University of Alberta

Role of Microbial Adhesion in Phenanthrene Biodegradation by *Pseudomonas fluorescens* LP6a

by

Hassan Abbasnezhad

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Chemical Engineering

Department of Chemical and Materials Engineering

Edmonton, Alberta

Fall 2009

Examining Committee

Dr. Murray R. Gray, Department of chemical and Materials Engineering

Dr. Julia M. Foght, Department of Biological Sciences

Dr. Zhenghe Xu, Department of chemical and Materials Engineering

Dr. Tony Yeung, Department of chemical and Materials Engineering

Dr. Andrew J. Daugulis, Department of Chemical Engineering

TO NAEIMEH, AYDA AND MY PARENTS

Abstract

Biodegradation of poorly water soluble hydrocarbons, such as n-alkanes and polycyclic aromatic hydrocarbons (PAHs) is often limited by the low availability of the pollutant to microbes. Adhesion of microorganisms to the oil-water interface can influence this availability. Our approach was to study a range of compounds and mechanisms to promote the adhesion of a hydrophilic PAH degrading bacterium, *Pseudomonas fluorescens* LP6a, to an oil-water interface and examine the effect on biodegradation of phenanthrene by the bacteria.

The cationic surfactants cetylpyridinium chloride (CPC), poly-L-lysine and chlorhexidine gluconate (CHX) and the long chain alcohols 1-dodecanol, 2-dodecanol and farnesol increased the adhesion of *P. fluorescens* LP6a to *n*-hexadecane from ca. 30% to ca. 90% of suspended cells adhering. The alcohols also caused a dramatic change in the oil-water contact angle of the cell surface, increasing it from 24° to 104°, whereas the cationic compounds had little effect. In contrast, cationic compounds changed the electrophoretic mobility of the bacteria, reducing the mean zeta potential from –23 to –7 mV in 0.01M potassium phosphate buffer, but the alcohols had no effect on zeta potential. This results illustrate that alcohols acted through altering the cell surface hydrophobicity, whereas cationic surfactants changed the surface charge density.

Phenanthrene was dissolved in heptamethylnonane and introduced to the aqueous growth medium, hence forming a two phase system. Introducing 1-dodecanol at concentrations of 217, 820 or 4100 mg/L resulted in comparable increases in

phenanthrene biodegradation of about 30% after 120 h incubation with non-induced cultures. After 100 h of incubation with LP6a cultures induced with 2-aminobenzoate, 4.5% of the phenanthrene was mineralized by cultures versus more than 10% by the cultures containing initial 1-dodecanol or 2-dodecanol concentrations of 120 or 160 mg/L. The production and accumulation of metabolites in the aqueous phase responded similarly to the addition of 1-dodecanol. Further experiments showed that the positive influence of the alcohols could not be attributed to the changes in surface and interfacial tension or increase in biomass concentration. The results suggest that enhanced adhesion of bacterial cells to the oil-water interface was the main factor responsible for the observed increase in phenanthrene biodegradation by *P. fluorescens* LP6a.

Acknowledgements

There are many people who made invaluable contributions to the progress of my project. It is not possible to acknowledge everyone here but I do thank them.

I am extremely thankful for the support I received from my supervisor, Dr. Murray Gray, for his guidance, support and a continuous encouragement throughout my project at the University of Alberta.

Thank you to Dr. Julia Foght from Biological Sciences, who welcomed me into her lab and were more than generous in sharing her experience as well as lab space and resources.

This project was funded by the Iranian Ministry of Science, Research and Technology, National Science and Engineering Research Council of Canada (NSERC) and Department of Chemical and Materials Engineering. I am grateful for their financial support during my program.

I am grateful to Loredana Dorobantu, who helped me in developing the adhesion and contact angle methods, to Kathrin Kirkwood for helping me on GC and to all the faculty member and staff in Chemical and Materials Engineering Department for all their help and support.

I would especially like to thank Kathy Semple and Sara Ebert, for their help on the development of the mineralization and other microbiological techniques. I would also like to thank the great help of summer students Manavdeep Reakhi and Nandlal Khatri.

Finally, I would like to thank my parents, my daughter, Ayda, and my amazing friends in Edmonton for all their emotional support and kindness. Last but not definitely least; I give my heartfelt thanks to my wonderful wife, Naeimeh Jodeiri Naghashkar, for her unfailing encouragement, support and patience throughout my project.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AFM: Atomic Force Microscopy

BH medium: Bushnell Haas Medium

BSTFA: N,O-Bis(trimethylsilyl)-trifluoroacetamide

CFU: Colony Forming Units

CHX: Chlorhexidine Gluconate

CPC: Cetylpyridinium Chloride

DCM: Dichloromethane

DMF: N,N-Dimethylformamide

dpm: Disintegrations Per Minute

GC: Gas Chromatography

HMN: 2,2,4,4,6,8,8-Heptamethylnonane

MATH: Microbial Adhesion to Hydrocarbons

OD_{600}: Optical Density at 600 nm

PAHs: Polycyclic Aromatic Hydrocarbons

PCA: Plate Count Agar

QCM: Quartz Crystal Microbalance

TSB: Tryptic Soy Broth

1. Introduction

1.1 Scope of the Research

Hydrocarbons are continuously introduced to the environment through spills and leakage during production, storage and transportation of fossil fuels. Many of these compounds can react with other systems in water or soil (Shen, et al. 2006) and can cause numerous unwanted effects (Boehm and Page 2007; Fuchsman and Barber 2000; Maliszewska-Kordybach and Smreczak 2000). Therefore it is vital to limit the discharge of these pollutants to the environment and to develop methods to clean up pollution and minimize their adverse effects on environment. Various remediation techniques have been used to remove these compounds from aquatic and soil environments. Among these techniques, bioremediation is of particular importance due to the fact that it is cost-effective, minimally invasive and successful in the removing many organic pollutants.

One factor that can limit bioremediation of hydrocarbons is their low solubility in aqueous environment. Microbes usually grow in the aqueous phase and when the solubility of contaminant in water is extremely low, as in the case of *n*-alkanes and higher molecular weight polycyclic aromatic hydrocarbons (PAHs), bioremediation becomes limited by the aqueous solubility of the contaminant. While bacteria generally prefer to metabolize substrates present in the aqueous phase, they also can take up the substrate if they are in close contact with the insoluble phase of the chemical (Bressler and Gray 2003; Efroymson and Alexander 1991). Consequently, the interface between the hydrocarbon phase and water becomes of supreme importance for bioremediation of these compounds.

The use of surfactants has been proposed as a promising method to enhance bioavailability and biodegradation of compounds such as PAHs by increasing their apparent solubility and/or desorption (Mulligan, et al. 2001; Volkering, et al. 1997). Many studies have been conducted to determine the effect of surfactants

on biodegradation of various contaminants. The conclusions reached in these studies have varied noticeably (Bramwell and Laha 2000; Chen, et al. 2000; Laha and Luthy 1991; Stelmack, et al. 1999). There have been several reports that bacterial adhesion to oil-water interfaces or to the surface of a solid organic compounds can be reduced or prevented in the presence of surfactants (Rodrigues, et al. 2006; Stelmack, et al. 1999). When the mechanism of microbial uptake is through direct contact to the surfaces of liquid or solid organic compounds, then the addition of surfactants may inhibit degradation by detaching the bacteria from the hydrophobic substrate and dispersing them into the aqueous phase. Adhesion of bacteria to the hydrocarbon phase, on the other hand, can offer some advantages in biodegradation of hydrocarbon degrading microorganisms (Hori, et al. 2002; Obuekwe, et al. 2007; Ortega-Calvo and Alexander 1994; Rosenberg and Rosenberg 1981).

Although there has been a great deal of research on microbial adhesion to hydrophobic surfaces, there have been very few attempts at the controlled use of microbial adhesion as a means to promote biodegradation of hydrocarbons. Use of some additives to promote microbial adhesion to hydrocarbons can offer several benefits for bioremediation of sparingly water soluble hydrocarbons. This dissertation endeavors to address this issue. The objectives of the research are outlined below.

1.2 Research Objectives and Thesis Overview

The main objective of this research was to increase our knowledge of bacterial adhesion to the oil-water interface and its influence on biodegradation of PAHs. More specific objectives were as follows:

1. To experimentally test different groups of chemicals to find appropriate compounds that can promote adhesion of the PAH degrading bacterium, *Pseudomonas fluorescens* LP6a, to the oil-water interface.

2. To determine the mechanism through which these compounds influence bacterial adhesion to the oil-water interface.

3. To investigate the effect of enhanced adhesion on biodegradation of the concerned PAH of interest, phenanthrene.

This thesis has been divided into five chapters. Chapter 2 covers the theoretical background information associated with the work. Chapter 3 comprises materials and methods, results and discussion about the research objective 1. In this chapter the influence of several compounds on adhesion of the bacteria to *n*-hexadecane-water interface is investigated. In addition, effect of some of these compounds on bacterial cell surface is examined in terms of changes in contact angle and zeta potential values. These effects are compared with one another and different mechanisms responsible for the observed changes are discussed. Chapter 4 describes the materials, experimental methods, results and discussion of the role of selected adhesion promoting agents on biodegradation and mineralization by *P. fluorescens* LP6a of phenanthrene. Chapter 5 summarizes the most important results and outlines potential directions for future research in this field.

.

1.3 References

1. **Boehm PD, Page DS (2007).** Exposure elements in oil spill risk and natural resource damage assessments: A review. *Hum Ecol Risk Assess* 13:418-448.
2. **Bramwell DAP, Laha S (2000).** Effects of surfactant addition on the biomineralization and microbial toxicity of phenanthrene. *Biodegradation* 11:263-277.
3. **Bressler DC, Gray MR (2003).** Transport and reaction processes in bioremediation of organic contaminants. 1. Review of bacterial degradation and transport. *Int J Chem React Eng* 1: R3:
4. **Chen P, Pickard MA, Gray MR (2000).** Surfactant inhibition of bacterial growth on solid anthracene. *Biodegradation* 11:341-347.
5. **Efroymson RA, Alexander M (1991).** Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic-solvent. *Appl Environ Microbiol* 57:1441-1447.
6. **Fuchsman PC, Barber TR (2000).** Spiked sediment toxicity testing of hydrophobic organic chemicals: Bioavailability, technical considerations, and applications. *Soil Sed Contam* 9:197-218.
7. **Hori K, Matsuzaki Y, Tanji Y, Unno H (2002).** Effect of dispersing oil phase on the biodegradability of a solid alkane dissolved in non-biodegradable oil. *Appl Microbiol Biotechnol* 59:574-579.
8. **Laha S, Luthy RG (1991).** Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. *Environ Sci Technol* 25:1920-1930.
9. **Maliszewska-Kordybach B, Smreczak B (2000).** Ecotoxicological activity of soils polluted with polycyclic aromatic hydrocarbons (PAHS) - Effect on plants. *Environ Technol* 21:1099-1110.
10. **Mulligan CN, Yong RN, Gibbs BF (2001).** Surfactant-enhanced remediation of contaminated soil: a review. *Eng Geol* 60:371-380.
11. **Obuekwe CO, Al-Jadi ZK, Al-Saleh ES (2007).** Sequential hydrophobic partitioning of cells of *Pseudomonas aeruginosa* gives rise to variants of increasing cell-surface hydrophobicity. *FEMS Microbiol Lett* 270:214-219.
12. **Ortega-Calvo JJ, Alexander M (1994).** Roles of bacterial attachment and spontaneous partitioning in the biodegradation of naphthalene initially present in nonaqueous-phase liquids. *Appl Environ Microbiol* 60:2643-2646.
13. **Rodrigues LR, Banat IM, van der Mei HC, Teixeira JA, Oliveira R (2006).** Interference in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicone rubber by rhamnolipid biosurfactants. *J Appl Microbiol* 100:470-480.

14. **Rosenberg M, Rosenberg E (1981).** Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *J Bacteriol* 148:51-57.

15. **Shen GQ, Lu YT, Hong JB (2006).** Combined effect of heavy metals and polycyclic aromatic hydrocarbons on urease activity in soil. *Ecotoxicol Environ Saf* 63:474-480.

16. **Stelmack PL, Gray MR, Pickard MA (1999).** Bacterial adhesion to soil contaminants in the presence of surfactants. *Appl Environ Microbiol* 65:163-168.

17. **Volkering F, Breure AM, Rulkens WH (1997).** Microbiological aspects of surfactant use for biological soil remediation. *Biodegradation* 8:401-417.

2. Literature Review

2.1. Introduction

The widespread production, processing and handling of fossil fuels has created serious environmental problems due to spills and leakage of crude oil and refined products. The use of microbes to clean up these spills offers an attractive approach in the remediation of polluted sites. Although bioremediation is generally considered a safe and inexpensive method for the removal of contaminants, our knowledge of the various alterations occurring in the process remains limited and bioremediation is still regarded as a developing field (Singh and Ward 2004). The ultimate ability of a microorganism to degrade a compound will depend on the genetic makeup of the organism and the expression of this genetic information. Assuming that genetically capable microorganisms exist, such organisms cannot effectively grow and degrade the compounds unless certain conditions are fulfilled. These can be loosely classified in three inter-related categories: (a) The conditions that are mostly related to the microorganism, (b) The environmental requirements to support the adequate growth of microorganisms, (c) The conditions that are related to the characteristics of the compounds to be degraded. Figure 2.1 is a schematic diagram of different issues involved in bioremediation.

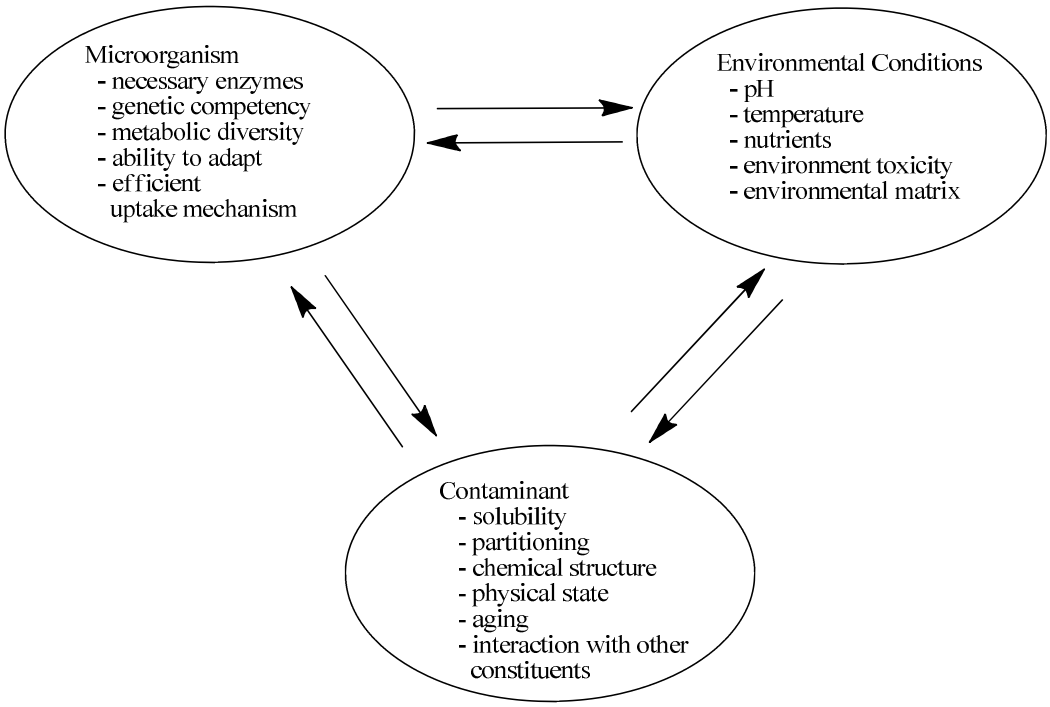

Figure 2.1: Schematic diagram demonstrating main determining issues in bioremediation.

One of the important factors in biological removal of hydrocarbons from contaminated environment is their bioavailability to an active microbial population. In this context, bioavailability is defined as the degree to which a contaminant can be readily taken up by the microorganism (Atlas and Philp 2005). Bioavailability of a contaminant is controlled by a number of factors such as hydrophobicity, water solubility, sorption to environmental matrix such as soil and diffusion. The mode of microbial uptake is also a key factor in determining bioavailability.

When contaminants have very low solubility in water, as in the case of *n*-alkanes and polyaromatic hydrocarbons, then the organic phase components will not partition efficiently into the aqueous phase that contains the microbes. In the case of soil, the organic phase will partition further to the soil organic matter and become even less bioavailable. The most common method to improve bioavailability of such hydrocarbons is addition of surfactant to enhance

desorption and solubilization of contaminants. The addition of surfactants has been reported to have variable effects, ranging from improving bioremediation, to no effect, to inhibition of removal of contaminants as reviewed below.

The challenge in reviewing this literature is the lack of robust experimental methods to define the controlling mechanism. For example, addition of surfactant can change several of the factors illustrated in Figure 2.1 simultaneously, giving multiple possible explanations for a change in the observable rate of biodegradation. One reason for the unpredictable effect of surfactants on biodegradation is that microbial adhesion to the oil-water interface is an important mechanism for increasing the access of microbes to the non-aqueous phase. The significance of microbial adhesion to bioavailability, and the impact of different environmental factors, is the subject of discussion in this chapter. I examine the literature on the role of adhesion in bioremediation of liquid hydrocarbons, with selected examination of the studies with solid hydrocarbons to illustrate how low-solubility compounds can interact with microorganisms in an aqueous environment.

Most studies on the nature of microbial adhesion and the forces involved have concentrated on the adhesion to solid surfaces. Since many of the challenges faced in these studies are also encountered in adhesion to liquids, I will briefly review some of these studies. The theory of Marshal and co-workers proposing a two step adhesion process has been used widely in subsequent studies (Marshal, et al. 1971). The first step involves the transport of the cells to the close vicinity of the interface to allow initial adhesion to occur. The forces involved in this stage are believed to be Lifshitz–Van der Waals and electrostatic forces (Meinders, et al. 1995; van Loosdrecht, et al. 1987). In this stage, the adhesion is reversible and there is a continuous exchange between free and adhering microorganisms. The next step is the irreversible adhesion as a bacterium robustly binds to the interface by various molecular mechanisms including production of exo-polysaccharides and/or specific structures, such as pili or fimbriae. The terms adhesion and

attachment are often used interchangeably. Some researchers, however, use "attachment" to describe only the initial stage of the microbial adhesion process (reversible adhesion). In this case attachment expresses a physical contact rather than the subsequent complicated chemical and cellular interactions (An and Friedman 1998). "Adhesion" is the term that has been used more frequently in scientific literature in recent years and it usually covers both the initial reversible and the second irreversible stages (Christensen, et al. 2000). In this thesis, we will use the term adhesion to refer to the overall process of microbial adhesion. If a distinction must be made between reversible and irreversible adhesion, then that distinction will be mentioned explicitly.

A critical examination of the methods of measurement used in past studies allows proper interpretation of the literature regarding the role of adhesion in microbial growth on and biodegradation of hydrocarbons. Some examples of adhesion-independent and adhesion-dependent biodegradation will be presented based on studies from the last two decades, followed by an examination of the opportunity to directly modify bacterial adhesion, and thereby enhance the rate of biodegradation of contaminants.

2.2 Challenges in investigating the role of adhesion in biodegradation

Adhesion to hydrophobic surfaces is one common strategy used by microorganisms to overcome bioavailability limitations of hydrocarbons (Bouchez-Naïtali, et al. 1999). The other natural microbial response to limited bioavailability is biosurfactant production (Johnsen and Karlson 2004). Biosurfactants and their role in biodegradation have attracted the bulk of the attention in the literature (Garcia-Junco, et al. 2003; Ron and Rosenberg 2002; Rosenberg, et al. 1999; Zhang and Miller 1994) to the point that in many cases the role of adhesion has been ignored. The adhesion of a microbe to the oil-water interface enhances the rate of transport of hydrocarbon from the non-aqueous

phase to the cell by reducing the distance between microorganism and its substrate. In an oil-water mixture, a further benefit is that the adhesion of bacterial cells to the oil-water interface can stabilize emulsions, and thereby increase the oil-water interfacial area (Dorobantu, et al. 2004).

In most hydrocarbon degrading organisms, the enzymes involved in the process are intracellular. Consequently, biodegradation requires the diffusion of the substrate from the oil phase through an aqueous phase and then across the cell wall and cell membrane. The adhesion of cells to the oil-water interface can minimize the diffusion distance and facilitate the diffusion of hydrophobic compounds to the cells. A closer examination of bacterial structure, however, exposes the complexity of this simple view. The exterior of the cell wall typically comprises proteins and/or polysaccharides, which influence the hydrophobicity and adhesion of the cell to interfaces (Doyle and Rosenberg 1990; Norman, et al. 2002). Additional extracellular structures can include exopolysaccharide layers, pili, and fimbriae which can extend a considerable distance from the cell wall and which may limit how closely the cell surface can approach the oil-water interface. These features depend on the expression of the corresponding genes during growth (Cooper and Kosaric 1982; Costerton, et al. 1974; Neufeld and Zajic 1984) and may have a significant effect on adhesion (Ofek and Doyle 1994). The interaction of the cell surface compounds and structures with the other chemicals present in the culture medium will be another factor adding to the challenges of how to characterize the microbial adhesion in this system. In the following sections these difficulties will be addressed in more detail.

2.2.1 Mechanisms of Microbial Adhesion

There are multiple factors involved in adhesion, including physicochemical factors such as shear, surface charge on the hydrocarbon; species-dependent factors like cell-surface composition and structures, biological factors within a species such as cell age and nutrition. One source of misunderstanding is the use of different terminologies in referring to microbial adhesion itself. Microbial

adhesion is usually defined as the process of transferring unbound, suspended cells from the aqueous phase to an interface (Hermansson 1999). Adhesion is generally measured as the numbers of cells bound to the interface after a washing and separation step, where unbound cells are removed. The process of adhesion can be studied as a function of time (in flow systems) or as a steady-state process after some defined time (static experiments). Researchers have frequently used the static systems because of their simplicity, however, well defined flow systems such as parallel plate flow chambers accompanied by on-line microscopic detection methods provide more information and are becoming more common (Busscher and van der Mei,Henny C. 2006; Meinders, et al. 1995; Seo and Bishop 2007).

