation,

volatilization, and/or leaching (Ewies et al., 1998; Hudak, 2004). Concentrations of TPH in the filtrate of blank and control batches did not vary significantly over time. The concentration of TPH in the aqueous phase of nutrient amended treatment flasks started from an initial value close to that of the other two batch types; however, during the last 20 days of the experiment, the aqueous TPH concentration increased dramatically from 2.3% to around 8.3% (p-value = 0.014). Since a filter with an average pore size of 8 μm was used, the TPH in the filtrate could be made up of both a solubilized fraction as well as a colloidal fraction. Surfactants can render fairly insoluble petroleum hydrocarbon compounds more soluble, but it is also possible that some compounds may have been mobilized or carried into the aqueous phase with surfactant micelles, which will form when surfactant concentrations increase beyond their CMC (Diallo et al., 1994; Miller, 1995b; Deziel et al., 1996; Hayes, 1996; Mulligan, 1998; Page et al., 1999). These micelles would have at least partially passed through the filter used to prepare the filtrate, thus the filtrate TPH may contain emulsions and micelles with diameters smaller than 8 μm.

It is clear that the increase in surfactant levels would result in an increase in the solubilization and mobilization of petroleum hydrocarbons in the NA batch; which would make these generally insoluble compounds more readily available for bio-mediated degradation processes. The increase in the aqueous TPH is more evidence for enhanced biosurfactant production in the nutrient amended batches. Furthermore the pattern of aqueous TPH over time resembles the CMD time profile.

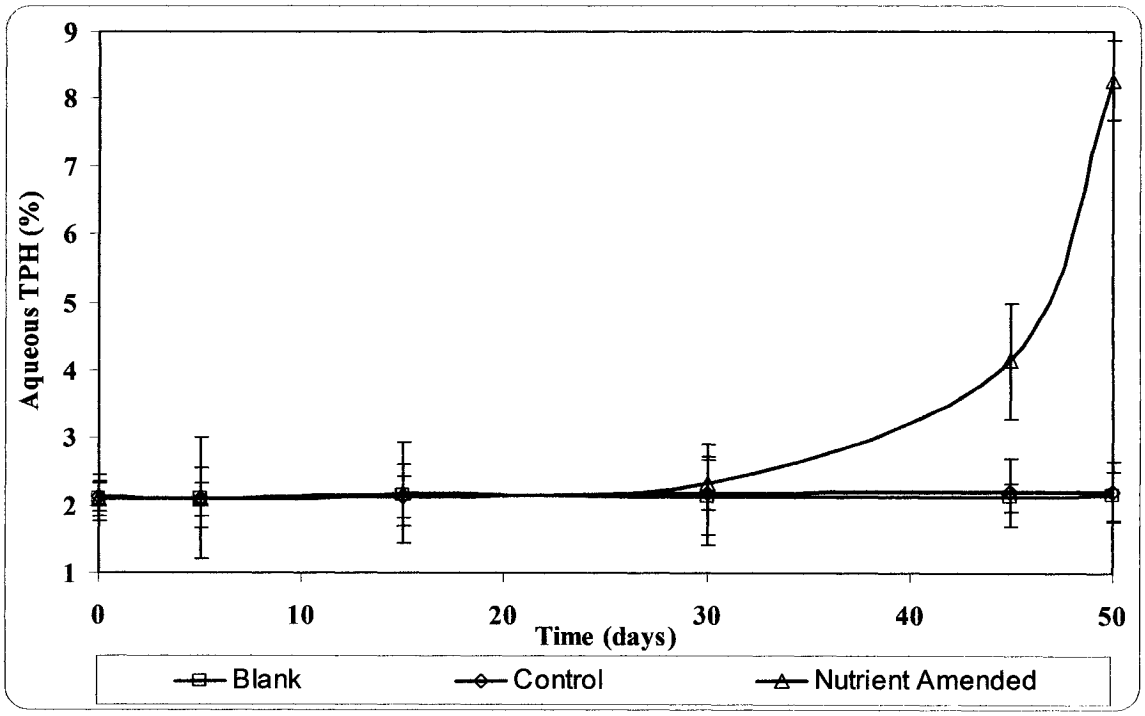

Figure 4.8: Average concentration of TPH in the batch filtered supernatants.

Figure 4.9 shows the percentage TPH biodegraded and mobilized into the liquid phase for the NA batch. Again the dynamic turnover of surfactant and petroleum hydrocarbon compounds should be considered when interpreting this graph. Even though little or no increase in mobilized TPH is evident within the first 30 days, the graph is actually documenting the net level of mobilized petroleum compounds, since biodegradation is taking place. The relative increase in aqueous phase TPH compounds seen after 30 days could imply that the rate of their mobilization into the aqueous phase is greater than their rate of biodegradation during this period. The onset of increased biosurfactant levels at day 30 would certainly cause a rise in aqueous phase TPH, and as evident by the increase in the biodegradation rate, an increase in the overall bioavailability to microorganisms. However, the increased rate of biodegradation was small comparing to the increase in the rate of mobilization, thus the mobilized TPH built up in the supernatant.