Few studies have examined the molecular mechanism of microbial adhesion to oil-water interfaces. Bacteria have been analyzed as living colloidal particles with a negative surface charge (i.e. zeta potential) and their adhesion was interpreted based on colloidal theories (Hermansson 1999; Skvarla 1993). The interfacial tensions involved when a bacterium approaches the interface are schematically shown in Figure 2.2. In this figure γ_{bo} is the tension at bacterium-oil interface, γ_{bw} that at the bacteria-water interface, and γ_{ow} that at the oil-water interface; then an equilibrium force balance equation can be written as:

$$\gamma_{bo} = \gamma_{bw} + \gamma_{ow} \cdot \cos\theta \qquad (1)$$

where θ is the contact angle between aqueous phase and bacterial surface. Equation (1), which is referred to as the Young equation, is based on a thermodynamic equilibrium between the three phases and assumes that the equilibrium value of the contact angle is constant and unique for the given system.

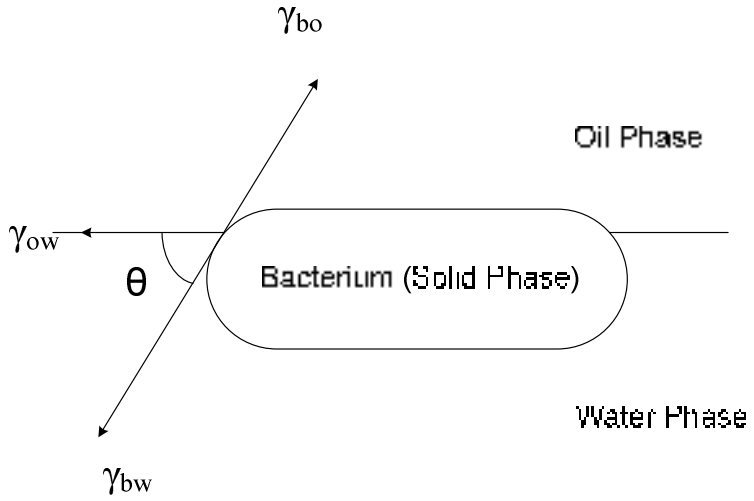

Figure 2.2: Schematic diagram of interfacial tensions acting on the interface between bacteria, oil and water.

In principle, θ and γ_{ow} can be measured experimentally, therefore an additional equation is required to obtain γ_{bo} and γ_{bw}. One method is to calculate the solid surface tensions by measuring the contact angle of particles against different liquids, as recently reviewed by Tavana and Neumann (2007). By measuring the contact angle of the bacterial surface and obtaining the interfacial tensions, it is possible to predict the positioning of bacteria with respect to the oil-water interface. Such criteria have been developed based on the three interfacial tensions listed in equation 1. (Albertsson 1986; Marshall 1986; Neufeld, et al. 1980). The limited number of papers in this area gives some interesting observations, for example, some very hydrophobic bacteria completely penetrate into the non-aqueous phase by crossing the interface (MacLeod and Daugulis 2005). These bacteria presumably carry some external water with them, but the role of extracellular structures such as fimbriae and pilli in modifying the liquid-liquid interface has not been reported. Measurement of the contact angle of bacteria is also challenging due to their small size, and the heterogeneity of the cell surface. These features suggest that the measured contact angle, θ, will exhibit hysteresis between advancing and receding liquid contact experiments, which gives

ambiguous results from equation (1). Further complications are introduced by changes that occur on the cell surface during sample preparation (Krekeler, et al. 1989; Neufeld and Zajic 1984). While most studies on the bacterial contact angle measurement have been conducted on water and air systems, it is also possible to determine the contact angles of cell layers in systems with two immiscible liquids (Neufeld, et al. 1980). Although the measurement and interpretation of contact angle data for microbial cells can be controversial, studies by many researchers (Reid, et al. 1992; van Merode, et al. 2006; van Oss, et al. 1975) suggest that data from contact angle measurements are valuable for predicting cellular interactions at interfaces.

Most studies of microbial adhesion measure the macroscopic properties of cell populations and overall number of cells that adhere, without considering the role of the microbial surface components (Bos, et al. 1999). Surface structures such as fibrils, fimbriae, capsules or flagella can vary within a bacterial community and each of these structures can potentially interact with an oil interface (Bihari, et al. 2007; Razatos, et al. 1998). Our limited knowledge of the variety of these structures and their role in the interaction of microorganisms with a liquid interface prevents a clear understanding of adhesion at the molecular level.

2.2.2 Challenges in adhesion measurement

A major problem in studying the effect of adhesion on biodegradation is the lack of appropriate methods to directly measure adhesion in situ. Methods for measuring adhesion can be divided into those based on adhesion to solid surfaces and those based on adhesion to liquids. Since it is easier to work with and characterize adhesion to solid surfaces, methods belonging to the first group are more prevalent and diverse. Early methods of this type included testing the adhesion of bacteria to low energy surfaces like polystyrene (Rosenberg 1981). In these methods the number of adhered bacteria was measured by direct counting. Newer methods belonging to this group study more specific interactions, often as a function of time (Busscher and van der Mei 2006).

The methods for determining adhesion to liquids were used mainly to test the adherence of microorganisms to hydrocarbons. Some early work used radiolabelled microorganisms to quantify the number of microbes in each phase (Zilber, et al. 1980). In 1980 the simple and convenient microbial adhesion to hydrocarbons (MATH) test was introduced (Rosenberg, et al. 1980). The method was based on removal of cells from aqueous solution as a result of mixing with a liquid hydrocarbon. Although this method was semi-quantitative, later studies showed that results of MATH usually (but not always) correlate with other measurements of cell surface properties (Jones, et al. 1996; Rosenberg and Kjelleberg 1986). MATH was used frequently in adhesion studies, thanks to the advantage of simplicity and lack of need for special preparation of cells, which can cause unknown changes in cell surface properties (Rosenberg 2006).

Most techniques for measuring microbial adhesion involve separating microorganisms from the growth medium and measuring their adhesion under standard conditions. Although this can provide useful information, it may not represent the adhesion of cells in the actual media. For instance, in some cases adhesion to solid surfaces is used to explain adhesion in liquid-liquid biodegradation experiments (Stelmack, et al. 1999). Adhesion tests such as MATH are usually performed in a specific environment with defined pH and ionic strength. These conditions are rarely the same as those that exist in the biodegradation experiment, but they can greatly change the microbial adhesion (Babu, et al. 1986). During biodegradation, the medium composition changes as substrates are transformed, nutrients are consumed and new compounds are produced. Some metabolites such as long chain alcohols can influence the adhesion of microorganisms even if they are present for a short period of time or at low concentration (Marchesi, et al. 1994a; Marchesi, et al. 1994b).

The ideal case would be measurement of bacterial adhesion in real time. Some new developments approach this ideal case, including recent studies (Kuehn, et al. 1998; Power, et al. 2007; Rodrigues, et al. 2003) on the real time visualization of

bacterial adhesion to solids using confocal laser scanning microscopy (CLSM). For instance, the growth of *Pseudomonas putida* was studied on solid phenanthrene and fluorene surfaces (Rodrigues, et al. 2005). Based on their previous work (Rodrigues, et al. 2003) on the colonization pattern of *P. putida* on phenanthrene and fluorene crystals, Rodrigues et al. investigated the role of physiological processes on the biodegradation of these PAHs. They also conducted zeta potential and MATH measurements to characterize bacterial surface properties. They concluded that *P. putida* was able to consume fluorene and phenanthrene with two different uptake strategies. While *P. putida* used only water soluble fluorene, it adhered to the solid phenanthrene and could degrade it at a higher rate despite the fact that it has lower water solubility than fluorene. Based on kinetic analysis of degradation data they proposed that greater adhesion of bacteria to phenanthrene crystals promoted the availability and uptake of this compound.

Another new method of online measurement of microbial adhesion is the quartz crystal microbalance (QCM) technique. It is again based on adhesion to solid surfaces. The use of QCM is based on the fact that any attachment or removal of the biomass to the crystal's electrode causes a change in frequency of oscillating quartz crystal, f, which is related to the mass change. Recent developments enable researchers to obtain information about the adhesion process including the changes in the viscoelasticity of the adhered layers during the process (Rodahl, et al. 1997). By coupling the QCM method and microscopic visualization of the adhered cells, it has been demonstrated that the QCM responses to cell adhesion to hydrophilic and hydrophobic surfaces could be discriminated (Fredriksson, et al. 1998a; Fredriksson, et al. 1998b). Although different cell species showed different QCM responses on a single surface, there was not a clear relationship between the QCM data and the adhesion mechanisms, and the biological properties (such as cell surface composition) of the adhered bacteria. The effect of flow conditions on the development and stability of biofilm formed by

Streptococus mutans was recently studied (Schofield, et al. 2007) by combining QCM with atomic force microscopy (AFM) measurements of surface coverage. They concluded that the continuous flow method could result in formation of a more floppy biofilm (loosely attached and more viscoelastic adsorbate). The QCM technique is limited to measuring the adhesion to solid surfaces, but it illustrates the desirable direction for investigation of the adhesion of cells to liquid-liquid interfaces. With the advancement in understanding of the relationship between the QCM response and physical properties of the adhered layer this method can be utilized more broadly in applications such as monitoring adhesion during microbial growth on hydrocarbons.

Recently the use of AFM has enabled measurements of adhesion forces and surface properties of cells directly (Ahimou, et al. 2002; Bolshakova, et al. 2004; Fang, et al. 2000; Wright and Armstrong 2006). AFM consists of a cantilever that can scan a planar substrate and has the capability of imaging surface topography under physiological conditions (which is not possible by other high resolution methods such as electron microscopy). AFM has also been used for measuring local physical properties such as adhesion forces and elasticity. The cantilever tip can be modified by attaching different compounds and particles, therefore, AFM can measure adhesion characteristics between variety of microorganisms and other surfaces. A major advantage of AFM is that, unlike other microscopic methods like scanning electron microscopy, it does not require an extensive pre-treatment of the microorganisms and it can be performed under physiological conditions. AFM provides much more detailed analysis of microbial cell surface hydrophobicity and gives information unobtainable by other methods. For example, AFM was employed to determine the heterogeneity of adhesion forces on individual bacterial cell surfaces (Dorobantu, et al. 2004). Detailed information provided by AFM can be used to better understand the various changes in surface properties of bacteria during the growth on organic compounds.

Although there have been significant advancements toward developing robust and facile measurement techniques, the available methods are still not mature enough to be applied in real complex situations such as microbial degradation of hydrocarbons in contaminated fields. Further developments in this regard will play a key role in all research fields dealing with microbial adhesion including the clarification of role of adhesion in biodegradation.

2.3 Adhesion and Microbial Growth on Hydrocarbons

Before discussing the effects of adhesion on the biodegradation process it is useful to look at a broader relationship between adhesion and microbial growth on hydrocarbons. Adhesion to an interface influences the bacterial activity in many different ways, as previously reviewed (Baror 1990; Rosenberg and Kjelleberg 1986; van Loosdrecht, et al. 1990). In an extensive review of adhesion to solids (van Loosdrecht, et al. 1990), the influence of the surface was divided into two distinct mechanisms: direct and indirect. The direct influence was the case when a change in a bacterial property, such as membrane permeability, was caused by adhesion. Other factors, such as changes in medium concentration and mass transfer were classified as indirect effects. The review concluded that none of the reported data could be attributed to direct influences. The indirect effects were not discussed in detail.

A number of studies considered the effect of adhesion on the growth of microbes on n-alkanes, and examined the mechanisms of hydrocarbon uptake. In these studies researchers tried to find specific patterns in growth kinetics amongst the microbial cultures that were attached to the oil-water interface. The mechanism of substrate uptake is a key point in any process involving sparingly soluble substrates. In some fungi extracellular enzymes can gain access to and start degradation of compounds outside of the microorganism boundaries (Krćmāŕ, et al. 1999; Novotný, et al. 2004), but in all other cases hydrocarbon compounds need to cross the cell membrane in order to contact membrane-bound or

cytoplasmic enzymes (Bressler and Gray 2003). Three major uptake modes can be proposed:

(a) Uptake through the aqueous phase in which the compound can only be used when it is dissolved in the aqueous environment. According to this mechanism, if substrate is present as a solid phase or as a pure organic liquid the solubility of compound in water will be the most important factor controlling the biodegradation rate. If the compound is present in another non-aqueous phase liquid (NAPL) the degradation rate will depend on the rate and extent of partitioning from the NAPL to the aqueous phase.

(b) Surfactant-mediated uptake where microorganisms excrete products that can either increase apparent solubility or emulsify the substrates, hence making them more available for microorganisms. For solid hydrocarbons, an increase in dispersion and dissolution by surfactants has been reported (Edwards, et al. 1991; Rosenberg 1981). For liquid hydrocarbons or hydrocarbons dissolved in NAPL, surfactants can emulsify the non-aqueous phase and increase the exposure of non-aqueous phase to the microorganisms.

(c) Direct contact mode which implies that cells attach to the substrate or the NAPL in which substrate is dissolved.

All these mechanisms have been reported for various microorganisms. The first uptake mode (which is also known as homogenous uptake mode) is common with, but not limited to, substrates that are completely water soluble or have relatively high solubility in the aqueous phase. Although surfactant-enhanced uptake can be very important in biodegradation of hydrocarbons, the discussion of this subject is beyond the scope of our discussion and hence it will not be covered in detail here. There are many papers devoted to study the different aspects of surfactants role on biodegradation. Interested readers can refer to several extensive review papers on this topic (Mulligan, et al. 2001; Singh, et al. 2007; Van Hamme, et al. 2006; Volkering, et al. 1997). In this chapter we will focus on the third mode, on the

role of microbial adhesion which has long been thought to be of great importance for direct uptake of substrate (Mudd and Mudd 1924).

As the solubility of the hydrocarbons being degraded approaches zero, the importance of the distance for transport through aqueous phase will increase to the point where a difference of nanometers can significantly affect the rate of uptake (Weisz 1973). The exterior surface of microorganisms presents macromolecular structures which may reduce the rate of diffusion from the oil phase to the cell by controlling the distance between the cell wall and the oil phase. Adhesion of cells to the interface will minimize the diffusion path from the oil phase to the cell interior. This minimum distance will vary depending on the characteristics of microorganisms and the surface they attach to. A basic diffusive mass transfer equation for the rate of compound transfer, Q_d (mass/time), to the microbial cell boundary can be written as (Harms and Bosma 1997):

$$Q_d = k(C_d - C_c) \qquad (2)$$

where C_d is the aqueous concentration of the substrate (mass/volume) at the oil-water interface, k is the mass transfer coefficient and C_c is the concentration at the cell surface. When a compound is being actively biodegraded, $C_c \approx 0$, while $C_d \approx C_{sol}x_{HC}$, the aqueous solubility of the component multiplied by its concentration in the oil phase. For a linear concentration gradient, the mass transfer coefficient can be written as (Koch 1990):

$$k = D_{eff}.A/L \qquad (3)$$

in which A is the surface area through which the diffusion occurs, L is the distance and D_{eff} (area/time) is the effective diffusivity. Combining these relationships, the maximum rate of diffusion of the substrate to the cell will be:

$$Q_{d,\max} \approx \frac{D_{eff} A C_{sol} x_{HC}}{L} \qquad (4)$$

Equation (4) shows that the mass flow will increase with a decrease in diffusion distance (L) and increase with the surface area (A). If $Q_{d,max}$ exceeds the rate of enzymatic conversion within the cells, then diffusion is not a constraint. For low solubility hydrocarbons, such as n-alkanes, the aqueous solubilities are so low that the rate of transport to a competent degrading cell will invariably control the rate of biodegradation.

The distance (L) and surface area (A) will depend on the nature of adhesion and the type of surface. For example, adhesion by interaction of fimbriae with the hydrocarbon surface can give a diffusion length of several micrometers, and an effective area that is less than the projected area of the cell onto the interface. If the cell wall interacts directly with the surface, then the diffusion distance could be tens of nanometers, whereas adhesion of a capsule layer to the hydrocarbon interface would give an intermediate diffusion length up to hundreds of nanometers. These short distances can be very important in determining the mass transfer rates when solubilities in water are of order 1 ppb. The second important factor in equation (4) is the actual surface area of hydrocarbon for transport to the microbial cell. When the substrate is in the solid state the interfacial area will be the section of microorganism that is in contact with solid surface. In the case of liquids the actual interfacial area can vary greatly depending on the interfacial tensions between the aqueous phase, the organic phase and the microbial cell surface. The orientation of the microorganism at the interface can range from complete immersion in organic phase to just barely touching the surface. All these cases were observed in laboratory studies (MacLeod and Daugulis 2005; Marshall 1980). Although it might appear that the complete immersion of microbes in the organic phase would maximize the contact area and provide the best degradation of compounds in organic phase, this situation can limit growth rate due to lack of access to ionic species from the aqueous phase. These aspects of micro-scale and nano-scale mass transfer determine the role of adhesion in biodegradation, but

there is an almost complete lack of discussion of this point in the literature on biodegradation processes.

Direct interfacial uptake has been mostly observed in cases of poorly soluble compounds such as long chain alkanes and polycyclic aromatic hydrocarbons (PAHs). It is often experimentally difficult to distinguish between the direct contact uptake and uptake through aqueous phase, therefore, kinetic behaviours of these two different mechanisms have been used as a means to differentiate which mechanism was involved in the biodegradation process. (Dunn 1968) conducted a simple analysis of the growth at an oil-water interface and in the aqueous phase, and considered the two extreme cases for each mechanism. When a microorganism only utilizes the hydrocarbon in its dissolved form (homogenous uptake) the growth is exponential with two extreme cases. At very low cell population the growth is only limited by the number of cells, so:

$$\frac{dX}{dt} = \mu.X \qquad (5)$$

with X being the cell concentration (gram of cells per volume of aqueous phase) and μ being the specific growth rate of the microorganism. At high cell density the hydrocarbon transfer from organic phase to the aqueous phase would be rate limiting and the equation would be:

$$\frac{dX}{dt} = k.A \qquad (6)$$

where k is a coefficient containing effects of many variables including mass transfer and the specific growth rate and A is the total oil-water interfacial area.

In the case of interfacial growth it was argued that cell division occurs while the cell is at the interface and the equation would be:

$$\frac{dX}{dt} = \mu.X_S \qquad (7)$$

where X_S is the cell concentration at the interface (the total number of cells that are located at interface). Writing equation 7 in terms of the fractional area coverage (σ) and the area occupied by unit mass of microorganism (a) gives:

$$\frac{dX}{dt} = \mu.(\frac{A}{a}).\sigma \qquad (8)$$

Assuming a Langmuir isotherm mode for the adsorption and desorption of cells to the interface gives:

$$\sigma = \frac{bX}{1+bX} \qquad (9)$$

where b is the adsorption equilibrium constant. Equation (8) can be rewritten as:

$$\frac{dX}{dt} = \mu.(\frac{A}{a}).(\frac{bX}{1+bX}) \qquad (10)$$

Considering the two limiting cases, for low cell concentration at the interface the increase in X with time will result in greater interfacial area coverage and the growth rate would be proportional to the both interfacial area and total cell concentration. Equation (10) would then reduce to:

$$\frac{dX}{dt} = \mu.(\frac{A}{a}).bX \qquad (11)$$

At high cell concentration, when the interface is fully covered, equation (10) would become:

$$\frac{dX}{dt} = \mu(\frac{A}{a}) \qquad (12)$$

In this case, the growth rate is proportional to the interfacial area and independent of the total cell concentration. The predictions of the two above mentioned models, illustrated in Figure 2.3, suggest that it is possible to distinguish between the two mechanisms using kinetic data. For the interfacial mechanism at lower

cell concentrations according to equation (11) the plot of growth rate versus X will be logarithmic and would change proportional to the interfacial area. For the homogeneous model, however, it will not depend on the area at similar cell concentration range, as indicated by the equation (5). It is crucial to consider that this distinction could only be made when the cell concentration is sufficiently low and all substrates are in high concentrations (to have a constant μ-value). Otherwise, the rate will change with interfacial area for both models according to equations (12) and (6). The other important point to consider is the fact that this distinction requires obtaining data for the initial rate of degradation which can be difficult in many cases.

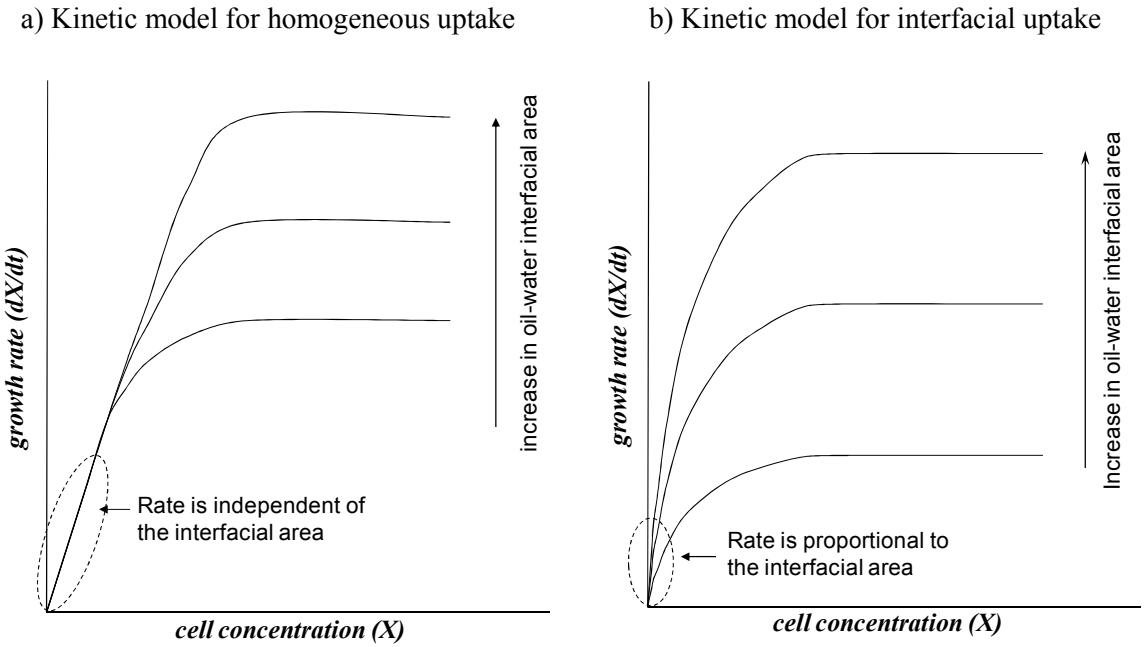

Figure 2.3: Schematic diagram of growth rate versus cell concentration: (a) homogeneous uptake: based on equation (5) and (6) for low and high cell concentrations, respectively and (b) interfacial uptake: based on equation (10) (adapted from (Dunn 1968))

Another experimental method to distinguish between interfacial and homogenous uptake modes is based on comparing cell growth at high cell populations. According to equations (6) or (12), at high cell concentrations the growth rate is

controlled by interfacial area and/or the mass transfer. The method is based on discriminating between these two factors, and was first proposed during a study of bacterial production of menthol, when the substrate was provided as an immiscible organic liquid (Westgate, et al. 1995). Two racemic isomers of menthyl acetate were provided to the bacteria, but only one of them was used as substrate and the other one was not metabolized. It was observed that the menthol production rate was proportional to the organic phase volume, both when substrate was provided as a pure isomer and when the organic phase was a racemic mixture of isomers. Comparing equal volumes of the racemic and single enantiomeric substrates, the interfacial area was the same but the rate of mass transfer of the actual substrate to the aqueous phase was expected to be less for the racemic mixture. They showed that the menthol production rate was proportional to the interfacial area (i.e. volume of the liquid for the actual substrate) and was independent of the enantiomer concentration. It was then concluded that the rate was controlled by the direct access of bacteria to the interface and not by mass transfer to the aqueous phase. The authors recommended a general method to distinguish between mass transfer and surface area controlled mechanisms based on the dilution of substrate with a structurally similar but non-metabolizable compound. This would provide an opportunity to set up two parallel experiments having the same interfacial area but different mass transfer rates. Comparable reaction rates under both cases would mean that interfacial uptake and not the mass transfer is the controlling step.

Other experiments were intended to clarify some features of uptake mechanisms, but failed to give discrimination. For example, addition of a co-solvent to increase the solubility of the organic substrate was used to determine the controlling mechanism in high cell concentration in the same experiment described above (Westgate, et al. 1995), but the authors were unable to completely rule out direct effects of the cosolvent on the microbes.

In some cases studying the interaction between growth on a water soluble compound and uptake of a sparingly water soluble organic substrate can also provide useful information. For instance, the effect of presence of an organic phase (both metabolizable and non-metabolizable) on bacterial consumption of glucose was used to demonstrate the affinity of bacteria toward a non-aqueous phase (MacLeod and Daugulis 2005). These experiments should be interpreted carefully since multiple factors can control the outcome while some of them (like NAPL inhibition of microbial growth, mixing and emulsification) are not controlled or monitored during these experiments. Different approaches are taken in applying the kinetic and mass transfer analysis to distinguish the controlling mechanism in biodegradation. The experimental conditions do not usually allow examination of all these criteria in a particular system. It is important to note that, although inferring these criteria can suggest the bacterial uptake mode, these criteria often do not provide sufficient evidence to prove a particular mechanism. The other point to consider is the fact that in an agitated culture cells are in a dynamic equilibrium between attached and planktonic phases (i.e. the Langmuir isotherm in equation is an equilibrium relationship). Therefore, as will be seen in some of the reviewed studies in sections 2.3 and 2.4, it is quite possible to have different uptake modes that prevail in different stages of growth. To sum up, it can be said that the interfacial growth mechanism is usually too complicated for analysis by macroscopic kinetics data. It is very important to combine microscopic observation with kinetic measurements and also examine all the assumptions of kinetic models before applying these models into any of the cases.

2.4 Reports of adhesion-independent biodegradation

In this section we give some examples of the reported studies in which microbial adhesion was not found to be a major factor. Table 2.1 shows a list of main references in this regard. These studies are classified into two groups: (a) studies on biodegradation of n-alkanes and (b) studies on biodegradation of PAHs. The effect of adhesion is believed to be much more important in case of n-alkanes

because of their extremely low solubility. For instance, tetradecane ($C_{14}H_{30}$) has an aqueous solubility that is more than 40 times lower than that of phenanthrene ($C_{14}H_{10}$) (Eganhouse and Calder 1976; Mackay, et al. 1975). Nevertheless, the absence of a major adhesion effect in n-alkane degradation was reported in some cases as will be discussed in this section. For papers grouped in part (a) adhesion was not a major factor, but it was mostly due to the presence of surface active compounds. The early results were qualitative (Chakravarty, et al. 1975; Yoshida, et al. 1971) but efforts continue to gain better insights.

Table 2.1: Studies in which microbial adhesion was reported not to be a major factor in biodegradation

References	Main substrate	Supply mode	Microorganism used	Main result and comments
(a) Papers showing absence of major adhesion effect for n-alkanes				
Yoshida, et al. 1971	n-alkanes (C_6-C_{18})	Liquid or vapor hydrocarbons	*Candida tropicalis*	Hydrocarbon uptake by direct contact mode was negligible; however, the paper lacks quantitative data to support the claim.
Chakravarty, et al. 1975	Mixture of dodecane and hexadecane	Liquid hydrocarbons	*Candida maltosa*	Biodegradation was modeled based on the uptake from aqueous phase. Effects of existing surface active metabolites on the cell surface and adhesion were not addressed.
Chrzanowski, et al. 2005	Dodecane and hexadecane	Liquid hydrocarbons	*Candida maltosa* EH 15	Correlation between adhesion and biodegradation was examined in presence of surfactants. The interference of surfactants with measurement of adhesion was ignored.

Pijanowska, et al. 2007	Dodecane and hexadecane	Liquid hydrocarbon	*Pseudomonas* & *Bacillus sp.*	Decrease in adhesion was correlated with the best biodegradation by some species. Experiments lacked proper controls.
Mohanty and Mukherji 2007	Diesel fuel containing n-alkanes	Liquid diesel fuel	*Exiguobacterium aurantiacum* and *Burkholderia cepacia*	Adhesion did not show a major impact on biodegradation. Adhesion data were not reported.
(b) Papers that demonstrate absence of major adhesion effect for PAHs (mostly due to their sufficient aqueous concentrations)				
Wodzinski and Coyle 1974	Phenanthrene	Solid crystals	*Pseudomonas sp.*	Phenanthrene was utilized in the dissolved state.
Bouchez, et al. 1995	Phenanthrene	Both solid crystal and dissolved in NAPL	*Pseudomonas sp, S Phe Na1*	No interfacial uptake was observed.
Efroymson and Alexander 1991	Naphthalene and hexadecane	Dissolved in NAPL	*Arthrobacterium sp.*	Adhesion was not an important factor for biodegradation of naphthalene.
Foght and Westlake 1988	Phenanthrene and anthracene	Dissolved in NAPL	*Pseudomonas fluorescens LP6a*	Hydrophilic microorganism that degrades variety of PAHs

Because of the presence of surface active compounds in most of the studies in part (a), the conclusions claimed in papers should be examined cautiously. In some cases the claims have to be disqualified due to lack of proper controls over interference of surfactants with experimental measurements. When the surface active compounds are present in a biodegradation experiment, their influence on the bacterial uptake mode should be considered along with their role on increasing solubility or dispersion of hydrocarbons. The failure to address these multiple effects can be observed in some of the papers in group (a). An example is a study examining the relationship between adhesion and n-alkane biodegradation by the yeast *Candida maltosa* (Chrzanowski, et al. 2005). In this study various types of natural and synthetic surfactants were investigated for their influence on growth, cell surface hydrophobicity and hydrocarbon degradation. The MATH method was used to characterize cell surface hydrophobicity. In the case of non-ionic synthetic surfactants it was demonstrated that the higher degradation rate corresponded to lower MATH values. This correlation was not true for natural surfactants. The authors failed to consider the effect of surfactants in the MATH tests and this point disqualifies the results of the study. The presence of surface active agents can reduce the hydrocarbon droplet size and during the MATH test could lead to higher hydrocarbon surface area for microbial adhesion. The outcome of MATH then will be determined not only by the cell surface hydrophobicity but also by the differences in hydrocarbon-water interfacial area in various surfactant concentrations. This factor has not been accounted for in the above mentioned paper. A contact angle measurement could have provided more useful information on this condition, since it could have measured the cell surface hydrophobicity distinct from interfacial area. The same group recently published another study on the effect of the phytogenic surfactant Quillaya saponin on the biodegradation rates of different bacterial strains (Pijanowska, et al. 2007). In this study dissimilar correlations between hydrophobicity and degradation rates were found for different bacteria, however, the same problem still existed in their study and no clear comments can be made in terms of effect of adhesion on biodegradation rates based on these results.

In a recent study (Mohanty and Mukherji 2007), two bacterial cultures, *Exiguobacterium aurantiacum* and *Burkholderia cepacia*, were investigated for their rate of diesel fuel biodegradation. Although the exact uptake mechanisms had not been revealed in the paper it was stated that neither bacteria produced any external biosurfactant or bioemulsifiers. It was also mentioned, but not proven, that both bacteria uptake oil through direct contact by enhancing cell surface hydrophobicity. The non-ionic surfactant Triton X-100 was used at twice the critical micelle concentration. This surfactant is a well known adhesion inhibitor (Efroymson and Alexander 1991; Stelmack, et al. 1999). It is generally believed that such a surfactant reduces or even prevents the biodegradation of extremely low solubility n-alkanes by hydrophobic cultures (Efroymson and Alexander 1991; Zhang and Miller 1994). None the less, in this study a significant enhancement in diesel oil biodegradation was observed in the presence of Triton X-100. The decay rate of all n-alkane components of diesel oil was dramatically increased in both cultures when Triton X-100 was used. The fact that the authors did not present any adhesion or emulsification measurements parallel to degradation tests makes it difficult to comment on the significance of different factors, including adhesion, on the final outcome. Nevertheless, it can be cautiously concluded that, even for hydrophobic microorganisms that use highly insoluble compounds, the effect of adhesion may not be vital, and even greater degradation rates could be achieved with less adherence if solubilization and emulsification are enhanced. As we will mention later this conclusion should not be generalized to other cases.

Papers in part (b) reported the absence of major influence of adhesion in biodegradation of PAHs. This lack of a role for adhesion is mainly due to sufficient aqueous concentration of the substrate in the studied cases. Generally speaking, when the same number of carbon atoms in a molecule is considered, PAHs have higher aqueous solubilities than n-alkanes, but their ubiquitous presence, their persistence in environment, and their potential dangers for human health have led to many studies of bioavailability. For example, in analyzing the effect of phenanthrene availability on biodegradation (Bouchez, et al. 1995),

different means of supplying the substrate (crystal versus dissolved in non aqueous solvents) were used and the kinetic of microbial growth and degradation were analyzed. The evaluation of growth curves showed two growth phases in all systems with the first exponential phase followed by a limited growth phase. The maximal specific growth rates (μ_{max}) were independent of the method of providing the substrate (crystals or dissolved in NAPL with different interfacial areas in each case), indicating that the transfer to the aqueous phase was not a limiting factor. This behavior is expected for homogeneous growth as it was shown in Figure 2.3a. The authors hypothesized that in the second growth phase, the biomass density increased so that the demand for substrate exceeded the rate of transfer to the aqueous phase and the rate was limited by dissolution. They conducted some additional experiments with various concentrations and compared the patterns of biodegradation kinetics with a mathematical model that was developed based on transfer-controlled growth. A close correspondence between growth kinetics and rate of substrate transfer in second phase was observed. The results demonstrated that in these experiments, phenanthrene had to be transferred to the aqueous phase for degradation to occur and the interfacial transfer of phenanthrene to bacteria (and hence the adhesion to organic phase) did not play an important role.

In some cases it was revealed that adhesion had a mixed effect on biodegradation. Efroymson and Alexander (1991) studied degradation of hexadecane and naphthalene using an *Arthrobacter* strain. They found that when the bacterial adhesion was prevented by addition of a non-ionic surfactant, the hexadecane mineralization was completely inhibited. In a separate experiment they showed that naphthalene degradation rate and extent was increased in presence of the same surfactant that prevents adhesion of cells to NAPL. In other words while adhesion to oil phase was necessary for hexadecane degradation, it had no positive impact on naphthalene degradation rate.

Many PAH-degrading bacteria are not hydrophobic and do not exhibit significant adhesion to the oil-water interface. For instance, *Pseudomonas fluorescens* strain

LP6a utilizes a variety of polycyclic aromatic hydrocarbons including phenanthrene and anthracene as sole carbon and energy source (Foght and Westlake 1996; Foght and Westlake 1988). This bacterium is proved to be a hydrophilic microorganism both in terms of MATH test and contact angle measurements (Dorobantu, et al. 2004). These hydrophilic bacteria, however, degrade the more soluble PAH compounds rather than n-alkanes. For compounds with extremely low aqueous solubility the microbes need a mechanism other than uptake from the aqueous solution in order to sustain biodegradation. If there is no adhesion, then the cells need a surface active agent that facilitates the compound availability.

2.5 Reports of adhesion-dependent biodegradation

The positive influence of adhesion on biodegradation has been reported in a number of different studies (Table 2.2). These studies are classified in three groups. Group (a) includes some of the earlier studies in this field. These papers mostly focused on studying adhesion as a characteristic of microbial populations without showing what, if any, role adhesion had in the biodegradation process. Group (b) consists of papers that followed the growth kinetics and specified how adhesion and interfacial uptake changed the pattern of biodegradation and growth. These papers usually did not focus on mechanisms of adhesion or microscopic schemes by which the adhesion stimulates biodegradation of hydrocarbons. Group (c) includes papers that mostly focused on the mechanism of biodegradation and its relation to adhesion. These papers deal with issues such as the specific changes in microbial cell membrane or interaction of cells with hydrocarbons due to adhesion.

Table 2.2: References reporting microbial adhesion as an important factor in biodegradation

References	Main substrate	Supply mode	Microorganism used	Main result
(a) Earlier papers focused on adhesion as a property specific to hydrocarbon degrading microorganisms				
Bos and de Boer, 1968	Unspecified hydrocarbons	Liquid hydrocarbons	Unspecified yeast	Nearly all cells were observed to be attached to the hydrocarbon droplets
Kennedy, et al. 1975	Various alkanes and alkenes	Liquid hydrocarbons	*Acinetobacter* sp.	Adhesion of microorganism to hydrocarbons was thought to be exclusive to hydrocarbon degraders.
Nakahara, et al. 1977	Hexadecane	Liquid hydrocarbon	*Candida lipolytica* ATCC 8662	More than half of cells were attached to large oil drops. Cells dropped the interfacial tension.
Miura, et al. 1977	*n*-Decane and *n*-tetradecane	Liquid hydrocarbons	Various yeasts	Hydrocarbon degrading cells showed more adhesion to hydrocarbons than non-degraders.
Nakahara, et al. 1981	Hexadecane	Liquid hydrocarbon	*Pseudomonas aeruginosa* & yeast	Surfactants did not affect degradation rate in the case of more adherent microorganism
(b) Papers that are based on analyses of kinetics data for growth and biodegradation				
Ortega-Calvo and	Naphthalene	Dissolved in NAPL	*Arthrobacter* sp.	Degradation occurred in two stages involving free

Alexander 1994				and attached microbes
Bouchez, et al. 1997	Pyrene and fluoranthene	Dissolved in NAPL	*Rhodococcus* sp.	Data indicated uptake by both adsorbed and free cells with the interfacial uptake being predominant
Bouchez-Naïtali, et al. 2001	Hexadecane	Dissolved in NAPL	*Rhodococcus equi*	Interfacial kinetics and effect of cell flocculation on kinetics pattern were explained.
MacLeod and Daugulis 2005	Various PAHs	Dissolved in NAPL	*Mycobacterium* PYR-1	Kinetic data confirmed the interfacial growth. Cells were also observed in organic phase.
(c) Papers that are focused on mechanisms of biodegradation or mechanism of adhesion influence				
Rosenberg and Rosenberg 1981	Hexadecane	Liquid hydrocarbons	*Acinetobacter calcoaceticus*	Adhesion was a prerequisite for biodegradation
Bouchez-Naïtali, et al. 1999	Hexadecane	Liquid hydrocarbon	Various alkane degraders	47% of the 61 isolates growing on hexadecane used exclusive direct contact uptake
Wick, et al. 2001	Anthracene	Solid crystals	*Mycobacterium* sp. *LB501T*	Substrate uptake occurred from aqueous solution, and through biomass formation.
Garcia-Junco, et al. 2001	Phenanthrene	Solid and dissolved in NAPL	*Pseudomonas aeruginosa* 19SJ	Biosurfactant production and attachment increased PAH biodegradation.

Wick, et al. 2002	Anthracene	Solid crystals	*Mycobacterium sp.* LB501T	Anthracene-grown cells was more adherent to hydrophobic surfaces
Johnsen and Karlson 2004	Phenanthrene, pyrene and fluoranthene	Solid crystals	Various PAH degrading bacteria	Biofilm formation were predominant mechanism for substrate uptake
Song, et al. 2006	Various PAHs and alkanes	Liquid hydrocarbons or dissolved in NAPL	Marine PAH and alkane degradaing bacteria	Biodegrdation of larger PAHs and C10-C36 alkanes was associated primarily with the oil films and predominant bacteria adhered to the oil-coated adsorbents during biodegradation
Bihari, et al. 2007	Hexadecane	Liquid hydrocarbon	*Acinetobacter haemolyticus* AR-46	Substrate uptake occurred through fimbriae-mediated cell contact with n-alkane droplet
Obuekwe, et al. 2007	Crude oil, hexadecane and phenanthrene	Liquid hydrocarbons	*Pseudomonas aeruginosa*	Cells exist as a mixture of hydrophilic and hydrophobic forms with different abilities to degrade hydrocarbons.
Obuekwe, et al. 2007	Crude oil, hexadecane and phenanthrene	Pure and mixed liquid hydrocarbons and solid crystals	*Paenibacillus sp.* R0032A and *Burkholderia cepacia*	Cells exist as a mixture of hydrophilic and hydrophobic forms with different abilities to degrade

Obuekwe, et al. 2008	Crude oil, hexadecane and phenanthrene	Pure and mixed liquid hydrocarbons and solid crystals	*Pseudomonas aeruginosa*	Hydrophobic subpopulations of the bacteria had greater hydrocarbon-utilizing ability than hydrophilic ones, or the parental strain.
Obuekwe, et al. 2009	Crude oil	Various hydrocarbon degrading bacteria	Various hydrocarbon degrading bacteria	Significantly high correlation between the ability to degrade crude oil and CSH was observed among 46 crude oil degrading isolates.

Early studies of yeast cells growing in a medium containing liquid hydrocarbons demonstrated that most of the cells attach to the hydrocarbon droplets (Bos and de Boer, 1968). The authors did not directly measure the adhesion of cells to hydrocarbon; however, based on microscopic observation of an unspecified lipid layer around attached yeast cells they concluded that the direct contact was the main mechanism of hydrocarbon up-take. Kennedy, et al. (1975) and Miura, et al. (1977) found that the ability of tested microbes to adhere to hydrocarbon correlated with their ability to utilize them. Other researchers, using different microorganisms, showed that degradation of hydrocarbons was not related to direct contact between microorganisms and hydrocarbons (Wodzinski and Coyle 1974; Yoshida, et al. 1971). These discrepancies raised questions about the mechanism of interactions between biodegradation and adhesion. Studies that analyze the kinetics patterns of biodegradation can provide some further insights into the uptake mode of hydrocarbons bay microorganisms.

The interfacial uptake mechanism was investigated for degradation of pyrene dissolved in NAPL by a *Rhodococcus* strain (Bouchez, et al. 1997) using oxygen consumption rate. The authors claimed that oxygen consumption corresponded with substrate uptake and that respirometric data can be used to track microbial growth and degradation kinetics. Two successive phases of exponential oxygen consumption during the degradation of pyrene were observed. The rate of substrate uptake was estimated through these data. In the first exponential phase the pyrene uptake rate was consistent with the rate of pyrene partitioning from NAPL to the aqueous phase under abiotic conditions. In the second exponential phase, however, the rate increased to values higher than expected from abiotic experiments. The specific growth rate (estimated using oxygen consumption data) in the second exponential phase was independent of pyrene concentration but increased with the volume of NAPL. These data suggest that increased interfacial area and hence the uptake from the interface increased the degradation rate. It was also concluded that the pyrene degradation cannot be solely explained by the abiotic partitioning data. A dynamic equilibrium was observed between the

adsorbed and planktonic cells with the interfacial uptake being predominant in the experiments. Authors investigated the degradation of fluoranthene in the same work and discovered a similar pattern.

Biodegradation of PAHs by a *Mycobacterium* PYR-1 was also investigated using kinetic data obtained in a two phase partitioning bioreactor (MacLeod and Daugulis 2005). The impact of agitation speed, substrate concentration and simultaneous presence of a water soluble substrate and PAHs were examined in this study. Higher agitation speed was found to favour the degradation rate due to the increase in surface area due to better mixing. No significant change was perceived in biodegradation rate when different concentrations of PAHs were used. When glucose was provided as a water soluble substrate along with a metabolizable organic solvent (Bis(ethylhexyl)sebacate), the uptake of glucose was inhibited. A non-toxic non-metabolized organic solvent (HMN) also hindered the glucose uptake, indicating affinity of microorganism toward the NAPL interface even when there was no substrate present in the NAPL. Microscopic examination of the bacterial culture using fluorescence microscopy revealed that bacteria not only adsorbed to the interface, but also appeared inside the organic phase. Contact angles of around 90° were measured for the cell surface. The high contact angle values were thought to be the main reason for bacterial adhesion and transfer to oil, and explained the reduction and inhibition of glucose degradation in presence of an organic phase. According to the kinetic behaviour previously described for interfacial growth models (Figure 2.3b), the positive influence of agitation rate, the inability to change linear growth rate with increasing substrate concentration, the negative effect of solvent on aqueous compound degradation along with very hydrophobic nature of *Mycobacterium* PYR-1, all suggested an interfacial mode of growth.