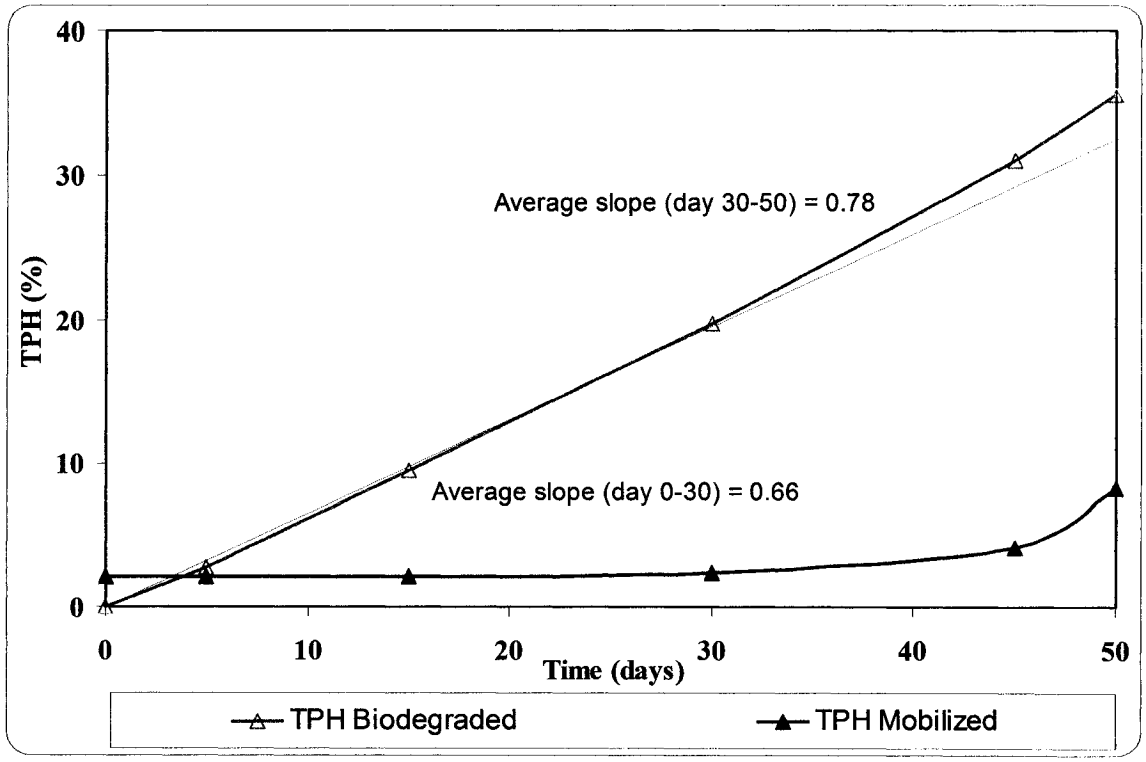

Figure 4.9: Mobilization and biodegradation of TPH in the nutrient amended batches over time and average biodegradation rates for 0 to 30 and 30 to 50 days.

Figures 4.5 and 4.9 reveal that the rate of biodegradation for nutrient amended treatment is not constant as it is in the case of the non-amended treatment. The percentage of TPH biodegraded over time was basically linear for the non-amended treatment with an average rate of 0.30% TPH per day for the 50 day treatment period (Figure 4.5). Whereas, the biodegradation rates for nutrient amended treatment started on average from 0.66% TPH per day in the first 30 days and reached an average of 0.78% TPH per day in the last 20 days. This increase can be attributed to the increase in the concentration of mobilized TPH that boosts the bioavailability of petroleum hydrocarbons, resulting in the increase of their biodegradation rates (Sabatini et al., 1995; Hayes, 1996; Volkering et al., 1998; Alexander, 1999; Zoller and Rubin, 2001; Cassidy et al., 2002a).

4.3.5 Mobilization of Metals

Figure 4.10 illustrates the change in the concentration of various metals in the filtered supernatants of nutrient amended batches over time. Initial concentration of metals in the filtered supernatant were low and in the order of zinc (1.1 mg/L), chromium (0.7 mg/L), copper (0.6 mg/L), and lead (0.2 mg/L), indicating that the soluble fraction of the metals have been removed by the extensive weathering the contaminants have undergone (Clevenger, 1990; Hong et al., 2002). The order of solubilized metals was similar to that observed in other studies. As explained earlier the mobility of metals primarily depends on their solubility in water and adsorption to soil particles. Zinc is most soluble and the least adsorbed metal among the metals studied making it the most mobile (Cameron, 1992; Herman et al., 1995; Hong et al., 2002; ATSDR, 2005c). Chromium and copper have moderate solubility but high adsorption to organic matter (Cameron, 1992; Barnhart, 1997; Hong et al., 2002; ATSDR, 2004, 2005b), while lead is almost insoluble, especially at neutral pHs. Lead also is very strongly adsorbed to soil making it the least mobile metal evaluated in this study (Cameron, 1992; Herman et al., 1995; Mulligan et al., 1999; Hong et al., 2002).

The levels of mobilized metals in the blank and control batches remained almost constant during the experiment. The metals mobilized into the liquid phase for the nutrient amended batches also remained at the same level as that for the blank and control batches for the first 30 days of the experiment. However, the concentrations of copper, zinc, chromium, and lead increased by a factor of 2.3, 2.1, 1.8, and 1.3, respectively in the NA batch liquid phase subsequent to this. Although relative increases in aqueous phase metal content averaged about 100%, the average relative changes were from about 2% of total

soil metal to about 4%. Since the actual levels of metals were low, an ANOVA test was performed to judge their significance. The p-values from a single factor ANOVA are presented in the Table 4.2. As stated previously, only if p-values are smaller than $\alpha = 0.05$ the differences are considered significant.