In a different study focused on kinetics of hexadecane degradation, the growth kinetics were analyzed for different strains of *Rhodococcus equi* (Bouchez-Naïtali, et al. 2001). None of the four studied strains were biosurfactant producers. In microscopic observation of cultures (without showing the data) authors stated

that most of the bacterial cells were found around oil drops or as aggregates in the oil phase. All strains, except one, showed adhesion of higher than 80% as measured by the MATH test. The one strain with 77% adhesion did not form many aggregates and was dispersed in the aqueous medium. The flocculating strains demonstrated a very short exponential growth phase (as characterized by oxygen consumption data) and the specific rate was not a function of the interfacial area. Although they showed a second linear phase of growth, the growth rate during this linear phase was not a function of interfacial area. In contrast, the non flocculating culture gave a longer exponential growth phase and the specific growth rate depended on the volume of NAPL (as in Figure 2.3b). For the second phase of the bacterial growth, linear growth was only observed for some NAPL volumes. No explanation was offered in this regard. The authors concluded that while the flocculating cultures did not behave according to an interfacial kinetic model, the non flocculating culture was in good agreement with that. This study suggests that factors such as flocculation can be as important in determining biodegradation kinetics as adhesion to the oil-water interface. Flocculation of the microbes will give much poorer transport of low-solubility substrates and this can counter the positive impact of adhesion to increase the mass transfer. In this case macroscopic kinetics will not be enough to identify the uptake mechanism and more detailed mechanistic analysis is required.

In investigating the interfacial uptake of hydrocarbons in oil-water systems, measurement and control of surface area poses an enormous challenge. The studies mentioned above dealt with interfacial area qualitatively by controlling volume of NAPL, mixing intensity or concentration of solid substrate. There are very few studies that conducted an accurate surface measurement during liquid-liquid biodegradation. One exception examined the effect of dispersing the oil phase on the biodegradation by *Acinetobacter sp.* of n-heneicosane($C_{21}H_{44}$) dissolved in non-biodegradable NAPL (pristane) was assessed in a stirred tank bioreactor (Hori, et al. 2002a). The primary goal of the paper was to investigate the role of impeller speed on the specific area of the oil phase and the biodegradation of NAPL dissolved n-heneicosane. The reactor was operated with

impeller speeds of 300, 600 and 900 rpm, and the growth and biodegradation were measured at various time intervals. The authors measured the droplet size distribution using an innovative sampling device they had developed earlier (Hori, et al. 2002b). This enabled them to calculate specific surface area of oil phase in each case. Parallel biodegradation experiments demonstrated that in spite of an increase in the specific surface area, both the growth rate of bacteria and the rate of n-heneicosane degradation decreased with the increase in impeller speed. The number of free cells and the number of attached bacteria was measured and control experiments ruled out the possibility of direct cell damage by shear stress. Analysis of the number of attached cells in different experiments also revealed that the ratio of adhered cells to total cells was higher at 300 rpm than at 900 rpm. Addition of surfactant at concentrations below the CMC inhibited the biodegradation rate. These results show that at high shear caused by enhanced impeller speed, adhesion is compromised and hence the biodegradation rate is reduced. It indicates that any effort to increase the specific surface area should also consider the possible role of cell adhesion and the uptake mode of bacterial population.

Another method to assess biodegradation mechanisms is to analyze the differences in cell surface properties of pure or mixed bacterial population. Bouchez and co-workers studied the relative distribution of substrate uptake modes among the long chain alkane degrading bacteria (Bouchez-Naïtali, et al. 1999). They measured cell hydrophobicity (by MATH test), interfacial and surface tensions and production of glycolipidic extracellular biosurfactants in order to characterize the hydrocarbon uptake mode. These properties were examined in cultures on an insoluble (hexadecane) and on a soluble (glycerol or succinate) carbon source for a 23 representative bacterial strains. It was assumed that substrate uptake from the aqueous solution was not possible because of the extremely low solubility of long chain alkanes. The direct contact and the biosurfactant-facilitated uptake modes were the only possible mechanisms to supply substrate to bacteria. Combination of the two mechanisms was also considered to be a likely case. The important point to consider about this paper is

the fact that all the uptake characterizations were based on measuring interfacial properties and were not coupled with the biodegradation measurements, i.e. the uptake was considered to be through direct interfacial mode only because of bacterial hydrophobicity and lack of surfactant production while no microscopic observation or biodegradation tests were conducted to support the inferred uptake mode. A somewhat similar study conducted recently in which biodegradation tests were conducted along with measurements of cell surface hydrophobicity (CSH) (Obuekwe, et al. 2009). Results showed a strong correlation between CSH and the ability to significantly degrade hydrocarbons. Out of 46 hydrocarbon degrading bacteria, 74% of isolates that degraded more than 40% of crude oil possessed high level of CSH. In fact the growth kinetics, as we mentioned before, can be based on much more complicated mechanisms and may not be predicable by measuring some macroscopic cell surface properties. The fact that a bacterial strain is characterized hydrophobic by some macroscopic measurements shuch as MATH test, does not always implies a particular uptake mode. For instance, in a recently published paper, the isolation of a hydrophilic alkane-degrading *Acinetobacter haemolyticus* strain was reported where the isolated cells were characterized as hydrophilic since a majority of cells were planktonic (Bihari, et al. 2007) and not associated with the alkane phase.

Existence of physiologically different sub-populations within a single culture was also pointed out during studies of axenic cultures (Ortega-Calvo and Alexander 1994). An interesting approach to both separate these different sub-populations and get a better insight in the effect of adhesion in their growth on hydrocarbons was offered using MATH test (Rosenberg and Rosenberg 1981). In this study authors performed sequential MATH tests to isolate a mutant of *Acinetobacter calcoaceticus* RAG-1 that was deficient in its ability to attach to hydrocarbons. In its natural state, RAG-1 adheres avidly to a wide range of hydrocarbons (Rosenberg, et al. 1980). The authors observed that sequential multi-step partitioning of bacterial cells in an octadecane-water system and successive enrichment of the cells remaining in the aqueous phase led to a hydrophilic strain, MR-481, which showed no significant adhesion to hydrocarbons. Later

experiments showed that mutant MR-481 was very similar to the wild type in terms of production of extracellular emulsifying agent during growth on ethanol, colony and cell morphology, and sensitivity to two specific bacteriophages. The two strains also showed very similar growth kinetics when grown on water soluble carbon sources. Nonetheless when hexadecane was used as sole carbon and energy source, the non-adhering mutant did not exhibit growth for 54 hours while RAG-1 grew with no significant lag. When an emulsifying agent was added to the medium, strain MR-481 started to grow on hexadecane after about six hours. Based on these observations authors concluded that adhesion was a prerequisite for growth on liquid hydrocarbons when there was limited emulsification.

A similar method was used by (Obuekwe, et al. 2007; Obuekwe, et al. 2007; Obuekwe, et al. 2008) to fractionate three different bacterial strains (*Paenibacillus sp.* R0032A and *Burkholderia cepacia* and *Pseudomonas aeruginosa*). Unlike the previous work (Rosenberg and Rosenberg 1981), four different variants were separated from each bacterial strain by performing sequential MATH tests. These variants were then used in degradation of crude oil, hexadecane and phenanthrene. The degradation experiments revealed that the ability of the variants to grow on all three hydrocarbons correlated their hydrophobicity. The more hydrophobic the variant and the better the adhesion, the higher the degradation rate. The study went further by looking at the structural differences in cell surface between variants of one bacterial strain, the *Pseudomonas aeruginosa* (Obuekwe, et al. 2007). Observation of cell surface by both scanning and transmission electron microscopy revealed that the most hydrophobic variant was densely covered by fimbriae whereas the hydrophilic variant was enclosed with an amorphous exopolymeric polysaccharide. The parent strain was covered by mixture of surface structures. The authors showed that when different cell fractions are regrown in nutrient broth (immediately after fractionation), they maintained their relative differences. Based on this fact, it was argued that these differences are sufficiently stable and support the existence of genetically diverse clones of the

organisms within parental strains. This study illustrates the significance of the extracellular structures on bacteria for their attachment to oil-water interfaces.

Bacterial strategies to promote bioavailability were also thoroughly investigated for uptake of PAH from crystals (Johnsen and Karlson 2004; Tongpim and Pickard 1996; Wick, et al. 2002; Wick, et al. 2001). Since the focus of our study is on adhesion to oil-water interface we do not review these papers in detail, but these studies showed that formation of biofilm on PAH crystals was a major mechanism to overcome low substrate bioavailability. As in the study by (Hori, et al. 2002a) of adhesion of bacteria to droplets in an agitated reactor, these studies found that biofilm formation depends on experimental conditions such as mixing.

The preceding discussion shows that microbial adhesion to the oil-water interface can play an important role in biodegradation of less soluble hydrocarbons. The importance of adhesion varies from case to case depending on the microorganism, substrate and experimental conditions. While each experiment approach reveals different features of the interactions between adhesion and biodegradation, more definitive results have been obtained by studies that are focused on microscopic mechanisms rather than macroscopic analyses of microbial growth.

2.6 Modifying microbial adhesion

The efforts to modify the cell-surface properties have been made for different reasons. In many medical applications it is desired to change these properties to prevent the adhesion to biomaterials and body implants (Jansen, et al. 1987; Katsikogianni and Missirlis 2004). There are few applications in which promoting microbial adhesion could offer some benefits. Increasing the adhesion of bacteria to oil has resulted in the development of two-phase water and oil mouthwashes that remove bacteria from the mouth (Goldberg and Rosenberg 1991). Recently bacterial cells have been seriously considered as flotation and flocculation agents in mineral processing. The fact that microorganisms can modify the surfaces of minerals made them a potential candidate for applications such as flotation collectors, flotation depressants and activators. These applications are based on

the selective adhesion of bacterial cells to the mineral particles. Separation of fine hydrophobic materials from hydrophilic mineral particles in coal production (Raichur, et al. 1996) and removing pyrite from mixtures of sulfide minerals (Nagaoka, et al. 1999) are just some of the many examples of these applications (Smith and Miettinen 2006). Modification of surface hydrophobicity of microorganisms can make them more suitable and specific for this separation processes and can offer significant improvement in dealing with these issues. Stimulation of microbial adhesion to the oil-water interface has also potential implications for bioremediation of less soluble hydrocarbons. In spite of extensive research on the possible relation between microbial adhesion and biodegradation of hydrocarbons, controlled enhancement of cell adhesion to promote oil biodegradation or bio-processing has received little attention.

While many compounds have been reported to inhibit microbial adhesion to hydrophobic surfaces (Babu, et al. 1986; Chen and Zhu 2004; Stelmack, et al. 1999), only a few have been found to promote this phenomenon. Some examples of the latter group are simple ionic compounds such as ammonium sulphate (Rosenberg 1984), cationic surfactants like cetylpyridinium chloride (CPC) (Goldberg, et al. 1990b), cationic polymers like poly-L-lysine or chitosan (Goldberg, et al. 1990a), and long chain alcohols like 1-dodecanol (Marchesi, et al. 1994a; Marchesi, et al. 1994b; Neumann, et al. 2006). Goldberg and coworkers (Goldberg, et al. 1990a; Goldberg, et al. 1990b) studied the effects of several cationic compounds on adhesion of disparate species of bacteria using the MATH test to characterize hydrophobicity of microbial surfaces. Research on the effect of long chain alcohols originated from a study conducted to investigate the adhesion of a microbial population to sediments in response to biodegradation of sodium dodecyl sulphate, SDS (Marchesi, et al. 1994a). During this study, Marchesi and coworkers noticed that the presence of 1-dodecanol, a primary intermediate of SDS biodegradation, increased the proportion of microorganisms adhered to hydrophobic sediments. The same group later used hydrophobic interaction chromatography and the MATH test to characterize hydrophobicity of the cell surface (Marchesi, et al. 1994b). They showed that a correlation exists between

adhesion and concentration of 1-dodecanol, but the effect of alcohols on bacterial cell surfaces was not systematically investigated in these studies.

Microbial adhesion can offer an interesting opportunity on selection and genetic modification of microorganisms for environmental applications. Advances in genetic engineering have encouraged researchers to design superior microorganisms for bioremediation purposes (Chen and Wilson 1997; Haro and de Lorenzo 2001; Pieper and Reineke 2000). With the current progresses in the identification of the functional genes and biological mechanisms that influence microbial adhesion (Robleto, et al. 2003; Tobe and Sasakawa 2002; Vacheethasanee, et al. 1998) this information can be combined with other genetic modifications to design engineered microorganisms for bioremediation of less soluble compounds. The large scale application of these genetically engineered microorganisms (GEMs) is still very restricted by civic regulations and hence it is better to concentrate at present on enrichment of naturally existing strains and/or chemical modification of cell surface.

Adhesion to hydrocarbons has to be taken into consideration when selecting appropriate strain for biodegradation of oil pollutions, as it has been shown the non-adherent variants of bacteria may not grow well on less soluble hydrocarbons (Obuekwe, et al. 2007; Obuekwe, et al. 2007; Rosenberg and Rosenberg 1981). This is especially important for in situ applications where mixing is very limited.

There are still many unexplored areas in the field of modifying microbial cells to enhance adhesion. These studies could provide a new approach towards a better understanding of how adhesion can affect different activities of microorganism, and also can open a new window to employ microbes in fields they have not been primarily thought to be useful.

2.7 Overall discussion on the importance of adhesion in biodegradation

Microbial adhesion is a multi-factorial phenomenon since it involves and depends on various biological, physical and chemical features. This chapter was aimed at analyzing the numerous studies devoted to the assessment of the microbial adhesion and its influence on the microbial activities on hydrocarbons. Different kinds of influences have been reported throughout various studies that make it impossible to draw a simple conclusion. In an early review book published in 1984 (Marshal 1984), the authors came to a conclusion that although attachment to a surface is an important factor in microbial activity the mechanism and extent of this factor is not completely known based on the knowledge at the time. In the context of hydrocarbon biodegradation and microbial adhesion, this statement still holds today. Although we now have much more information about adhesion to oil-water interface and to the solid hydrocarbons, as is usually the case in science, new questions are arising while we try to answer the old ones. For the sake of our discussion, some of the facts about the role of adhesion in biodegradation can be outlined as follows:

a. Adhesion to hydrocarbons is not limited to hydrocarbon degrading microorganisms.

b. In most cases, adhesion to hydrocarbons is not a prerequisite for hydrocarbon degradation. Different hydrocarbon utilising bacteria have been found that have very poor adhesion to their substrates and are able to use poorly water soluble compounds.

c. Detaching the microorganism cells from the oil-water interface can reduce the rate of growth and degradation.

d. Microbial adhesion can benefit growth on and degradation of hydrocarbons, especially in case of very poorly water soluble hydrocarbons such as n-alkanes and larger PAHs. The effect of adhesion has been shown to be more profound in environments with limited

emulsification. In the absence of significant emulsification agents, adhesion is expected to be a necessary condition for growth on extremely low solubility hydrocarbons.

e. Natural populations of hydrocarbon degrading bacteria are found to be heterogeneous regarding their cell surface hydrophobicity and adhesion characteristics. As a result, in a condition of low solubility and low emulsification, the cells with higher adhesion ability can become dominant. As a result the role of more adherent bacteria can increase in bioremediation of less soluble hydrocarbons, perhaps beyond what is already predicted from laboratory experiments based on overall bacterial population.

f. Although some correlations have been found for the growth and biodegradation kinetics of adherent bacteria, reliable conclusions can not be derived by the presence or lack of these kinetic evidences unless they are coupled with either direct observation of bacteria at the interface or accurate measurement of bacterial adhesion to oil-water interface.

The actual importance of adhesion in biodegradation processes may have been downplayed in many studies. Most studies have been focused on the role of surface active compounds to increase mass transfer, without considering their counter effect on spreading the contaminant in the environment and possibility of detaching cells from the interface. Laboratory experiments are usually conducted at small scale with adequate mixing, however, in large scale bioremediation applications this is rarely the case. At a contaminated site, the contaminant is usually spread over large area and the economic considerations prevent using any artificial mixing facilities. These conditions amplify the importance of adhesion role in the process. In other words, in large scale projects for hydrocarbon bioremediation, the microbial adhesion may offer a better approach to overcome bioavailability limitations and play much more important role in increasing the overall mass transfer of hydrocarbons to the microorganisms. This fact reveals the

value of conducting more research in developing bioremediation strategies that consider using the adhesion as an asset to benefit the process.

New and innovative techniques to measure and characterize microbial adhesion continue to emerge. Differentiation of the various biological and physico-chemical transformations that take place at the same time as the adhesion process is still a significant challenge. The development of new techniques in future will enhance our knowledge of adhesion mechanisms and their interaction with other biological and physical processes. There is a great need to sharpen the focus on details of the microbial adhesion and obtain more deterministic data about the role it plays in different processes, and to enable the development of innovative approaches to bioremediation and bioprocessing of hydrocarbons.

2.8 References

1. **Ahimou F, Denis FA, Touhami A, Dufrene YF (2002).** Probing microbial cell surface charges by atomic force microscopy. *Langmuir* 18:9937-9941.

2. **Albertsson PA (1986).** Partition of Cell Particles and Macromolecules New York:Wiley & Sons, Inc. 346 p.

3. **An YH, Friedman RJ (1998).** Concise review of mechanisms of bacterial adhesion to biomaterial surfaces. *J Biomed Mater Res* 43:338-348.

4. **Atlas RM, Philp J (2005).** Bioremediation; applied microbial solutions for real-world environmental cleanup. Washington, D.C.:ASM Press. 366 p.

5. **Babu JP, Beachey EH, Simpson WA (1986).** Inhibition of the interaction of Streptococcus sanguis with hexadecane droplets by 55- and 60-kilodalton hydrophobic proteins of human saliva. *Infect Immun* 53:278-284.

6. **Baror Y (1990).** The Effect of Adhesion on Survival and Growth of Microorganisms. *Experientia* 46:823-826.

7. **Bihari Z, Pettko-Szandtner A, Csanadi G, Balazs M, Bartos P, Kesseru P, Kiss I, Mecs I (2007).** Isolation and characterization of a novel n-alkane-degrading strain, Acinetobacter haemolyticus AR-46. *Z Naturforsch , C, J Biosci* 62:285-295.

8. **Bolshakova AV, Kiselyova OI, Yaminsky IV (2004).** Microbial surfaces investigated using atomic force microscopy. *Biotechnol Prog* 20:1615-1622.

9. **Bos P, de Boer, Willemina E. (1968).** Some aspects of the utilization of hydrocarbons by yeasts. *Antonie Van Leeuwenhoek* 34:241-243.

10. **Bos R, van der Mei,Henny C., Busscher HJ (1999).** Physico-chemistry of initial microbial adhesive interactions – its mechanisms and methods for study. *FEMS Microbiol Rev* 23:179-229.

11. **Bouchez M, Blanchet D, Vandecasteele JP (1997).** An interfacial uptake mechanism for the degradation of pyrene by a Rhodococcus strain. *Microbiology* 143:1087-1093.

12. **Bouchez M, Blanchet D, Vandecasteele JP (1995).** Substrate Availability in Phenanthrene Biodegradation - Transfer Mechanism and Influence on Metabolism. *Appl Microbiol Biotechnol* 43:952-960.

13. **Bouchez-Naïtali M, Blanchet D, Bardin V, Vandecasteele JP (2001).** Evidence for interfacial uptake in hexadecane degradation by Rhodococcus equi: the importance of cell flocculation. *Microbiology* 147:2537-2543.

14. **Bouchez-Naïtali M, Rakatozafy H, Marchal R, Leveau JY, Vandecasteele JP (1999).** Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. *J Appl Microbiol* 86:421-428.

15. **Bressler DC, Gray MR (2003).** Transport and Reaction Processes in Bioremediation of Organic Contaminants. 1. Review of Bacterial Degradation and Transport. *Int j chem react eng* 1: R3:

16. **Busscher HJ, van der Mei,Henny C. (2006).** Microbial Adhesion in Flow Displacement Systems. *Clin Microbiol Rev* 19:127-141.

17. **Chakravarty M, Singh HD, Baruah JN (1975).** A kinetic model for microbial growth on liquid hydrocarbons. *Biotechnol Bioeng* 17:399-412.

18. **Chen G, Zhu H (2004).** Bacterial deposition in porous medium as impacted by solution chemistry. *Res Microbiol* 155:467-474.

19. **Chen SL, Wilson DB (1997).** Genetic engineering of bacteria and their potential for Hg2+ bioremediation. *Biodegradation* 8:97-103.

20. **Christensen GD, Simpson WA, Anglen JO, Gainor BJ (2000).** Methods for Evaluating Attached Bacteria and Biofilms: An Overview. In: An YH and Friedman RJ, editors. Handbook of bacterial adhesion : principles, methods, and applications. Totowa, N.J.:Humana Press. p 213-234.

21. **Chrzanowski Ł, Kaczorek E, Olszanowski A (2005).** Relation between *Candida maltosa* Hydrophobicity and Hydrocarbon Biodegradation. *World J Microb Biot* 21:1273-1277.

22. **Cooper DG, Kosaric N (1982).** Surface Activity of the Cells and Extracellular Lipids of Corynebacterium fascians CF15. *J Ferment Technol* 60:19-24.

23. **Costerton JW, Ingram JM, Cheng KJ (1974).** Structure and function of the cell envelope of gram-negative bacteria. *Microbiol Mol Biol Rev* 38:87-110.

24. **Dorobantu LS, Yeung AKC, Foght JM, Gray MR (2004).** Stabilization of Oil-Water Emulsions by Hydrophobic Bacteria. *Appl Environ Microbiol* 70:6333-6336.

25. **Doyle RS, Rosenberg M (1990).** Microbial cell surface hydrophobicity.

26. **Dunn IJ (1968).** An interfacial kinetics model for hydrocarbons oxidation. *Biotechnol Bioeng* 10:891-894.

27. **Edwards DA, Luthy RG, Liu Z (1991).** Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. *Environ Sci Technol* 25:127-133.

28. **Efroymson RA, Alexander M (1991).** Biodegradation by an Arthrobacter Species of Hydrocarbons Partitioned into an Organic-Solvent. *Appl Environ Microbiol* 57:1441-1447.

29. **Eganhouse RP, Calder JA (1976).** Solubility of Medium Molecular-Weight Aromatic-Hydrocarbons and Effects of Hydrocarbon Co-Solutes and Salinity. *Geochim Cosmochim Acta* 40:555-561.

30. **Fang HHP, Chan KY, Xu LC (2000).** Quantification of bacterial adhesion forces using atomic force microscopy (AFM). *J Microbiol Methods* 40:89-97.

31. **Foght JM, Westlake, D. W. S. (1996).** Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of Pseudomonas fluorescens. *Biodegradation* 7:353-366.

32. **Foght JM, Westlake DWS (1988).** Degradation of Polycyclic Aromatic-Hydrocarbons and Aromatic Heterocycles by a Pseudomonas Species. *Can J Microbiol* 34:1135-1141.

33. **Fredriksson C, Khilman S, Kasemo B, Steel DM (1998a).** In vitro real-time characterization of cell attachment and spreading. *J Mater Sci Mater Med* 9:785-788.

34. **Fredriksson C, Kihlman S, Rodahl M, Kasemo B (1998b).** The Piezoelectric Quartz Crystal Mass and Dissipation Sensor: A Means of Studying Cell Adhesion. *Langmuir* 14:248-251.

35. **Garcia-Junco M, Gomez-Lahoz C, Niqui-Arroyo J-, Ortega-Calvo J- (2003).** Biosurfactant- and Biodegradation-Enhanced Partitioning of Polycyclic Aromatic Hydrocarbons from Nonaqueous-Phase Liquids. *Environ Sci Technol* 37:2988-2996.

36. **Garcia-Junco M, De Olmedo E, Ortega-Calvo J (2001).** Bioavailability of solid and non-aqueous phase liquid (NAPL)-dissolved phenanthrene to the biosurfactant-producing bacterium Pseudomonas aeruginosa 19SJ. *Environ Microbiol* 3:561-569.

37. **Goldberg S, Doyle RJ, Rosenberg M (1990a).** Mechanism of enhancement of microbial cell surface hydrophobicity by cationic polymers. *J Bacteriol* 172:5650-5654.

38. **Goldberg S, Konis Y, Rosenberg M (1990b).** Effect of cetylpyridinium chloride on microbial adhesion to hexadecane and polystyrene. *Appl Environ Microbiol* 56:1678-1682.

39. **Goldberg S, Rosenberg M (1991).** Bacterial desorption by commercial mouthwashes vs two-phase oil:water formulations *Biofouling* 3:193-198.

40. **Harms H, Bosma TNP (1997).** Mass transfer limitation of microbial growth and pollutant degradation. *Journal of Industrial Microbiology and Biotechnology* 18:97-105.

41. **Haro MA, de Lorenzo V (2001).** Metabolic engineering of bacteria for environmental applications: construction of Pseudomonas strains for biodegradation of 2-chlorotoluene. *J Biotechnol* 85:103-113.

42. **Hermansson M (1999).** The DLVO theory in microbial adhesion. *Colloids Surf B* 14:105-119.

43. **Hori K, Matsuzaki Y, Tanji Y, Unno H (2002a).** Effect of dispersing oil phase on the biodegradability of a solid alkane dissolved in non-biodegradable oil. *Appl Microbiol Biotechnol* 59:574-579.

44. **Hori K, Yamaji M, Tanji Y, Unno H, Ishikawa Y (2002b).** A new device for in situ sampling of dispersion for image analysis. *Measurement Science and Technology* 13:N27-N29.

45. **Jansen B, Peters G, Pulverer G (1987).** Mechanisms and Clinical Relevance of Bacterial Adhesion to Polymers. *J Biomater Appl* 2:520-543.

46. **Johnsen AR, Karlson U (2004).** Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. *Appl Microbiol Biotechnol* 63:452-459.

47. **Jones DS, Adair CG, Mawhinney WM, Gorman SP (1996).** Standardisation and comparison of methods employed for microbial cell surface hydrophobicity and charge determination. *Int J Pharm* 131:83-89.

48. **Katsikogianni M, Missirlis YF (2004).** Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cell Mater* 8:37-57.

49. **Kennedy RS, Finnerty WR, Sudarsanan K, Young RA (1975).** Microbial Assimilation of Hydrocarbons .1. Fine-Structure of a Hydrocarbon Oxidizing Acinetobacter Sp. *Arch Microbiol* 102:75-83.

50. **Koch AL (1990).** Diffusion - the Crucial Process in Many Aspects of the Biology of Bacteria. *Adv Microb Ecol* 11:37-70.

51. **Krćmāŕ P, Kubātovā A, Votruba J, Erbanovā P, Novotnŷ Ć, Vāclav Š (1999).** Degradation of polychlorinated biphenyls by extracellular enzymes of *Phanerochaete chrysosporium* produced in a perforated plate bioreactor. *World Journal of Microbiology and Biotechnology* 15:269-276.

52. **Krekeler C, Ziehr H, Klein J (1989).** Physical methods for characterization of microbial cell surfaces. *Cellular and Molecular Life Sciences (CMLS)* 45:1047-1055.

53. **Kuehn M, Hausner M, Bungartz H, Wagner M, Wilderer PA, Wuertz S (1998).** Automated Confocal Laser Scanning Microscopy and Semiautomated Image Processing for Analysis of Biofilms. *Appl Environ Microbiol* 64:4115-4127.

54. **Mackay D, Shiu WY, Wolkoff AW (1975).** Gas Chromatographic Determination of Low Concentrations of Hydrocarbons in Water by Vapor Phase Extraction. *ASTM Spec Tech Publ* 573:251-258.

55. **MacLeod CT, Daugulis AJ (2005).** Interfacial effects in a two-phase partitioning bioreactor: degradation of polycyclic aromatic hydrocarbons (PAHs) by a hydrophobic Mycobacterium. *Process Biochem* 40:1799-1805.

56. **Marchesi JR, Owen SA, White GF, House WA, Russell NJ (1994a).** SDS-degrading bacteria attach to riverine sediment in response to the surfactant or its primary biodegradation product dodecan-1-ol. *Microbiology* 140:2999-3006.

57. **Marchesi JR, White GF, House WA, Russell NJ (1994b).** Bacterial cell hydrophobicity is modified during the biodegradation of anionic surfactants. *FEMS Microbiol Lett* 124:387-392.

58. **Marshal KC (1984).** Microbial adhesion and aggregation. Berlin, Springer-Verlag. 423p.

59. **Marshal KC, Stout R, Mitchell R (1971).** Mechanism of the initiai events in the sorption of marine bacteria to surfaces. *J Gen Microbiol* 68:337-348.

60. **Marshall KC (1986).** Adsorption and adhesion processes in microbial growth at interfaces. *Adv Colloid Interface Sci* 25:59-86.

61. **Marshall KC (1980).** Microorganisms and Interfaces. *Bioscience* 30:246-249.

62. **Meinders JM, van der Mei HC, Busscher HJ (1995).** Deposition Efficiency and Reversibility of Bacterial Adhesion under Flow. *J Colloid Interface Sci* 176:329-341.

63. **Miura Y, Okazaki M, Hamada S, Murakawa S, Yugen R (1977).** Assimilation of liquid hydrocarbon by microorganisms. I. Mechanism of hydrocarbon uptake. *Biotechnol Bioeng* 19:701-714.

64. **Mohanty G, Mukherji S (2007).** Effect of an emulsifying surfactant on diesel degradation by cultures exhibiting inducible cell surface hydrophobicity. *J Chem Technol Biotechnol* 82:1004-1011.

65. **Mudd S, Mudd BH (1924).** The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. *J Exp Med* 40:633-645.

66. **Mulligan CN, Yong RN, Gibbs BF (2001).** Surfactant-enhanced remediation of contaminated soil: a review. *Eng Geol* 60:371-380.

67. **Nagaoka T, Ohmura N, Saiki H (1999).** A Novel Mineral Flotation Process Using Thiobacillus ferrooxidans. *Appl Environ Microbiol* 65:3588-3593.

68. **Nakahara T, Erickson LE, Gutierrez JR (1977).** Characteristics of hydrocarbon uptake in cultures with two liquid phases. *Biotechnol Bioeng* 19:9-25.

69. **Nakahara T, Hisatsuka K, Minoda Y (1981).** Emulsification on Growth and Respiration of Microorganisms in Hydrocarbon Media. *J Ferment Technol* 59:415-418.

70. **Neufeld RJ, Zajic JE, Gerson DF (1980).** Cell Surface Measurements in Hydrocarbon and Carbohydrate Fermentations. *Appl Environ Microbiol* 39:511-517.

71. **Neufeld RJ, Zajic JE (1984).** The surface activity of *Acinetobacter calcoaceticus* sp. 2CA2. *Biotechnol Bioeng* 26:1108-1113.

72. **Neumann G, Cornelissen S, van Breukelen F, Hunger S, Lippold H, Loffhagen N, Wick LY, Heipieper HJ (2006).** Energetics and Surface Properties of Pseudomonas putida DOT-T1E in a Two-Phase Fermentation System with 1-Decanol as Second Phase. *Appl Environ Microbiol* 72:4232-4238.

73. **Norman RS, Frontera-Suau R, Morris PJ (2002).** Variability in Pseudomonas aeruginosa Lipopolysaccharide Expression during Crude Oil Degradation. *Appl Environ Microbiol* 68:5096-5103.

74. **Novotný Č, Svobodová K, Erbanová P, Cajthaml T, Kasinath A, Lang E, Šašek V (2004).** Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biology and Biochemistry,* 36:1545-1551.

75. **Obuekwe CO, Al-Jadi ZK, Al-Saleh E (2007).** Insight into heterogeneity in cell-surface hydrophobicity and ability to degrade hydrocarbons among cells of two hydrocarbon-degrading bacterial populations. *Can J Microbiol* 53:252-260.

76. **Obuekwe CO, Al-Jadi ZK, Al-Saleh ES (2008).** Comparative hydrocarbon utilization by hydrophobic and hydrophilic variants of Pseudomonas aeruginosa. *J Appl Microbiol* 105:1876-1887.

77. **Obuekwe CO, Al-Jadi ZK, Al-Saleh ES (2007).** Sequential hydrophobic partitioning of cells of Pseudomonas aeruginosa gives rise to variants of increasing cell-surface hydrophobicity. *FEMS Microbiol Lett* 270:214-219.

78. **Obuekwe CO, Al-Jadi ZK, Al-Saleh ES (2009).** Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. *Int Biodeterior Biodegrad* 63:273-279.

79. **Ofek I, Doyle RJ (1994).** Bacterial Adhesion to Cells and Tissues. New York:Chapman & Hall Inc. 578 p.

80. **Ortega-Calvo JJ, Alexander M (1994).** Roles of bacterial attachment and spontaneous partitioning in the biodegradation of naphthalene initially present in nonaqueous-phase liquids. *Appl Environ Microbiol* 60:2643-2646.

81. **Pieper DH, Reineke W (2000).** Engineering bacteria for bioremediation. *Curr Opin Biotechnol* 11:262-270.

82. **Pijanowska A, Kaczorek E, Chrzanowski Å, Olszanowski A (2007).** Cell hydrophobicity of Pseudomonas spp. and Bacillus spp. bacteria and hydrocarbon biodegradation in the presence of Quillaya saponin. *World J Microbiol Biotechnol* 23:677-682.

83. **Power L, Itier S, Hawton M, Schraft H (2007).** Time Lapse Confocal Microscopy Studies of Bacterial Adhesion to Self-Assembled Monolayers and Confirmation of a Novel Approach to the Thermodynamic Model. *Langmuir* 23:5622-5629.

84. **Raichur AM, Misra M, Bukka K, Smith RW (1996).** Flocculation and flotation of coal by adhesion of hydrophobic Mycobacterium phlei. *Colloids Surf B* 8:13-24.

85. **Razatos A, Ong Y, Sharma MM, Georgiou G (1998).** Molecular determinants of bacterial adhesion monitored by atomic force microscopy. *Proc Natl Acad Sci U S A* 95:11059-11064.

86. **Reid G, Cuperus PL, Bruce AW, van der Mei,H C., Tomeczek L, Khoury AH, Busscher HJ (1992).** Comparison of contact angles and

adhesion to hexadecane of urogenital, dairy, and poultry lactobacilli: effect of serial culture passages. *Appl Environ Microbiol* 58:1549-1553.

87. **Robleto EA, Lopez-Hernandez I, Silby MW, Levy SB (2003).** Genetic analysis of the AdnA regulon in Pseudomonas fluorescens: Nonessential role of flagella in adhesion to sand and biofilm formation. *J Bacteriol* 185:453-460.

88. **Rodahl M, Hook F, Fredriksson C, Keller CA, Krozer A, Brzezinski P, Voinova M, Kasemo B (1997).** Simultaneous frequency and dissipation factor QCM measurements of biomolecular adsorption and cell adhesion. *Faraday Discuss* 107:229-246.

89. **Rodrigues AC, Wuertz S, Brito AG, Melo LF (2005).** Fluorene and phenanthrene uptake by Pseudomonas putida ATCC 17514: Kinetics and physiological aspects. *Biotechnol Bioeng* 90:281-289.

90. **Rodrigues AC, Wuertz S, Brito AG, Melo LF (2003).** Three-dimensional distribution of GFP-labeled Pseudomonas putida during biofilm formation on solid PAHs assessed by confocal laser scanning microscopy. *Water Sci Technol* 47:139-142.

91. **Ron EZ, Rosenberg E (2002).** Biosurfactants and oil bioremediation. *Curr Opin Biotechnol* 13:249-252.

92. **Rosenberg M, Kjelleberg S (1986).** Hydrophobic Interactions: Role in Bacterial Adhesion. *Adv microb ecol* 9:353-393.

93. **Rosenberg M, Rosenberg E (1981).** Role of adherence in growth of Acinetobacter calcoaceticus RAG-1 on hexadecane. *J Bacteriol* 148:51-57.

94. **Rosenberg E, Barkay T, Navon-Venezia S, Ron EZ (1999).** Role of Acinetobacter bioemulsans in petroleum degradation. In: Fass R, et al., editors. Novel Approaches for Bioremediation of Organic Pollution. New York:Kluwer Academic/Plenum Publishers. p 171-180.

95. **Rosenberg M (2006).** Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. *FEMS Microbiol Lett* 262:129-134.

96. **Rosenberg M (1981).** Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. *Appl Environ Microbiol* 42:375-377.

97. **Rosenberg M, Gutnick D, Rosenberg E (1980).** Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 9:29-33.

98. **Rosenberg M (1984).** Ammonium sulphate enhances adherence of Escherichia coli J-5 to hydrocarbon and polystyrene. *FEMS Microbiol Lett* 25:41-45.

99. **Schofield AL, Rudd TR, Martin DS, Fernig DG, Edwards C (2007).** Real-time monitoring of the development and stability of biofilms of Streptococcus mutans using the quartz crystal microbalance with dissipation monitoring. *Biosens Bioelectron* 23:407-413.

100. **Seo Y, Bishop PL (2007).** Influence of nonionic surfactant on attached biofilm formation and phenanthrene bioavailability during simulated surfactant enhanced bioremediation. *Environ Sci Technol* 41:7107-7113.

101. **Singh A, Van Hamme JD, Ward OP (2007).** Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnol Adv* 25:99-121.

102. **Singh A, Ward OP (2004).** Biotechnology and bioremediation- An Overview. In: Singh A and Ward OP, editors. Biodegradation and Bioremediation. New York:Springer. p 1-15.

103. **Skvarla J (1993).** A physicochemical model of microbial adhesion. *J Chem Soc , Faraday Trans* 89:2913-2921.

104. **Smith RW, Miettinen M (2006).** Microorganisms in flotation and flocculation: Future technology or laboratory curiosity? *Miner Eng* 19:548-553.

105. **Song RH, Hua ZZ, Li HZ, Chen J (2006).** Biodegradation of petroleum hydrocarbons by two Pseudomonas aeruginosa strains with different uptake modes. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 41:733-748.

106. **Stelmack PL, Gray MR, Pickard MA (1999).** Bacterial adhesion to soil contaminants in the presence of surfactants. *Appl Environ Microbiol* 65:163-168.

107. **Tavana H, Neumann AW (2007).** Recent progress in the determination of solid surface tensions from contact angles. *Adv Colloid Interface Sci* 132:1-32.

108. **Tobe T, Sasakawa C (2002).** Species-specific cell adhesion of enteropathogenic Escherichia coli is mediated by type IV bundle-forming pili. *Cell Microbiol* 4:29-42.

109. **Tongpim S, Pickard MA (1996).** Growth of Rhodococcus S1 on anthracene. *Can J Microbiol* 42:289-294.

110. **Vacheethasanee K, Temenoff JS, Higashi JM, Gary A, Anderson JM, Bayston R, Marchant RE (1998).** Bacterial surface properties of clinically

isolated Staphylococcus epidermidis strains determine adhesion on polyethylene. *J Biomed Mater Res* 42:425-432.

111. **Van Hamme JD, Singh A, Ward OP (2006).** Physiological aspects - Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnol Adv* 24:604-620.

112. **van Loosdrecht MC, Lyklema J, Norde W, Schraa G, Zehnder AJ (1987).** Electrophoretic mobility and hydrophobicity as a measured to predict the initial steps of bacterial adhesion. *Appl Environ Microbiol* 53:1898-1901.

113. **van Loosdrecht MC, Lyklema J, Norde W, Zehnder AJ (1990).** Influence of interfaces on microbial activity. *Microbiol Mol Biol Rev* 54:75-87.

114. **van Merode AEJ, van der Mei,Henny C., Busscher HJ, Krom BP (2006).** Influence of Culture Heterogeneity in Cell Surface Charge on Adhesion and Biofilm Formation by Enterococcus faecalis. *J Bacteriol* 188:2421-2426.

115. **van Oss CJ, Gillman CF, Neumann AW (1975).** Phagocytic engulfment and cell adhesiveness as cellular surface phenomena. New York:M. Dekker. 160 p.

116. **Volkering F, Breure AM, Rulkens WH (1997).** Microbiological aspects of surfactant use for biological soil remediation. *Biodegradation* 8:401-417.

117. **Weisz PB (1973).** Diffusion and Chemical Transformation: An interdisciplinary excursion. *Science* 179:433-440.

118. **Westgate S, Bell G, Halling PJ (1995).** Kinetics of uptake of organic liquid substrates by microbial cells: A method to distinguish interfacial contact and mass-transfer mechanisms. *Biotechnol Lett* 17:1013-1018.

119. **Wick LY, de Munain, R. A., Springael D, Harms H (2002).** Responses of Mycobacterium sp. LB501T to the low bioavailability of solid anthracene. *Appl Microbiol Biotechnol* 58:378-385.

120. **Wick LY, Colangelo T, Harms H (2001).** Kinetics of Mass Transfer-Limited Bacterial Growth on Solid PAHs. *Environ Sci Technol* 35:354-361.

121. **Wodzinski RS, Coyle JE (1974).** Physical State of Phenanthrene for Utilization by Bacteria. *Appl Environ Microbiol* 27:1081-1084.

122. **Wright CJ, Armstrong I (2006).** The application of atomic force microscopy force measurements to the characterisation of microbial surfaces. *Surf Interface Anal* 38:1419-1428.

123. **Yoshida F, Yamane T, Yagi H (1971).** Mechanism of uptake of liquid hydrocarbons by microorganisms. *Biotechnol Bioeng* 13:215-228.

124. **Zhang Y, Miller RM (1994).** Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. *Appl Environ Microbiol* 60:2101-2106.

125. **Zilber IK, Rosenberg E, Gutnick D (1980).** Incorporation of 32P and Growth of Pseudomonad UP-2 on n-Tetracosane. *Appl Environ Microbiol* 40:1086-1093.

3. Improvement of Microbial Adhesion to the Oil-Water Interface[1]

3.1 Introduction

Studies on microbial adhesion to the oil-water interface date back to over 80 years, when Mudd and Mudd published their work in 1924 (Mudd and Mudd 1924a; Mudd and Mudd 1924b). There are many applications for microbial attachment, but most studies to date on this field have focused on the medical and biofilm areas. In spite of extensive research on microbial adhesion, controlled enhancement of this phenomenon to promote oil biodegradation or bio-processing has not received significant attention. The physico-chemical and biological factors involved in adhesion are not yet clearly defined (Busscher, et al. 1995; Hermansson 1999; Ishii, et al. 2006; Katsikogianni and Missirlis 2004; van der Mei, et al. 1995). Factors affecting this process can be grouped into three categories: characteristics of microbial cells (including charge, hydrophobicity and composition of cell surface), properties of the oil surface, and environmental factors like ionic strength, pH and presence of specific compounds such as surfactants. In order to control the microbial adhesion, one needs to understand contribution of these factors to the forces between bacterial cells and the oil-water interface.

Modifying the cell surface without harming its biological function is a difficult task. While many compounds have been reported to inhibit microbial adhesion to hydrophobic surfaces (Babu, et al. 1986; Chen and Zhu 2004; Stelmack, et al. 1999) only a few have been found to promote this phenomenon. Some examples of the latter group are simple ionic compounds such as ammonium sulphate (Rosenberg 1984), cationic surfactants like cetylpyridinium chloride (CPC; Goldberg, et al. 1990b), cationic polymers like poly-L-lysine or chitosan

[1] A version of this chapter has been published: Abbasnezhad H, Gray MR, Foght JM (2008), Colloids and Surfaces B: Biointerfaces, 62:36-41.