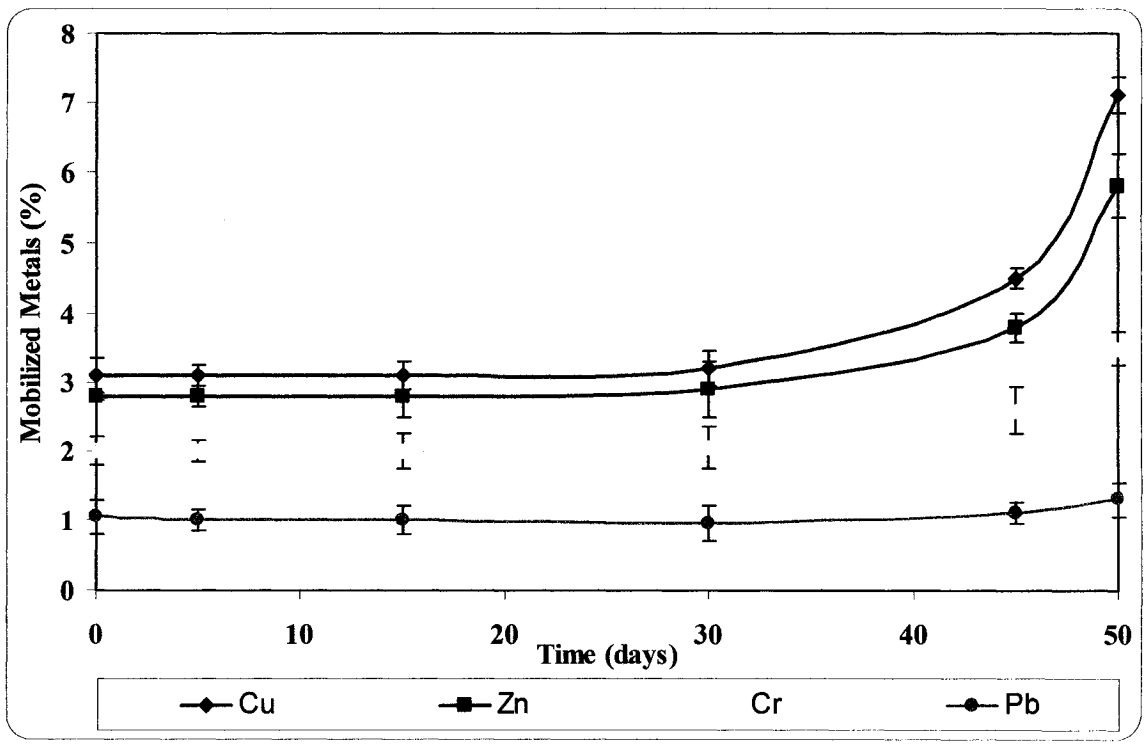

Figure 4.10: Time profile for the concentration of metals in the filtered supernatants of nutrient amended batches.

Table 4.2: Calculated p-values from the ANOVA single factor significance test for the initial and final concentration of metal in the nutrient amended batches.

Metal	Cu	Zn	Cr	Pb
P-value	0.003	0.022	0.042	0.553

The ANOVA results indicate that the increase in concentrations of for Cu, Zn, and Cr were significant, but that for Pb was not. The spike in increased Cu, Zn, and Cr concentrations matched an increase in the rate of net biosurfactant production (Figure 4.7). These increases are believed to be due to mobilization into the liquid phase by the action of biosurfactant. Again, the term mobilization is used instead of solubilization since the filtrate used to test for metals was prepared using a filter with an average pore size of 8 μm, and although solubilization may have been the mechanism involved, the occurrence of mobilized metallic colloids or metallo-surfactant micelles cannot be ruled out. The fact that lead was not significantly mobilized into the liquid phase is not surprising given that it is considered a recalcitrant metal due to its extremely low solubility, especially at around neutral pH (Cameron, 1992; Herman et al., 1995; Hong et al., 2002; ATSDR, 2005b).

4.4 Phase Two Results: Enhanced Biosurfactant Production

Phase two of this study evaluated the effect of stimulating biosurfactant production by nitrogen limitation on bioremediation of the sample soil. Batches were initially supplemented with inorganic nitrogen (nitrate) and phosphorous (phosphate) within the first 20 days, whereupon, only phosphorous was provided, resulting in subsequent nitrogen limitation conditions. The nitrate and phosphate supplement schedule for phase two of this study is in Appendix.

Figure 4.11 shows surface tension, nitrate-nitrogen concentration, microbial density, and CMD profile for the batch liquid phase over time. The microbial density increased greatly up to day 20, during the same time period nitrate-nitrogen levels decreased steadily from

an initial concentration of 91.4 mg/L. In the same time period surface tension decreased slowly, signifying net production of biosurfactant. At around day 20 the nitrate-nitrogen concentrations decreased to below detection and the microbial population showed no net gain (stationary growth phase); furthermore, surface tension exhibited a sharper decline up to day 25 where it reached its minimum value indicating that the CMC for biosurfactant had been reached or surpassed. This suggests that, as in the phase one, of the study the microorganisms in the system are producing biosurfactant under growth-limiting conditions. The CMD value for day 25 indicated that the CMC had been surpassed by a factor of almost 2. The nitrate-nitrogen levels remained below detection until the end of the experiments at day 30, where the CMD was greater than 4.