(Goldberg, et al. 1990a), and long chain alcohols like 1-dodecanol (Marchesi, et al. 1994b; Owen, et al. 1997). However, none of these studies clearly demonstrated the mechanism of adhesion. Goldberg and coworkers (1990a; 1990b) studied the effects of several cationic compounds on adhesion of disparate species of bacteria using the microbial adhesion to hydrocarbons (MATH) method to characterize hydrophobicity of microbial surfaces. Research on the effect of long chain alcohols originated from a study conducted to investigate the adhesion of a microbial population to sediments in response to biodegradation of sodium dodecyl sulphate (SDS; Marchesi, et al. 1994a). During this study, Marchesi and coworkers noticed that the presence of 1-dodecanol, a primary intermediate of SDS biodegradation, increased the proportion of microorganisms adhered to hydrophobic sediments. The same group later used hydrophobic interaction chromatography and the MATH method to characterize hydrophobicity of the cell surface (Marchesi, et al. 1994b). They showed that a correlation exists between adhesion and concentration of 1-dodecanol, but the effect of alcohols on cell surfaces was not systematically investigated in these studies.

Previous studies altering cell surface hydrophobicity have focused on different behaviours of disparate species, mainly using adhesion-based techniques to characterize cell surface hydrophobicity. Our approach in this study was to use different compounds to promote adhesion of the hydrophilic bacterium *Pseudomonas fluorescens* LP6a to the oil-water interface. To define the effect of these compounds on the bacterial surface, we have used both MATH and contact angle methods, along with electrophoretic mobility measurements to understand the mechanism of the enhancement of adhesion to an oil-water interface in this model system.

3.2 Materials and Methods

3.2.1 Microorganisms, growth conditions

Pseudomonas fluorescens LP6a was used as the test microorganism for this study. It is known to have a hydrophilic surface (Dorobantu, et al. 2004). This Gram-negative bacterium uses a variety of polyaromatic hydrocarbons (PAHs) and was

originally isolated from a petroleum condensate-contaminated soil (Foght and Westlake, 1996). *Acinetobacter venetianus* RAG-1 (Reisfeld, et al. 1972), a Gram-negative bacterium, was also used for comparison. RAG-1 is reported to have a hydrophobic surface in its early stationary growth phase (Dorobantu, et al. 2004).

The cultures were grown at 28°C in tryptic soy broth (TSB; Sigma-Aldrich, Chemical Co.) on a rotary shaker at 200 rpm; then cells were harvested at the beginning of their stationary phase by centrifugation at 12,000 x g. Cells were washed twice and resuspended in potassium phosphate buffer at pH 7 at the specified molarity. Bushnell Haas (BH) Medium (g/l: magnesium sulfate 0.2; ammonium nitrate 1.0; ferric chloride 0.05, potassium hydrogen mono phosphate 1.0, potassium dihydrogen phosphate 1.0, calcium chloride 0.02; pH adjusted to 7–7.2) was used for degradation experiments with specified additives as sole carbon source.

3.2.2 Chemicals

n-Hexadecane (≥ 99%), chlorhexidine gluconate (CHX; 20 % aqueous solution), and poly-L-lysine (0.1% w/v in water) were purchased from Sigma-Aldrich Chemical Co. CPC (96.01-101.0%), 1-dodecanol (98.0 %), 2-dodecanol (99.0%) and farnesol (mixture of isomers of $C_{12}H_{28}O$, 96%) were purchased from Acros Organics (NJ, USA). N,N-Dimethylformamide (DMF) was purchased from Anachemia (Montréal, QC, Canada). N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Company (Rockford, IL, USA).All other reagents were laboratory grade, purchased from Fisher Scientific Co.

3.2.3 Degradation and toxicity measurements

In order to test the degradability and toxicity of cationic compounds, solutions of each compound in water were prepared individually and sterilized using 0.22 micrometer pore sized filters (Millipore Corp, Billerica, MA) before addition to the cultures. Solutions of long chain alcohols, 1-dodecanol, 2-dodecanol and

fsarnesol, in DMF were prepared for concentrations lower than 820 mg/L. For higher concentrations (1g/L to 10 g/L) the alcohols were added directly to the culture in a volume corresponding to the required concentration (all alcohol concentrations were calculated based on the total volume of the culture media which included both organic and aqueous phases). The experiments were conducted in a series of 250 ml Erlenmeyer flasks.

For degradation tests (where additives served as the sole carbon and energy sources), BH Medium was used. To investigate the toxicity of additives, TSB medium was used with the additives serving as supplemental carbon and energy sources or toxicants. Cultures were acidified at the end of each experiment and were extracted thrice with dichloro methane. All extracts were analyzed using an Agilent Technologies 5890 gas chromatograph. An HP-1 capillary column (25 m x 0.322 mm x 0.17 μm) was used to determine concentration alcohols in the extract (organic phase). When alcohols were present, the samples were derivatized with BSTFA in order to get sharp peaks for alcohols. Tetradecanol served as extraction and internal standards for all alcohols.

3.2.4 Adhesion Experiments

Washed bacterial suspensions were adjusted with phosphate buffer to an optical density at 600nm (OD_{600}) of 0.6 for use in the MATH test, slightly modified from the original method (Rosenberg, et al. 1980). When required, cationic compounds were dissolved in milli-Q water at different concentrations and a constant volume of 0.4 mL was added to 1.2 mL cell suspension. Undiluted 1-dodecanol, 2-dodecanol or farnesol in a volume corresponding to the required concentration were added directly to cell suspensions and mixed for 1 min (i.e. 20 μL 1-dodecanol was added to 90 mL cell suspension to obtain the final concentration of 182.2 mg/L; 1.2 mL of this suspension was used for MATH test). When killed cells were required in adhesion test, $HgCl_2$ was added to the cell suspension at concentrations of either 0.1mM or 0.2mM and incubated for 1 h before performing the MATH test.

In the MATH test, 1.0 mL of *n*-hexadecane was added to each 13 mm x 150 mm test tube containing 1.2 mL cell suspension and the mixtures were incubated at 30°C for 10 min then mixed by vortex at maximum speed for 2 min. After 15 min settling time, 0.2 mL of aqueous phase was transferred to one well of a 96-well microplate and OD_{600} was measured spectrophotometrically. Adhesion percent was expressed as the difference in the turbidity of aqueous phase before and after mixing with *n*-hexadecane (i.e. $\frac{OD_0 - OD_t}{OD_0} \times 100$; subscripts 0 and t indicate conditions before and after mixing, respectively). Three pseudoreplicates (i.e., three aliquots of a single washed cell preparation) were used for each MATH test. The order in which compounds were added was an important factor in the adhesion test. In all experiments alcohols were added to and mixed with the cell suspension prior to addition of *n*-hexadecane.

3.2.5 Contact Angle Measurement

Contact angle was measured for LP6a cells treated with 1-dodecanol or CPC and for untreated cells. In the case of 1-dodecanol or CPC, the cell suspension was mixed with a given concentration of these compounds for 10 min before the measurement. Washed cell suspension (30 mL) was filtered through a 0.22 μm pore size polyvinylidene difluoride membrane filter (Millipore, Fisher Scientific Ltd., Nepean, Ontario) leaving a smooth bacterial lawn deposited on the filter paper. The cell deposit was dried at room temperature for about an hour, a time determined in previous trials to be sufficient to obtain a constant value for contact angle measurement. The filter was then mounted on a Teflon support and immersed in a hexadecane bath. Immediately after placing a 20-μL sessile drop of milli-Q water on the bacterial lawn, the contact angle at both left and right sides was measured using a Krüss drop shape analysis system (DSA 10-MK2; Krüss USA, Charlotte, N.C.).

The contact angle was measured through the water phase at 1-sec intervals for 1 min. The average value was recorded as the surface contact angle. Measurements were made on at least two separate locations for each sample, and on at least three

independently deposited layers for each experimental series (Dorobantu, et al. 2004; Neufeld, et al. 1980). Parallel control measurements were conducted on filter papers without bacterial cells present.

3.2.6 Zeta Potential Measurement

To investigate the effect of buffer molarity and different pre-treatments on surface charges of cells, *P. fluorescens* LP6a cells were suspended at ~10^7 colony-forming units (cfu)/mL in 10 mM potassium phosphate buffer (pH 7). The lower molarity was used to prevent interference between the electrical field and presence of ions (Li and McLandsborough 1999). This helped us to obtain more reliable data. The CPC solution or 1-dodecanol was added to the cell suspension. Zeta potentials then were measured immediately at room temperature by laser Doppler velocimetry using a Zetaphoremeter 4.20 (CAD Instrumentation, Les Essarts-le-Roi, France) equipped with a rectangular glass electrophoresis cell, a CCD camera and a viewing system (Li and McLandsborough 1999).

3.3 Results

The effect of ionic strength on bacterial adhesion to *n*-hexadecane was investigated by washing and re-suspending LP6a and RAG-1 in buffers of different molarities. In the case of *P. fluorescens* LP6a, adhesion increased from 8 % in 10 mM potassium phosphate buffer to about 30% in 250 mM buffer (Table 3.1). For *A. venetianus* RAG-1 the change in buffer molarity and, consequently, in ionic strength did not result in a large change: adhesion to *n*-hexadecane was greater than 90% in both buffers. To clarify the effect of ionic strength on adhesion, NaCl or $MgSO_4$ were used to raise the ionic strength of 10 mM buffer to the same value as for the 250 mM buffer. Results in Table 3.1 illustrate the increased adhesion of *P. fluorescens* LP6a with increased ionic strength. Whereas addition of $MgSO_4$ resulted in a slightly higher adhesion than NaCl (40.5% ± 7.95 compared to 32.1% ± 6.12 for NaCl), results were not significantly different from the value for 250 mM buffer medium (33.2% ± 2.92). Further investigation of LP6a cells under the microscope showed that bacteria formed aggregates in higher buffer molarities, therefore, the higher ionic strength promoted adhesion of cells

not only to the oil but also to each other (Figure 3.1). For the remainder of the experiments in this study, 250 mM potassium phosphate buffer was used as the washing and resuspending medium.

Table 3.1: Effect of ionic strength of the suspending aqueous medium on adhesion of *P. fluorescens* LP6a or *A. venetianus* RAG-1 cells to *n*-hexadecane.

Media used for washing and re-suspending cells	Ionic Strength (M)	*P. fluorescens* LP6a Adhesion (%)[a]	*A. venetianus* RAG1 Adhesion (%)[a]
250 mM potassium phosphate buffer (pH = 7)	0.575	33.2 ± 2.92	98.98 ± 1.44
10 mM potassium phosphate buffer (pH = 7)	0.019	8.3 ± 3.74	90.15 ± 2.202
10 mM buffer amended with NaCl	0.575	32.1 ± 6.12	-------[b]
10 mM buffer amended with $MgSO_4$	0.575	40.5 ± 7.95	--------[b]

a, measured by the MATH test (see Methods for details); mean ± standard deviation (n=3).
b, not tested

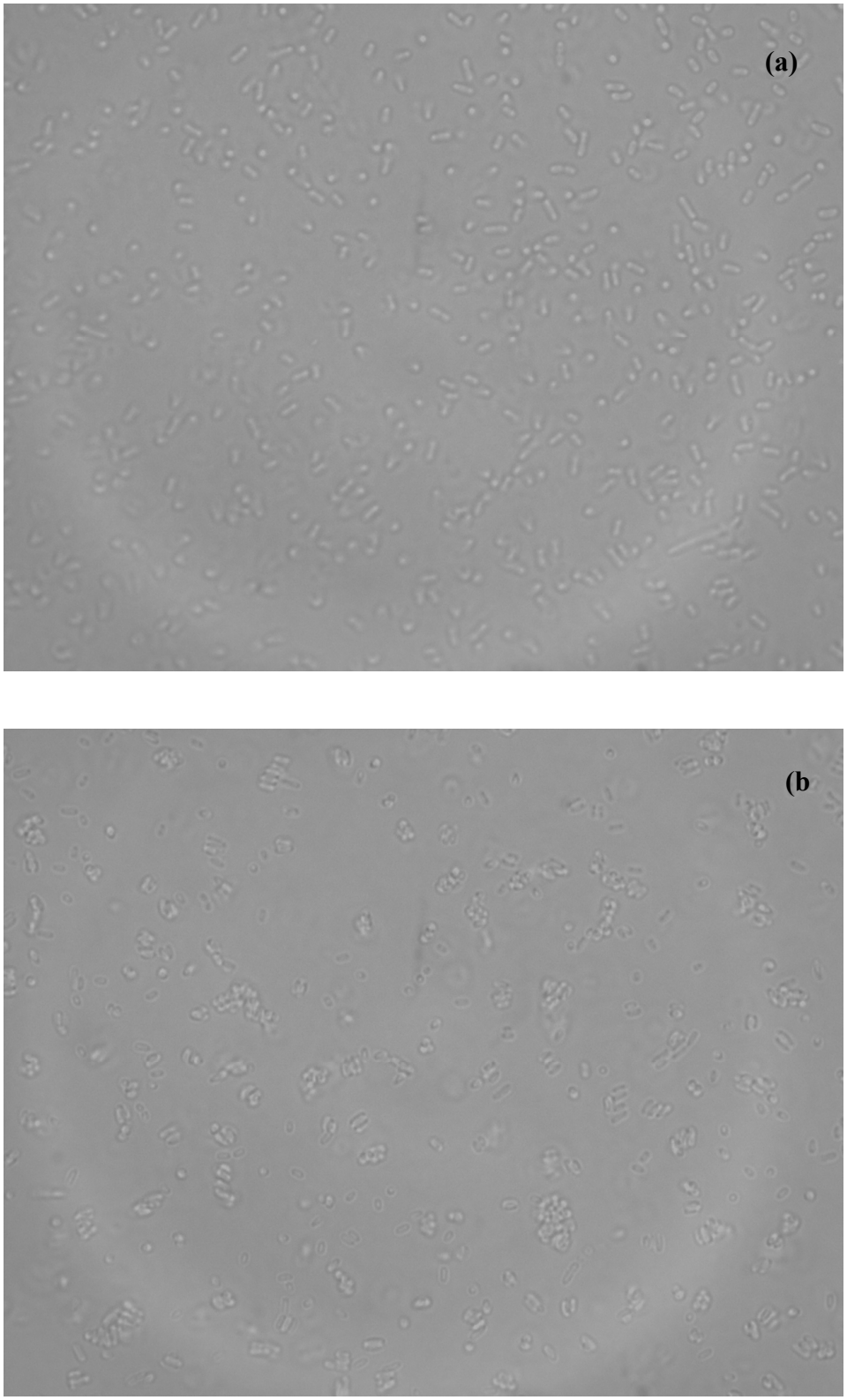

Figure 3.1: Micrograph of *Pseudomonas fluorescens* LP6a in two different phosphate buffers (a) 10 mM phosphate buffer and (b) 250 mM phosphate buffer

The addition of several types of cationic compounds enhanced the adhesion of LP6a to *n*-hexadecane. The effect of CPC, poly-L-lysine and CHX on the adhesion of a dilute suspension of *P. fluorescens* LP6a in 250 mM phosphate buffer is shown in Figure 3.2. In all cases, the presence of these cationic surfactants increased cell adhesion to *n*-hexadecane over a wide range of concentrations. Poly-L-lysine was less effective than the other two compounds. All these compounds completely inhibited the growth of *P. fluorescens* LP6a in TSB over the same range of concentrations (See appendix A for detail).

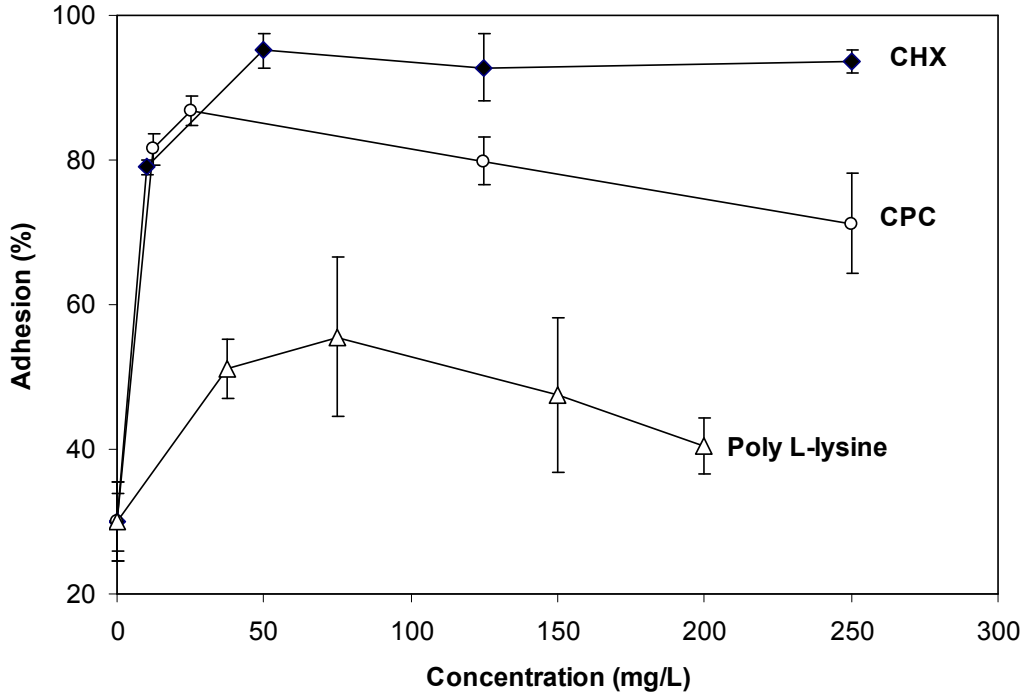

Figure 3.2: Effect of cell pre-treatment with different concentrations of CPC, CHX or poly-L-lysine on adhesion of *P. fluorescens* LP6a cells to *n*-hexadecane in the MATH test. Data points represent the mean of three pseudoreplicates and error bars show the standard deviation.

The effect of 1-dodecanol, 2-dodecanol and farnesol on adhesion is shown in Figure 3.3. Both 1-dodecanol and 2-dodecanol dramatically increased adhesion in

250 mM buffer over a wide range of concentrations. Farnesol also increased adhesion for all tested concentrations above 180 mg/L. At lower concentrations (50 mg/L) farnesol was not very effective and only increased the adhesion by 10% compared to the control (Figure 3.3). In growth experiments none of these compounds inhibited growth of *P. fluorescens* LP6a in TSB medium over a range of concentrations from 100 mg/L to 10 g/L. However, after 14 days incubation, 1-dodecanol and 2-dodecanol were completely degraded by LP6a both in the presence of TSB and as sole carbon source when provided at 0.1% v/v in BH medium. At the same concentration and time scale farnesol was only 40% degraded in the presence of TSB and was not degraded as sole carbon source in BH medium (See appendix A). Figure 3.2 and Figure 3.3 depict similar performance for alcohols and cationic compounds in terms of adhesion based on the MATH test.

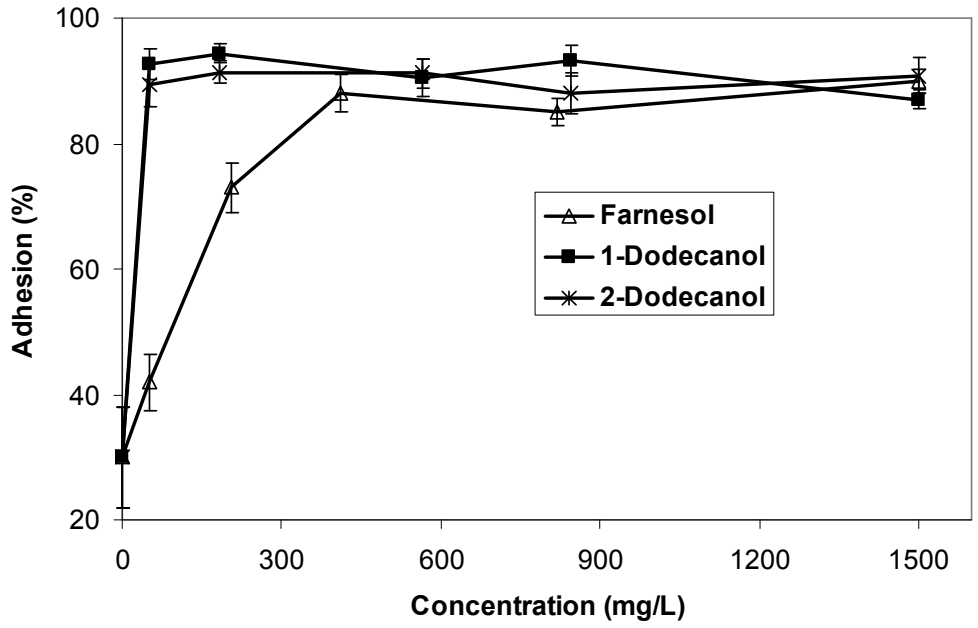

Figure 3.3: Effect of cell pre-treatment with different concentrations of 1-dodecanol, 2-dodecanol or farnesol on adhesion of *P. fluorescens* LP6a cells to *n*-hexadecane in the MATH test. Data points represent the mean of three pseudoreplicates and error bars show the standard deviation.

Images of contact angle measurement for *P. fluorescens* LP6a, without pre-treatment or after treating with CPC or 1-dodecanol are shown in Figure 3.4. There was a large difference in the CPC and 1-dodecanol effects: cells treated with 1-dodecanol showed a dramatic increase in contact angle (θ) from its original value of 24.5° to 104.4°. In contrast, treatment with CPC slightly increased θ to 42.1°. Control measurements did not show any significant changes of contact angle on the filter treated with the same solutions but lacking cells.

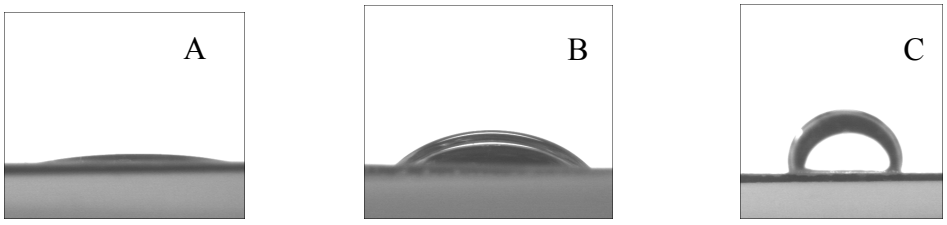

Figure 3.4: Effect on three phase contact angle of pre-treating *P. fluorescens* LP6a with CPC or 1-dodecanol. Contact angle was measured by placing a drop of water onto a lawn of cells submerged in *n*-hexadecane. A, Untreated (control) cell lawn, θ = 24.5°; B, Cells pre-treated with 200 mg/L CPC (θ = 42.1°); C., Cells pre-treated with 200 mg/L 1-dodecanol (θ = 104.4°).

The effects of different treatments on electrophoretic mobility of *P. fluorescens* LP6a are shown in Figure 3.5. Values for zeta potentials were calculated using the Smoluckowski equation (Hunter 1981). LP6a suspended in 0.01M phosphate buffer was negatively charged with a mean zeta potential of -23.58 ± 2.38 mV. This value was increased to -7.54 ± 2.12 mV by treating cells with CPC in the same buffer. 1-Dodecanol-treated cells had almost the same mean zeta potential (-23.92 ± 3.92) as non-treated cells in buffer. The mobility graphs in Figure 3.5 show that treating cells with CPC significantly altered the bacterial zeta potential, while 1-dodecanol had almost no effect.

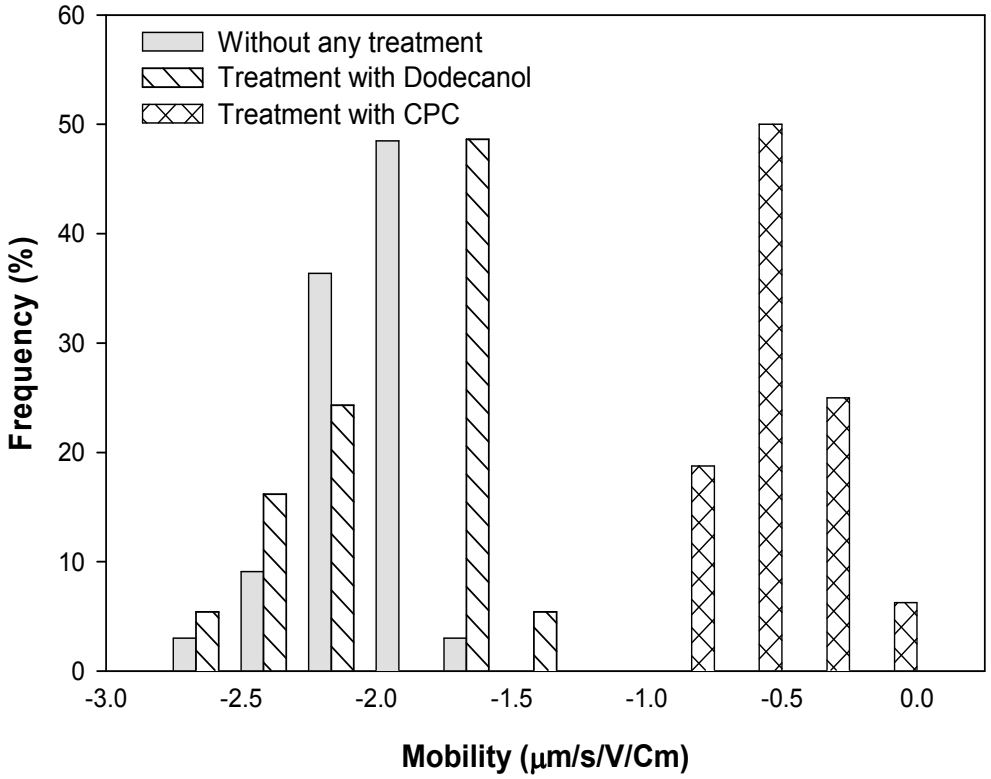

Figure 3.5: Electrophoretic mobility of *P. fluorescens* LP6a cells suspended in 0.01M potassium phosphate buffer, untreated (control, mean zeta potential of -23.58 ± 2.38 mV) or pre-treated with 200 mg/L of 1-dodecanol (mean zeta potential of -23.92 ± 3.92 mV) or 200 mg/L of CPC (mean zeta potential of -7.54 ± 2.12 mV).

Series of experiments were conducted to examine the effect of 1-dodecanol on killed LP6a cells. In order to kill the cells, cultures of LP6a grown on TSB were incubated with lethal concentrations of $HgCl_2$ (0.2 mM) for one hour just before harvesting cells for the MATH test. $HgCl_2$ is known to lead to cell death without having an effect on the physical and chemical properties of cell surfaces (Neumann, et al. 2006). $HgCl_2$ concentration of 0.2 mM led to complete loss of viability of the cells (as confirmed by streaking loopfuls of culture onto Plate Count Agar (PCA) and incubating for 48 hours at 30°C). 1-Dodecanol was added to the cells after they were harvested and washed by centrifugation as described before. Results for these experiments are reported in table 3.2. After treating the living cells with 410 mg/L of 1-decanol, the adhesion of cells to *n*-hexadecane increased drastically, from 29% to 87%. Similarly, dead cells showed the same

behavior after treatment with 1-dodecanol which increased adhesion to 86%. Treating cells with 820 mg/L of 1-dodecanol resulted in a similar increase in adhesion for both living and dead cells. In the absence of 1-dodecanol, treating cells with $HgCl_2$ did not have a significant influence on adhesion of LP6a to *n*-hexadecane. The results clearly show that the effect of 1-dodecanol on the adhesion of *P. fluorescens* LP6a to *n*-hexadecane does not depend on metabolic activity of cells and 1-dodecanol is equally effective in increasing the adhesion of both living and dead cells.

Table 3.2: Effect of pre-incubation with $HgCl_2$ on the adhesion of *P. fluorescens* LP6a cells to *n*-hexadecane in presence and absence of 1-dodecanol. Data points represent the mean of three pseudoreplicates ± standard deviation (n=3).

Concentration of $HgCl_2$ (mM)	Concentration of 1-dodecanol (mg/L)	*P. fluorescens* LP6a Adhesion (%)[a]
0	0	29.4 ± 3.4
0.2	0	37.0 ± 2.9
0.2	820	89.9 ± 2.3
0.2	410	86.2 ± 3.6
0	820	91.1 ± 2.5
0	410	87.4± 3.2

a, measured by the MATH test (see Methods for details)

3.4 Discussion

This study demonstrates that the adhesion of Gram-negative *P. fluorescens* LP6a to an oil-water interface is influenced by the ionic strength of the suspension medium. Therefore, electrostatic forces play an important role in adhesion of this strain to the oil-water interface. Investigation of LP6a cells under the microscope confirmed that the bacteria form aggregates in higher buffer molarities (Figure 3.1). On the other hand, the hydrophobic strain *A. venetianus* RAG-1 adhered to the hydrophobic surfaces irrespective of the electrostatic interactions (Table 3.1). These observations are clearly in agreement with other reports (Skvarla 1993) indicating that when a bacterium adheres to a hydrophobic surface to a greater extent (in this case RAG-1) this adhesion is not affected by electrical interactions. Clearly, the importance of electrostatic forces on bacterial adhesion depends on the relative magnitude of all forces involved in the adhesion process. Adjusting the ionic strength of the suspending medium by adding either a monovalent or divalent salt illustrates that in this case adhesion was not specifically related to a certain cation but rather dependent on the ionic strength of the medium.

Pre-treating the *P. fluorescens* LP6a cells with either cationic compounds or long chain alcohols led to a dramatic enhancement in adhesion (Figures 3.2 and 3.3). For cationic compounds, similar results have been reported before (Goldberg, et al. 1990a; Goldberg, et al. 1990b; Neu 1996). In those studies the authors concluded, with little evidence, that CPC acts by binding directly to the cell surface via its positively charged end group, so that the hydrophobic tail could introduce a hydrophobic moiety to the cell surface. This conclusion contradicts other studies on interaction of CPC with cell membranes, which report that the alkyl chain in CPC penetrates into the membrane leaving a positively charged group located at cell surface (Thorsteinsson, et al. 2003). A schematic description of these two mechanisms is shown in Figure 3.6.

Our results on contact angle measurement (Figure 3.4) did not show a significant change in cell surface hydrophobicity due to treatment with CPC. Instead, it caused a significant shift in the mean zeta potential of the bacterium (Figure 3.5).

The fact that the cell surface was not hydrophobic after treatment with CPC cannot be explained by the first mechanism mentioned above. The charge neutralization caused by CPC was the most likely reason for enhanced adhesion to the oil interface. These experiments also indicated that results of MATH tests should not be interpreted as measures of hydrophobicity because adhesion can be determined by other colloidal forces.

In the case of the long chain alcohols tested, the adhesion effects were very different. As expected, 1-dodecanol did not change zeta potential, but it had a significant effect on the three phase contact angle of the cells. This result showed that 1-dodecanol changed the surface hydrophobicity of *P. fluorescens* LP6a, without imposing a change on the charge density at the cell surface. The solubility of 1-dodecanol in water is only around 3 mg/L (Krause and Lange, 1965), in all experiments there will be 1-dodecanol in non-aqueous phase. Since 1-dodecanol is soluble in *n*-hexadecane in all concentrations tested (Chanami, et al., 2002), it is expected that the non-soluble portion of 1-dodecanol to partition into the lipid part of bacterial cell membrane and the organic phase (*n*-hexadecane in adhesion tests and HMN in biodegradation experiments).

Results in Table 3.2 clearly indicate that the observed increase in the adhesion of LP6a cells to *n*-hexadecane after treatment with 1-dodecanol is not dependent on the cells biological activity and is just a physicochemical effect which is observed for both dead and living cells. This contradicts the results obtained in another study (Neumann, et al. 2006) in which the positive effect of 1-decanol on *Pseudomonas putida* DOT-T1E cell surface hydrophobicity was observed only for living cells. In addition to using a different bacterial strain and a different alcohol, the mentioned study also used a much higher concentration of decanol (82.9 g/L) compared to our study with 1-dodecanol ($\leq$ 820 mg/L).

Stimulation of microbial adhesion to the oil-water interface has potential implications for bioremediation. Because of their expected high toxicity to microorganisms (and observed toxicity to LP6a in this study), cationic surfactants

are probably not good candidates for this application, even though they have found some other uses, i.e., some antimicrobial formulations (Goldberg and Rosenberg 1991). On the other hand, long chain alcohols are not toxic, as confirmed with *P. fluorescens* LP6a. The solubility and biodegradability of such alcohols can also be controlled by changing the attached alkyl groups. Furthermore, as was shown in this study for 1-dodecanol and 2-dodecanol, they are effective at very low concentrations in significantly changing the surface hydrophobicity of hydrophilic Gram-negative cells.

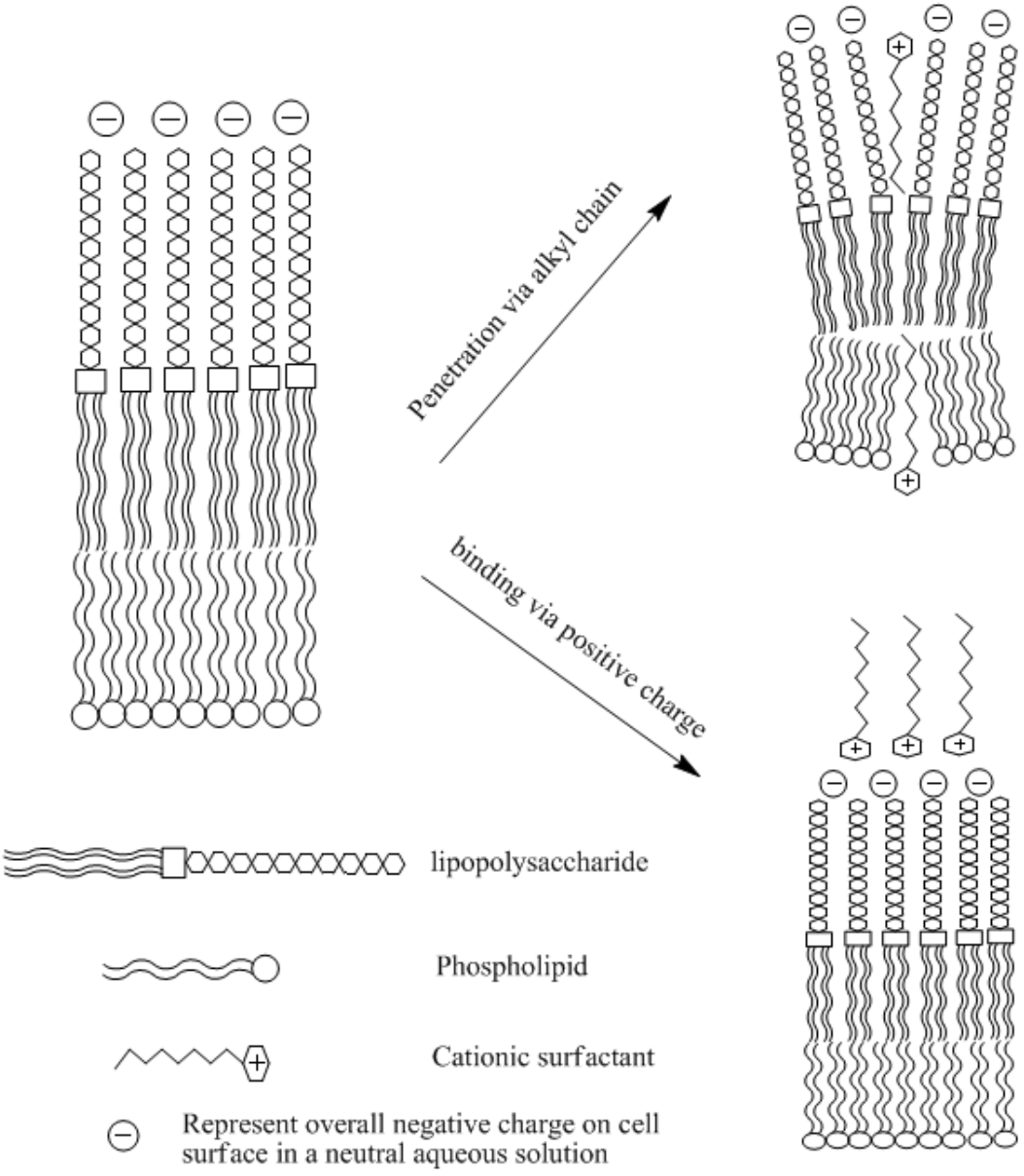

Figure 3.6: Representation of two different mechanisms of cationic surfactant effects on Gram negative outer membrane (Negative charges shown on the cell surface are not meant to display the actual location of the charged groups but rather they are representing the overall negative charge on the cell surface in a neutral solution).

Assessment of the mechanism of adhesion for microorganisms of interest, as well as direct use of adhesion enhancers for bioremediation studies, could lead to a better understanding of how adhesion can affect biodegradation of low-solubility hydrocarbons. Studies on this aspect of project are discussed in Chapter 4 of this thesis. To minimize other influences by long chain alcohols (such as possible reduction in surface tension or serving as an extra carbon and energy source for microorganism), it was decided to use as small concentration of alcohol as possible. Since, in lower concentrations, farnesol is not as effective as the other two alcohols, it is not used for biodegradation and mineralization tests and only 1-dodecanol and 2-dodecanol are considered for further biodegradation experiments.

3.5 References

1. **Babu JP, Beachey EH, Simpson WA (1986).** Inhibition of the interaction of Streptococcus sanguis with hexadecane droplets by 55- and 60-kilodalton hydrophobic proteins of human saliva. *Infect Immun* 53:278-284.

2. **Busscher HJ, Vandebeltgritter B, Vandermei HC (1995).** Implications of microbial adhesion to hydrocarbons for evaluating cell-surface hydrophobicity 1. Zeta-potentials of hydrocarbon droplets. *Colloids Surf B* 5:111-116.

3. **Chanamai R, Horn G, McClements DJ (2002).** Influence of oil polarity on droplet growth in oil-in-water emulsions stabilized by a weakly adsorbing biopolymer or a nonionic surfactant. *J Colloid Interface Sci* 247:167-176.

4. **Chen G, Zhu H (2004).** Bacterial deposition in porous medium as impacted by solution chemistry. *Res Microbiol* 155:467-474.

5. **Dorobantu LS, Yeung AKC, Foght JM, Gray MR (2004).** Stabilization of oil-water emulsions by hydrophobic bacteria. *Appl Environ Microbiol* 70:6333-6336.

6. **Foght JM, Westlake, DW (1996).** Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of Pseudomonas fluorescens. *Biodegradation* 7:353-366.

7. **Goldberg S, Doyle RJ, Rosenberg M (1990a).** Mechanism of enhancement of microbial cell surface hydrophobicity by cationic polymers. *J Bacteriol* 172:5650-5654.

8. **Goldberg S, Konis Y, Rosenberg M (1990b).** Effect of cetylpyridinium chloride on microbial adhesion to hexadecane and polystyrene. *Appl Environ Microbiol* 56:1678-1682.

9. **Goldberg S, Rosenberg M (1991).** Bacterial desorption by commercial mouthwashes vs two-phase oil:water formulations *Biofouling* 3:193-198.

10. **Hermansson M (1999).** The DLVO theory in microbial adhesion. *Colloids Surf B* 14:105-119.

11. **Hunter RJ (1981).** Zeta potential in colloid science: Principles and applications. New York:Academic Press. 386 p.

12. **Ishii S, Unno H, Miyata S, Hori K (2006).** Effect of cell appendages on the adhesion properties of a highly adhesive bacterium, *Acinetobacter* sp. Tol 5. *Biosci Biotechnol Biochem* 70:2635-2640.

13. **Katsikogianni M, Missirlis YF (2004).** Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cell Mater* 8:37-57.

14. **Krause FP, Lange W (1965).** Aqueous solubilities of *n*-dodecanol, *n*-hexadecanol, and *n*-octadecanol by a new method. *J Phys Chem* 69:3171-3173.

15. **Li J, McLandsborough LA (1999).** The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. *Int J Food Microbiol* 53:185-193.

16. **Marchesi JR, Owen SA, White GF, House WA, Russell NJ (1994a).** SDS-degrading bacteria attach to riverine sediment in response to the surfactant or its primary biodegradation product dodecan-1-ol. *Microbiology* 140:2999-3006.

17. **Marchesi JR, White GF, House WA, Russell NJ (1994b).** Bacterial cell hydrophobicity is modified during the biodegradation of anionic surfactants. *FEMS Microbiol Lett* 124:387-392.

18. **Mudd S, Mudd BH (1924a).** Certain interfacial tension relations and behavior of bacteria in film *J Exp Med* 40:647-660.

19. **Mudd S, Mudd BH (1924b).** The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. *J Exp Med* 40:633-645.

20. **Neu TR (1996).** Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol Rev* 60:151-166.

21. **Neufeld RJ, Zajic JE, Gerson DF (1980).** Cell surface measurements in hydrocarbon and carbohydrate fermentations. *Appl Environ Microbiol* 39:511-517.

22. **Neumann G, Cornelissen S, van Breukelen F, Hunger S, Lippold H, Loffhagen N, Wick LY, Heipieper HJ (2006).** Energetics and surface properties of *Pseudomonas putida* DOT-T1E in a two-phase fermentation system with 1-decanol as second phase. *Appl Environ Microbiol* 72:4232-4238.

23. **Owen SA, Russell NJ, House WA, White GF (1997).** Re-evaluation of the hypothesis that biodegradable surfactants stimulate surface attachment of competent bacteria. *Microbiology* 143:3649-3659.

24. **Reisfeld A, Rosenberg E, Gutnick D (1972).** Microbial degradation of crude oil: Factors affecting the dispersion in sea water by mixed and pure cultures. *Appl Microbiol* 24:363-368.

25. **Rosenberg M, Gutnick D, Rosenberg E (1980).** Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 9:29-33.

26. **Rosenberg M (1984).** Ammonium sulphate enhances adherence of *Escherichia coli* J-5 to hydrocarbon and polystyrene. *FEMS Microbiol Lett* 25:41-45.

27. **Skvarla J (1993).** A physicochemical model of microbial adhesion. *J Chem Soc , Faraday Trans* 89:2913-2921.

28. **Stelmack PL, Gray MR, Pickard MA (1999).** Bacterial adhesion to soil contaminants in the presence of surfactants. *Appl Environ Microbiol* 65:163-168.

29. **Thorsteinsson T, Loftsson T, Masson M (2003).** Soft antibacterial agents. *Curr Med Chem* 10:1129-1136.

30. **van der Mei HC, van de Belt-Gritter B, Busscher HJ (1995).** Implications of microbial adhesion to hydrocarbons for evaluating cell surface hydrophobicity 2. Adhesion mechanisms. *Colloids Surf B* 5:117-126.

4. Influence of Enhanced Adhesion on Biodegradation and Mineralization of Phenanthrene

4.1 Introduction

The presence of polycyclic aromatic hydrocarbons (PAH) in the environment is a concern due to their toxic nature, potential carcinogenic effects and potential endocrine-disrupting properties (Gillesby, et al. 1997; Safe 1998). The US Environmental Protection Agency (EPA) has classified several PAHs, including phenanthrene, as priority pollutants (EPA 2002). Some of the PAHs act as procarcinogens once absorbed and metabolically activated by organisms (Douben, Peter E. T. 2003; Skarpheoinsdottir, et al. 2007). PAHs with three or more rings are even more important, because of their recalcitrance to biodegradation. The persistence of PAHs in the environment is mostly due to their hydrophobic character and low water solubility (Volkering, et al. 1992; Woo and Park 2004).