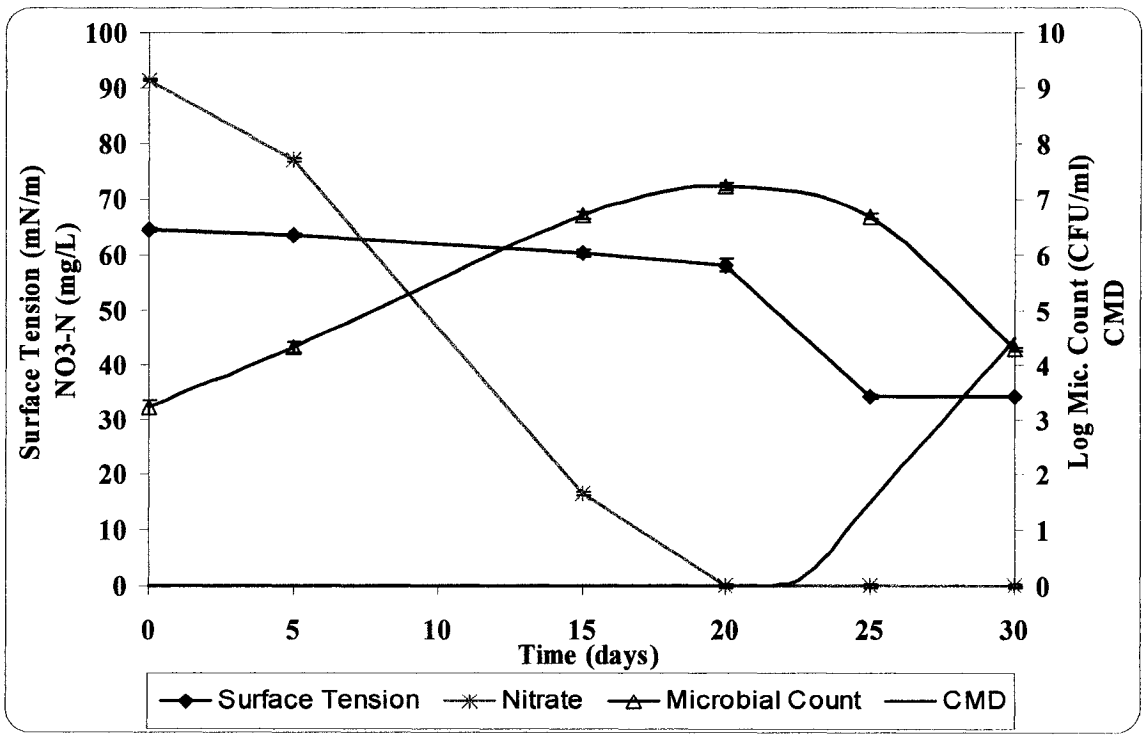

Figure 4.11: Time profile of surface tension, nitrogen-nitrate, microbial population, and CMD of the filtered supernatants of phase two patches.

The phase one nutrient amended results and the phase two nitrogen limiting (NL) results display some interesting differences. Both microbial densities reached levels of about 10^7 CFU/mL by day 20, but as nitrate-nitrogen levels reached nil in the NL batches a decline in microbial density occurred therein. The NA batch's microbial density had just about reached a stationary growth phase by this time, which was maintained to the end of the experiment. Nitrogen levels were maintained throughout the test period for the NA batch. Increase in net biosurfactant production in the NL batch, compared to that for the NA batches, is evident upon examination of the respective CMD values. In NA batch the CMD for the first 30 days was zero, increased to 1 by day 45, and reached 3.25 by the end of the experiment. In the NL batch the CMD reached a value greater than 1 in just 25 days and had a final CMD of 4.5 by the end of the experiment on day 30. This result indicates that nitrogen limitation followed by a period of microbial growth stimulates biosurfactant production and achieves higher levels of biosurfactant in a shorter period of time (Cassidy and Irvine, 2000; Cassidy et al., 2002a; Yateem at al., 2002; Hudak and Cassidy, 2004). Moreover, because of reduced microbial population as well as nutrient limited conditions of the media, the produced biosurfactants will be less subjected to microbial biodegradation; resulting in their long-term stability (Hudak and Cassidy, 2004).

Figure 4.12 illustrates the percent of TPH mobilized into the liquid phase and removed over time. Taking into account that the microbial density increased and nitrate-nitrogen decreased, the reduction in TPH indicates aerobic biodegradation. If we subtract the blank abiotic TPH loss factor from the previous batch tests, an estimated 12% of the removal by day 30 will be the result of biodegradation. If we compare this to the

estimated percent TPH biodegraded in the NA batch at day 30 of about 19% it would indicate that biodegradation of petroleum hydrocarbons was slowed down by the removal of nitrogen from the system. Indeed the cessation of TPH removal from day 20 onward in the NL batch is evident in Figure 4.12. This is plausible if not expected since the microbial density on day 20 was about the same for both but then decreased from day 20 to day 30 in the NL batch, due to nitrogen depletion, but not in the NA batch.

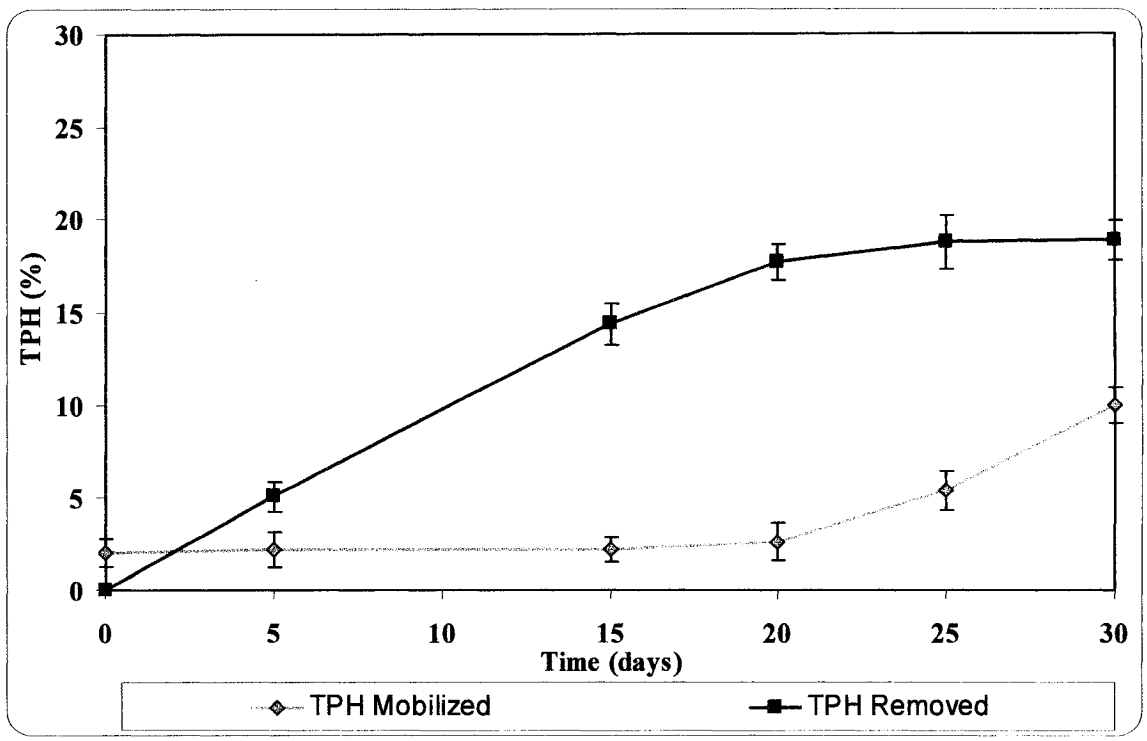

Figure 4.12: The percentage of mobilized and removed TPH over time for the phase two batches.

As in the NA batches the increase in the concentration of aqueous phase biosurfactants increased the concentration of mobilized TPH in the filtered supernatants. Initial aqueous TPH levels of 2% in NL batches were comparable to that for NA batches and reached a level of 10% by day 30. Results of a single factor ANOVA test show that the final concentration of TPH in the supernatant is significantly different than the initial

(p-value = 0.025). By day 30 the liquid phase TPH levels were not much more than initial values in the NA batches, but reached levels of about 8% by day 50. Thus it would appear that the NL batch was able to generate higher levels of liquid phase TPH within a shorter time frame due to the earlier onset of biosurfactant production at elevated levels.

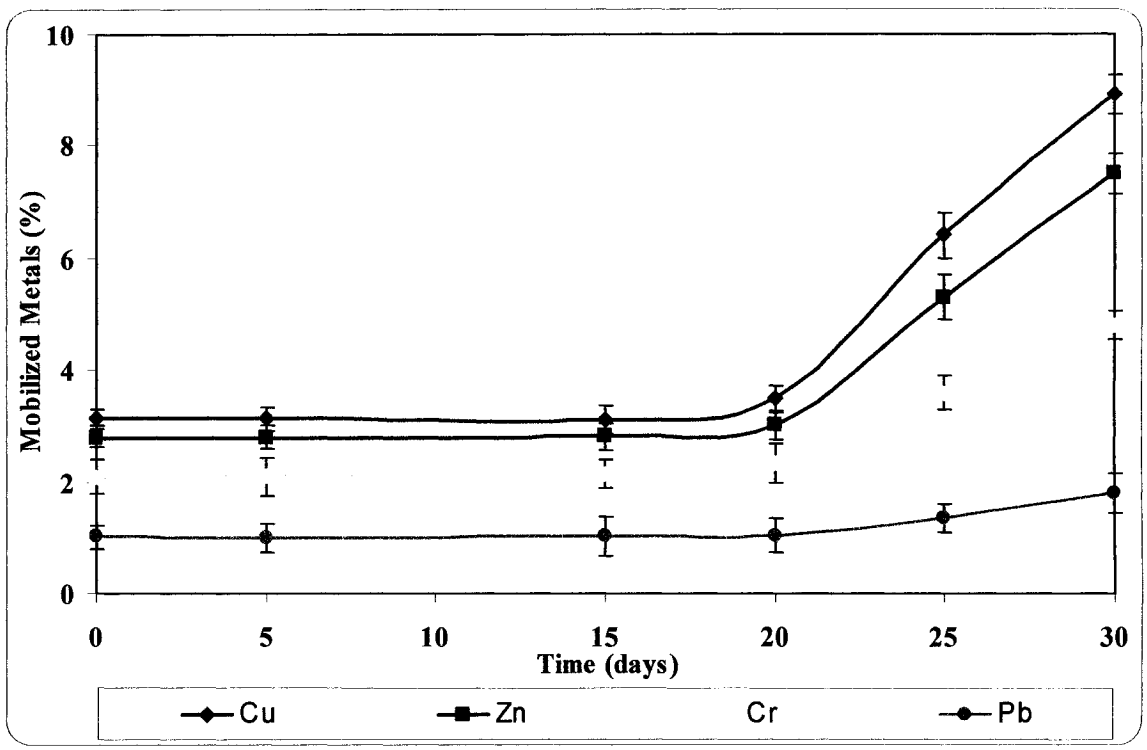

Figure 4.13: Changes in the concentration of metals in phase two experiments over time.

Figure 4.13 shows the concentrations of metals mobilized into the liquid phase over time for the NL batch. Cu, Zn, Cr, and Pb concentrations in the aqueous phase increased by a factor of 2.9, 2.7, 2.3, and 1.8, respectively; however, after single factor ANOVA calculation (Table 4.3) it was determined that lead's final concentration is not significantly different than its initial concentration.

Table 4.3: Calculated p-values from the ANOVA single factor significance test for initial and final concentration of metals in phase two.

Metal	Cu	Zn	Cr	Pb
P-value	0.003	0.006	0.020	0.185

This result again suggests that the biosurfactant production is associated with an increase in the aqueous phase metal contents of Cu, Zn, and Cr. The average increase factor for the three metals was 2.6 for the NL batch and 2.0 for the NA batch. Under NL conditions these higher metal levels were found after 30 days compared to 50 days for the NL batches. Finally, the removal of Pb from co-contaminated soil may prove to be problematic under these conditions using biosurfactant alone.

4.5 Discussion

The initial stated goal of this study was to advance current knowledge in the area of soil remediation by investigating in-soil production of biosurfactant by indigenous microbial populations in co-contaminated soils. Using a soil that was heavily contaminated with petroleum hydrocarbons and metals, phase one of this study examined the growth of the native microbial population, the biodegradation of petroleum hydrocarbons, the production of biosurfactant, and the mobilization of TPH and metals into the aqueous phase.

This study was performed on both soil and soil amended with nutrients (N and P). Both soils promoted microbial growth and displayed a characteristic initial rise in microbial density over time, followed by a more stationary growth phase. The microbial density of the nutrient amended soil however was about 100 times that of the non-amended soil

throughout the stationary phase. Certainly, this was expected because of high concentrations of petroleum hydrocarbons, which are known to be deficient in nutrients, making the whole environment nutrient limiting with respect to the available biodegradable material (Providenti et al., 1993b; Baker 1994; Lepo and Cripe, 1999; Gallego et al., 2001). Biodegradation of petroleum hydrocarbons was observed in both soils; however, the nutrient amended soil exhibited greater biodegradation for petroleum hydrocarbons, where 36% of TPH was biodegraded by the end of the experiments comparing to 15% for non-amended batches (Miller, 1995b; Head and Swannell, 1999; Gallego et al., 2001). Thus, with respect to maximizing microbial growth and biodegradation, nutrient addition is required; however, even without nutrient addition, the biodegradation of TPH was still feasible, although at a lower rate (Baker 1994; Rosenberg, and Ron, 1996; Alexander, 1999; Atlas and Unterman, 1999).

Net biosurfactant production was evident in both treatments, but was extremely low in the non-amended one; whereas, biosurfactant levels in the nutrient amended treatment reached concentrations greater that the CMC, having a CMD of 3.25 by the end of the experiment. Furthermore, the onset of the greatest increases in biosurfactant levels occurred at day 30, coinciding with the stationary growth phase of microbial population in nutrient amended batches. Thus, indicating that microorganisms produced biosurfactants under growth-limiting conditions, as is suggested by other authors (Cassidy and Irvine, 1997, 2000; Cassidy et al., 2000, 2002a; Cassidy and Hudak, 2001, 2002; Yateem et al., 2002; Hudak and Cassidy, 2004).

Also, the increase in biosurfactant levels was associated with a corresponding increase in petroleum hydrocarbons and metal mobilization into the liquid phase (Diallo et al., 1994; Miller, 1995b; Deziel et al., 1996; Hayes, 1996; Mulligan, 1998; Page et al., 1999; Ochoa-Loza et al., 2001; Hong et al., 2002). In the non-amended treatment batches, where biosurfactant levels remained low those levels remained almost constant. In nutrient amended batches the concentration of aqueous TPH quadrupled at the end of the day 50, while at the same time the average metal content in the filtered supernatant doubled. Furthermore, based on the results of this experiment as well as many other studies, the increase in aqueous TPH enhanced the bioavailability of petroleum hydrocarbons, resulting in increased biodegradation rate: from 0.66% TPH per day in the first 30 days of experiments to 0.79% TPH per day in the remaining 20 days (Sabatini et al., 1995, 1998; Hayes, 1996; Volkering et al., 1998; Alexander, 1999; Zoller and Rubin, 2001; Cassidy et al., 2002a).

The first phase of experiments thus demonstrated the feasibility of using indigenous microbial populations amended with nutrients to facilitate bioremediation of a co-contaminated soil through enhancing TPH biodegradation, and TPH and metal mobilization, through increased biosurfactant production. The second phase of this study investigated further enhancement of biosurfactant levels using nitrogen limitation.

Previous studies have shown that nitrogen limitation is the most effective way to increase biosurfactant production in pure cultures (Mulligan and Gibbs, 1989; Yateem at al., 2002). Nutrient amended batches were prepared and after a short growth period (20 days) batches were deprived of nitrogen. The microbial growth curve displayed a phase of

decreasing microbial density after reaching levels similar to those in the previously discussed phase one study, which is attributed to the increased nutrient requirement of the now greatly enhanced population. Once deprived of nitrogen the population, unable to meet its nitrogen requirement will decrease (Hudak, 2004; Hudak and Cassidy, 2004). It was during this declining phase that surfactant levels began to increase dramatically, as their own rate of biodegradation is reduced (Desia and Banat, 1997; Cassidy et al., 2002a; Hudak and Cassidy, 2004). By day 30 the CMD of the liquid phase was 4.5, which as in phase one was accompanied by increases in concentrations of mobilized TPH and metal contaminants into the liquid phase (Diallo et al., 1994; Mulligan, 1998; Page et al., 1999; Cassidy et al., 2000; Ochoa-Loza et al., 2001; Hong et al., 2002; Hudak and Cassidy, 2004). The rate for %TPH removal for the nitrogen limited treatment was somewhat linear up until day 20 when TPH biodegradation stopped. Again, this corresponded to the depletion of nitrogen in the aqueous solution below detection, and consequently the onset of the phase of microbial density decrease (Hudak and Cassidy, 2004). The maximum percentage of TPH biodegraded by this time was about 12%.

Limitation in nitrogen following growth of indigenous, mixed culture microorganisms in batch experiments enhanced the production of biosurfactants (Cassidy and Irvine, 2000; Cassidy et al., 2002a; Hudak and Cassidy, 2004). The maximum CMD level for the nitrogen limited batches was 38% higher than the maximum nutrient amended CMD. Moreover, the maximum levels of mobilized contaminants occurred on day 30 for the nitrogen limited treatment, while in the nutrient amended treatment it occurred after 50 days. The ratio of maximum average mobilized metals and TPH for two treatments (NL mobilized/NA mobilized) was 1.3 and 1.2, respectively. Indicating that the enhanced

mobilization of metal and TPH was directly related to the increase in CMD of the filtered supernatant, as observed by others (Mulligan, 1998; Cassidy and Irvine, 2000; Cassidy et al., 2002a; Hong et al., 2002; Hudak and Cassidy, 2004).

5 CONCLUSIONS

In conclusion, this study demonstrated the feasibility of biosurfactant production in co-contaminated soil in order to facilitate bioremediation. In the nutrient amended treatment study biosurfactant levels were elevated, biodegradation of TPH was enhanced, and TPH and metal contaminants were mobilized into the aqueous compartment.

- NA treatment removed 43% of TPH in the soil.
- Biosurfactants were produced in concentrations up to 3 times their CMC in NA batches.
- Produced biosurfactants increased the concentration of mobilized TPH and metals by a factor of 4 and 2, respectively.
- Increased concentration of TPH in the aqueous phase increased their biodegradation rate from 0.66 to 0.79% TPH per day.

The second study found that even greater biosurfactant levels could be achieved in shorter times using nitrogen limitation; which resulted in more mobilized TPH and metal contaminants. As well, since the microbial densities dropped and biodegradation effectively stopped, when nitrogen was depleted, the possibility of degradation of produced biosurfactants was greatly reduced.

- Nitrogen limitation produced higher concentration of biosurfactant in shorter time period.

- The maximum concentration of produced biosurfactants reached to more than 4 times their CMC concentrations.

- The produced biosurfactants increased the concentration of mobilized TPH and metals by a factor of 5 and 2.5, respectively.

- TPH removal at day 30 for NA and NL treatments were 26% and 20%, respectively.

The potential for remediation of a co-contaminated soil, based on biosurfactant production by soil indigenous microorganisms using organic pollutants as substrate and subsequent flushing and collection of mobilized contaminants, using the nitrogen limited method is promising. However, based on the percentages of mobilized metal and TPH observed in this study (which each amounted to about 10% of total), it is unlikely that complete remediation of a heavily co-contaminated soil could be achieved in one treatment cycle. Also, in co-contaminated soil where the actual mass of biodegradable contaminants is low, but which nonetheless represent a serious hazard due to their extreme toxicity, substrate addition in conjunction with nutrient addition may be required.

6 FUTURE WORK

Based on the results obtained the future studies should include:

- Evaluate longer experimental periods for nutrient amended batches to determine the maximum potential for biosurfactant production.

- Examination of the effects of pH, substrate addition, mixing, and other factors involved in microbial growth and/or biosurfactant production to optimize the rate and extent of biosurfactants production and contaminant removal.

- Investigate multiple cycle remediation of nitrogen limited studies to enhance metal removal from co-contaminated soils.

- Performing column studies to evaluate the potential of in-situ biosurfactant production by indigenous soil microorganisms and contaminant flushing.

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APPENDIX

Nutrient Addition

Each batch contained 2 g of air dried soil, with a TPH concentration of 11.1%. Therefore, each batch has 0.222 g of TPH.

$$1\ \text{Batch} \longrightarrow 2\ \text{g soil} \xrightarrow{11.1\%\frac{g\ TPH}{g\ Soil}} 0.222\ \text{g TPH}$$

Literature review suggests that the 80-90% of TPH is carbon (Alexander, 1999). If a level of 90% carbon is chosen then:

$$0.222\ \text{g TPH}\ \ 0.1998\ \text{g C} \approx 200\ \text{mg C}$$

Concentrations of nitrogen and phosphorous were selected based on ratios of:

$$\text{C:N:P} \xrightarrow{(Cookson, 1995)} 100{:}10{:}1$$

Nitrogen

Sodium nitrate ($NaNO_3$) was selected as the source of inorganic nitrogen.

$$\text{C:N} \longrightarrow 100{:}10$$

$$200\ \text{mg C} \xrightarrow{10:1} 20\ \text{mg N}$$

$$MW_{NaNO3} = 85.0\ \text{g/mol} \xrightarrow{\frac{14\,g\,N/mol}{85\,g\,NaNO3/mol}} \text{Fraction of nitrogen in sodium nitrate} = 16.5\%$$

$$1\ \text{Batch} \xrightarrow{require} 20\ \text{mg N} \xrightarrow[16.5\%\frac{g\ N}{g\ NaNO3}]{} 121.2\ \text{mg } NaNO_3/\text{Batch}$$

A stuck solution of 24.25 g sodium nitrate was added to a 1 L flask and filled to volume with distilled water. Each nutrient amended batch requires 5 mL of this stuck solution to achieve complete degradation of organic soil contaminants. However, single application of nutrient may be inhibitory for microorganism's growth (Walworth and Reynolds, 1995); therefore, for phase one experiments the stock solution was added in 1 mL increments at day 0, 15, and 40. The dates above were approximated from the Trial Run results and were fine tuned using trial and error to produce a non-limiting environment. For the second nitrogen limiting phase of the study only 1 mL of the stock solution (20% of the calculated required nitrogen) was added to batches at the start of the experiment.

Fraction of N = 24.25 g/L x 0.005 L = 0.1212 g $NaNO_3$ x 0.165 N / $NaNO_3$ = 0.2 g N

Phosphorous

Monobasic and dibasic potassium phosphates were added as the source of inorganic phosphorous. To account for the precipitation of phosphorous compounds to soil particles (Walworth and Reynolds, 1995) the amount of phosphorous added was doubled:

$$C{:}P \longrightarrow 100{:}1$$

$$200\ \text{mg C} \xrightarrow{100:1} 2\ \text{mg P} \xrightarrow{\times 2} 4\ \text{mg P}$$

A mixture of monobasic and dibasic potassium phosphates was also used as a buffering solution, having a pH of 7.5. For this, solutions of equal molarity (0.026 M) monobasic

and dibasic potassium phosphates were mixed with K_2HPO_4:KH_2PO_4 ratio of 84:16 (v/v) (total volume of 200 mL) and final pH was adjusted of 7.5

$$MW_{K2HPO4} = 174.2 \text{ g/mol} \xrightarrow{\frac{31 g\,P\,g/mol}{174.2 g\,K2HPO4\ g/mol}} \text{Fraction of phosphorous in monobasic potassium phosphate} = 17.8\%$$

$$MW_{KH2PO4} = 136.1 \text{ g/mol} \xrightarrow{\frac{31 g\,P\,g/mol}{136.1 g\,KH2PO4\ g/mol}} \text{Fraction of phosphorous in dibasic potassium phosphate} = 22.8\%$$

The prepared stock solution (V = 200 mL) containing 761 mg K_2HPO_4 and 113 mg KH_2PO_4. Again, 5 mL of the solution will be required for total degradation of all organic compounds in the soil. For both phases of the study inorganic phosphorous was added in the same manner as nitrogen was supplemented in phase one.

$$\text{Mass}_{(K2HPO4)} \text{ in solution} = 2 \times 84 ml \times 0.026 \frac{mol}{L} \times 174.2 \frac{g}{mol} = 761 \text{ mg}$$

$$\text{Mass}_{(KH2PO4)} \text{ in solution} = 2 \times 16 ml \times 0.026 \frac{mol}{L} \times 136.1 \frac{g}{mol} = 113 \text{ mg}$$

$$5 \text{ mL solution} \xrightarrow{\frac{5}{200}\left(761 mg\,K2HPO4\,x\,17.8\%\frac{g\,P}{g\,K2HPO4} + 113 mg\,KH2PO4\,x\,22.8\%\frac{g\,P}{g\,KH2PO4}\right)} 4 \text{ mg P/Batch}$$

Sodium Azide

Suggested effective concentration of sodium azide is 1 mmol/L (Pramer et al., 1972). As stated in the Material and Methods section, 30 times this concentration was used for each blank batch (V = 0.1L) in order to ensure abiotic conditions.

$$MW_{NaN3} = 65.0 \text{ g/mol}$$

Required mass of NaN_3 in each batch = $30\frac{mmol}{L} \times 65.0\frac{g}{mol} \times 0.1L$ = 195 mg ≈ 200 mg