Because of their low water solubilities, PAHs are often found either in non-aqueous phase liquids (NAPLs) or strongly sorbed to soil organic matter (Breedveld and Karlsen 2000; Weissenfels, et al. 1992). Surfactants have long been used to enhance bioavailability and biodegradation of PAHs by increasing their apparent solubilities and promoting mobilization by increasing desorption (Edwards, et al. 1991; Mulligan, et al. 2001; Volkering, et al. 1997). While the use of surfactants is a prominent method to enhance the biodegradation of less soluble compounds, reports on the impact of surfactants on bioremediation are not all positive (Bramwell and Laha 2000; Chen, et al. 2000; Laha and Luthy 1991). Bacterial adhesion to oil-water interfaces or to the surface of solid organic compounds can be reduced or prevented in the presence of surfactants (Rodrigues, et al. 2006; Stelmack, et al. 1999). When the mechanism of bacterial uptake of low-solubility hydrophobic compounds is through direct cell attachment to the surfaces of liquid or solid organic compounds, then the addition of surfactants may inhibit degradation by dispersing the bacteria into the aqueous phase. Adhesion of hydrocarbon degrading bacteria to the oil phase, on the other hand,

can enhance biodegradation (Hori, et al. 2002; Ortega-Calvo and Alexander 1994). When *Acinetobacter venetianaus* RAG-1 was mutated to minimize its adherence to oil-water interface, it lost the ability to grow on liquid hexadecane (Rosenberg and Rosenberg 1981; Vaneechoutte, et al. 1999). Similarly, strains of three bacteria that adhered to oil-water interfaces exhibited faster rates and higher extent of biodegradation with increasing hydrophobicity as characterised by the microbial adhesion to hydrocarbons (MATH) test, hydrocarbon interaction chromatography and a salting out-aggregation test (Obuekwe, et al. 2007a; Obuekwe, et al. 2007b; Obuekwe, et al. 2008).

Despite extensive research on microbial adhesion to oil, enhancement of cell adhesion to promote oil biodegradation has received very little attention. A few compounds have been reported to increase adhesion of bacterial cells to the oil-water interface (Goldberg, et al. 1990; Marchesi, et al. 1994a; Marchesi, et al. 1994b). In the previous chapter we demonstrated that the addition of 1-dodecanol, 2-dodecanol, and farnesol to a suspension of *Pseudomonas fluorescens* LP6a cells increased their adhesion to an oil-water interface. In the present chapter, we evaluate the effect of enhanced adhesion of *Pseudomonas fluorescens* LP6a to an oil-water interface by adding various concentrations of 1-dodecanol and 2-dodecanol. Farnesol was not used because lower concentrations of this compound did not influence LP6a adhesion to the oil-water interface (Figure 3.3). The use of additives effective at lower concentrations is beneficial both from application point of view and because of experimental considerations. Higher concentrations of additives can introduce other factors into the biodegradation experiments and complicate the results. With higher concentrations, the influence of additive in reducing interfacial tension and increasing the biomass density may dominate the adhesion effects. In our experiments biodegradation or mineralization of phenanthrene dissolved in the non-aqueous phase was monitored. Changes due to biomass density and reduction in interfacial tensions were also examined in order to confirm the observed kinetics of transformation of phenanthrene.

4.2 Materials and Methods

4.2.1 Chemicals

2,2,4,4,6,8,8-Heptamethylnonane (HMN) with purity of 98% was purchased from Aldrich Chemical Co. and was used as non-degradable water immiscible oil phase. 1-Dodecanol (98.0 %) and 2-dodecanol (99%) were purchased from Acros Organics (NJ, USA). Phenanthrene (98% pure) and *o*-terphenyl were purchased from Aldrich Chemical Company (St. Louis, MO, USA). The inducers, salicylic acid and 2-aminobenzoic acid were purchased from Analar (British Drug Houses Ltd, Poole, England). Naphthalene was obtained from Sigma Chemical Company (St. Louis, MO, USA). [9-^{14}C]Phenanthrene was used to measure mineralization of phenanthrene and quantify its metabolites in the radiolabeled transport assays (96.5% radiochemical purity; 19.3 mCi mmol-1; Amersham, Arlington Heights, IL, USA). N,N-Dimethylformamide (DMF) was purchased from Anachemica (Montréal, QC, Canada). N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Company (Rockford, IL, USA). All other reagents (unless stated otherwise) were laboratory grade, purchased from Fisher Scientific Co. Aqueous liquid scintillation fluor was obtained from Amersham Ltd. Dichloromethane (DCM), high performance liquid chromatography (HPLC) grade, were purchased from Fisher Scientific.

4.2.2 Microorganism and Growth Conditions

Pseudomonas fluorescens LP6a was used because of its positive response to adhesion enhancement by long chain alcohols, as described in the previous chapter, and its ability to utilize a variety of PAHs such as phenanthrene as a sole source of carbon and energy (Foght and Westlake 1991). The cultures were grown overnight at 28°C in tryptic soy broth (TSB) on a rotary shaker at 150 rpm. The cells were harvested by centrifugation at 12000 x g and washed twice with 10 mM phosphate buffer. Washed cells were then resuspended in Bushnell Haas (BH) medium (g/l: magnesium sulfate 0.2; ammonium nitrate 1.0; ferric chloride 0.05, potassium hydrogen mono phosphate 1.0, potassium dihydrogen phosphate 1.0, calcium chloride 0.02; pH adjusted to 7–7.2). This suspension was used as

inoculum for biodegradation and mineralization experiments. In all biodegradation and mineralization experiments HMN was used as non-aqueous phase in which phenanthrene was dissolved.

4.2.3 Alcohols Degradation and Toxicity Tests

Degradability and toxicity of 1-dodecanol and 2-dodecanol was assessed according to section 3.2.3 of the previous chapter.

4.2.4 Phenanthrene Biodegradation Assay

To prepare culture flasks, 5 mL of *P. fluorescens* LP6a inoculum (prepared as described in section 4.2.2) was added to 45 mL of cooled autoclaved BH medium in a 250 mL Erlenmeyer flask. One millilitre of HMN containing an appropriate amount of dissolved phenanthrene was then added to the culture flask to give the required concentration of phenanthrene (phenanthrene concentration ranged from 80 to 891 mg/L, calculated based on the total volume of culture medium). The ratio of two phase volumes (volume of organic phase (HMN) to volume of aqueous phase (BH medium and inoculum)) was kept constant throughout the experiments to eliminate any surface area effects due to change of NAPL volume. 1-Dodecanol and 2-dodecanol were dissolved in DMF when they were used at concentrations lower than 180 mg/L (based on total volume of culture medium). Two hundred and fifty microliter of this solution was added to the flasks. At higher concentrations the alcohols were added directly to the medium in a volume corresponding to the required concentration. Alcohols were added to and mixed with the contents of flasks prior to addition of organic phase (HMN with dissolved phenanthrene). Due to the high solubility of the alcohols in alkane solvents, the resulting mixture consisted of two phases; an aqueous phase and a non-aqueous phase containing most of the phenanthrene and alcohol.

At the end of each experiment, a sufficient volume of concentrated HCl was added to the flask to achieve pH < 1 and kill the cells. Two hundred and fifty microliter *o*-terphenyl was added to each flask in DMF solution to serve as an extraction and internal GC standard for phenanthrene. Tetradecanol dissolved in DMF was used as the extraction standard as well as internal GC standard for 1-

dodecanol and 2-dodecanol. The flasks were stored at 4°C until they were extracted at room temperature. The extraction was performed thrice with 25 mL HPLC grade dichloromethane. The pooled solvent extract was collected in an Erlenmeyer flask and an aliquot of it was filtered through sodium sulphate to remove the remaining water. To reduce the polarity of alcohols and produce sharp GC peaks, all the samples containing 1-dodecanol were derivatized with BSTFA before analysing using GC. Two hundred microlitter of BSTFA solution was added to 1 ml of extract. The solution was mixed, capped tightly with Teflon liners and heated at 70°C for 15 minutes. The vials were cooled down to room temperature and analyzed by GC. All extracts were analyzed using an Agilent Technologies 5890 GC. An HP-1 capillary column (25 m x 0.322 mm x 0.17 μm) was used to determine the concentration of residual phenanthrene and alcohol.

4.2.5 Culture Induction

Phenanthrene degradation by *P. fluorescens* LP6a can be induced by compounds such as salicylate, naphthalene and 2-aminobenzoate (Foght and Westlake 1996). In order to decrease the lag phase and enhance the rate of phenanthrene utilization, induced cultures were used for all the mineralization experiments. Among three inducers tested, salicylate, naphthalene and 2-aminobenzoate, the latter was used because unlike the other two, it is not metabolized by the bacteria but it can induce activity of all necessary enzymes. Induction by other two compounds is reported in Appendix B. Stock solution of 2-aminobenzoate (0.5 M) in 95 % ethanol was prepared and 250 μL of this solution was added to the 50 mL of cultures in BH medium to obtain a final 2-aminobenzoate concentration of 2.5 mM. Phenanthrene dissolved in HMN was then added to the culture as a growth substrate.

4.2.6 Mineralization Experiments

The phenanthrene mineralization activity of bacterial cells was measured based on $^{14}CO_2$ evolution from [9-^{14}C]phenanthrene (Ulrich, et al. 2009). Mineralization experiments were carried out in 250-mL biometer flasks containing 25 mL of culture medium. Two hundred and fifty microliter of ^{14}C phenanthrene dissolved

in HMN was added to the biometer flask at time zero, giving the desired phenanthrene concentration and 100,000 disintegrations per minute (dpm). Cumulative $^{14}CO_2$ production was monitored as previously described (Ulrich, et al. 2009). Samples of aqueous phase (0.7 mL) were removed through the syringe connected to the main flask opening. This sample was microfuged to pellets the cells and 0.5 mL of the supernatant was transferred to 10 mL ACS fluor (Amersham Biosciences, UK Ltd., Little Chalfont, England) for scintillation counting using a Beckman LS3801 liquid scintillation counter with automatic quench correction. Samples were dark adapted for 30 min before counting to reduce chemiluminescence. The dpm measured for each sample after subtracting the blank was then used to determine the fraction of recovered label, relative to the ^{14}C added at time zero. Both the aqueous phase and KOH (CO_2 trap) were sampled at time zero and used as the blank for the corresponding measurements. Cumulative production of $^{14}CO_2$ was reported as a percentage of the original radiolabel added, after correcting for background radiation (typically 30 dpm).

4.2.7 Surface and Interfacial Tension Measurements

To determine any influence of 1-dodecanol on interfacial properties of the culture, media surface and interfacial tensions of cell free medium were measured both in the presence and absence of 1-dodecanol using a Single Fibre Process Tensiometer (K14, Krüs, USA). *P. fluorescens* LP6a cells were grown on TSB for 48 h and 250 µL of 1-dodecanol dissolved in DMF were added to cultures to obtain final concentrations of 50, 100 or 150 mg/L 1-dodecanol. Cells were mixed with 1-dodecanol for 10 min in a rotary shaker. The bacterial cells were removed from the culture broth by filtering through a 0.22 µm pore size filter (Millipore). Surface and interfacial tensions were measured by the De Nouy ring method (McInerney, et al. 1990). Interfacial tension was measured against a thin layer of *n*-hexadecane poured on the surface of the sample liquid. All measurements were performed with the fresh interface at the room temperature.

4.2.8 Adhesion Measurement

Microbial adhesion was measured using the MATH method, as described in Chapter 3. The only difference was the use of HMN as the oil phase for adhesion tests. HMN was used to simulate the condition of biodegradation tests where HMN was the carrier of phenanthrene and composed the organic phase in the culture medium (with dissolved phenanthrene). Three subsamples from each flask were assayed using the MATH test.

4.3 Results

4.3.1 Bacterial Adhesion in Presence of 1-Dodecanol

The influence of 1-dodecanol on the adhesion of induced cultures of LP6a to the HMN-water interface was assessed during the biodegradation of phenanthrene dissolved in HMN. The cultures were incubated for 100 h with or without 1-dodecanol (180 mg/L). At each time point, samples were taken from the aqueous phase then the MATH test was conducted on the samples. The cells incubated with 1-dodecanol showed an average of 86 ± 6 % adhesion to the oil phase in the MATH test, remaining fairly constant during the biodegradation period (Figure 4.1). The cells incubated without 1-dodecanol started with much lower adhesion (35%), but it increased as biodegradation proceeded. After 60 h adhesion reached 82%, comparable to the samples with 1-dodecanol. The results indicate that incubating LP6a cells with 1-dodecanol increases the cell surface hydrophobicity as characterized by MATH test.

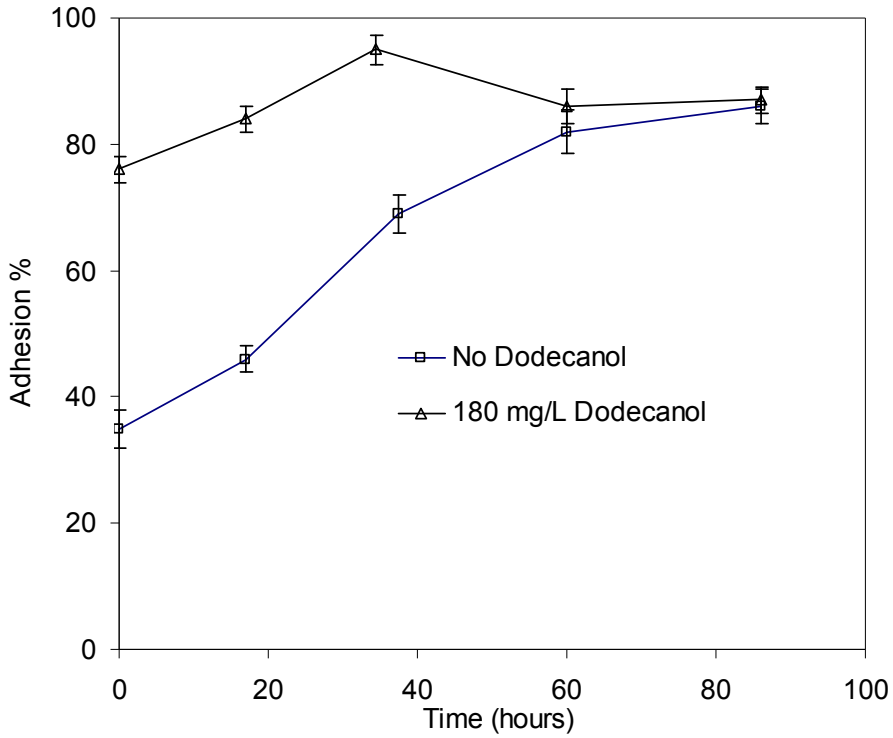

Figure 4.1: Effect of 1-dodecanol on the adhesion of *P. fluorescens* LP6a cells to HMN during the biodegradation period. Adhesion reported as percentage values according to the MATH method. Data points in both cases represent the mean of three pseudo-replicates and error bars show the standard deviation.

4.3.2 Toxicity and Degradability of Dodecanol

In growth experiments, 1-dodecanol and 2-dodecanol did not inhibit the growth of *P. fluorescens* LP6a on TSB medium over a range of concentrations from 50 mg/L to 10 g/L. Both of these compounds were completely degraded by LP6a in presence of TSB and also as sole carbon source when provided at 0.1% v/v in BH medium after 14 days incubation (See Appendix A for details). The average rate of biodegradation by LP6a of 1-dodecanol was about 130 mg/d when it was provided at initial concentration of 4100 mg/L along with initial HMN with dissolved phenanthrene concentration of 890 mg/L (data in Appendix A). These experiments demonstrate that neither 1-dodecanol nor 2-dodecanol is toxic or inhibitory for LP6a and they can be easily degraded by the microorganism.

4.3.3 Biodegradation Experiments with Non-Induced Cultures

Figure 4.2 demonstrates the effect of 1-dodecanol on the biodegradation of phenanthrene measured after 120 h incubation with different initial 1-dodecanol concentrations and equivalent initial phenanthrene concentration of 891mg/L (Figure 4.2a) or different phenanthrene concentrations and constant 1-dodecanol concentration (Figure 4.2b). Without 1-dodecanol present, the LP6a degraded only 5% percent of the phenanthrene initially present. Addition of as little as 217 mg/L of 1-dodecanol to cultures improved phenanthrene biodegradation to 33%. Initial 1-dodecanol concentrations of 820 and 4100 mg/L had a similar positive effect on phenanthrene biodegradation.

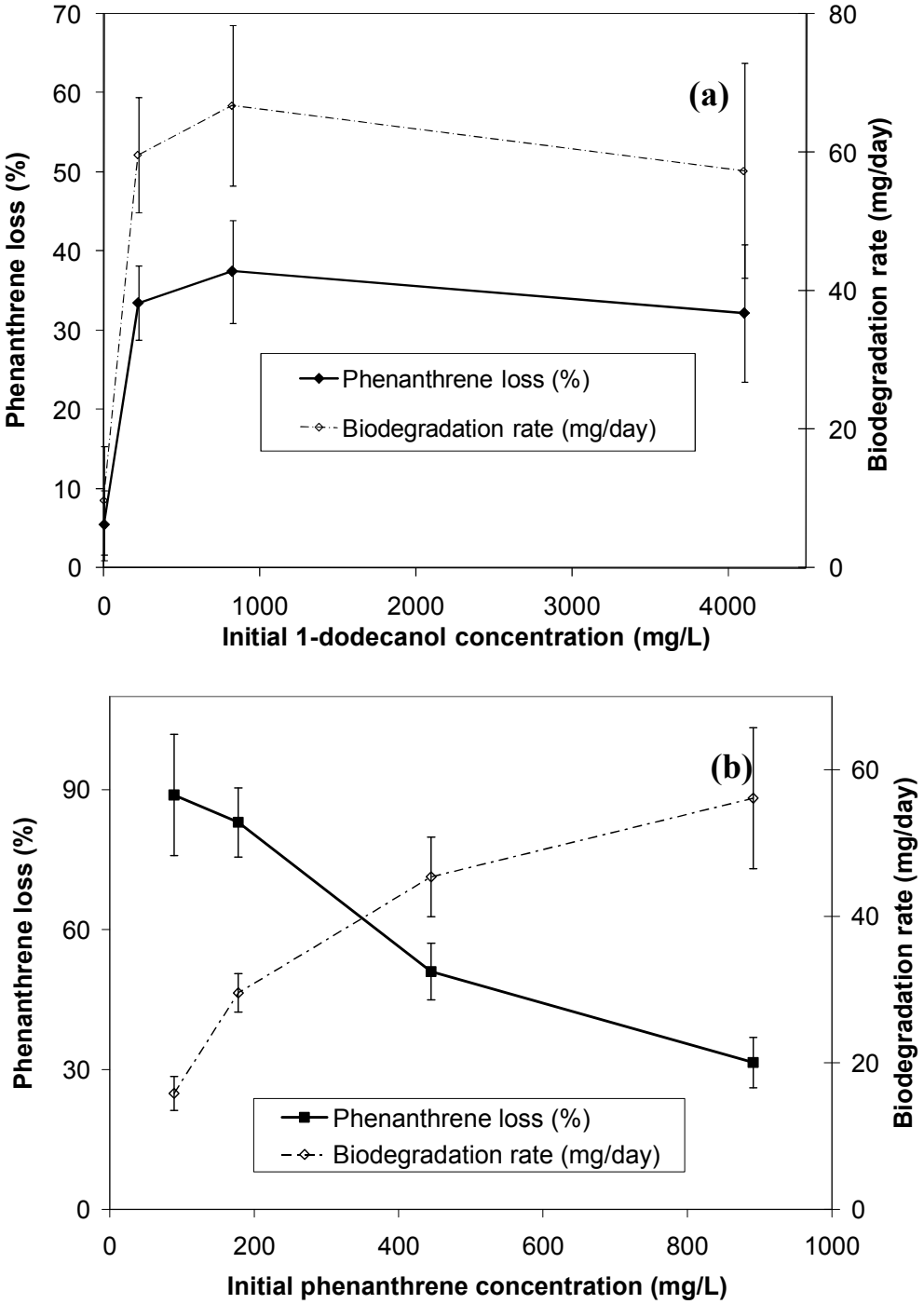

Figure 4.2: Effect of 1-dodecanol on the biodegradation of phenanthrene after 120 h incubation with non-induced LP6a. (a) Samples with different initial concentrations of 1-dodecanol. Phenanthrene concentration was 5 mM (891 mg/L) in all samples. (b) Samples with different initial phenanthrene concentrations. 1-Dodecanol concentration was 217 mg/L in all samples.

Figure 4.2b shows the degradation results for samples with different initial phenanthrene concentrations but at constant volume ratio of oil:water, and an initial 1-dodecanol concentration of 217 mg/L. As the phenanthrene concentration increased from 89 to 891 mg/L the extent of biodegradation dropped from circa 90% to 32% after 120 h, but the average rate of biodegradation increased from 16 to 56 mg/d. These data illustrate that at constant 1-dodecanol concentration, the rate of biodegradation increases with the initial concentration of phenanthrene.

To examine the effect of 1-dodecanol on phenanthrene biodegradation over a longer time period, three sets of cultures containing 0, 820 or 4100 mg/L 1-dodecanol were incubated with uninduced LP6a for up to 360 h (Figure 4.3). The addition of 1-dodecanol increased the extent of biodegradation for the incubation times of 120 h and 240 h, but after 360 h incubation all of the cultures gave equivalent conversion in the range 53-60%. The effect of 1-dodecanol at 820 and 4100 mg/L was equivalent, consistent with the results given in Figure 4.2a. At this condition the 1-dodecanol biodegradation rate was about 6.5 mg/L/h so at an initial 1-dodecanol concentration of 820 mg/l all the 1-dodecanol was converted after 126 h. When the initial concentration of 1-dodecanol was 4100 mg/L, about 43% of 1-dodecanol remained after 360 h. These experiments show that at longer incubation times, the cultures approach a comparable level of phenanthrene degradation (55 ± 15% after 360 h) regardless of initial 1-dodecanol concentrations.

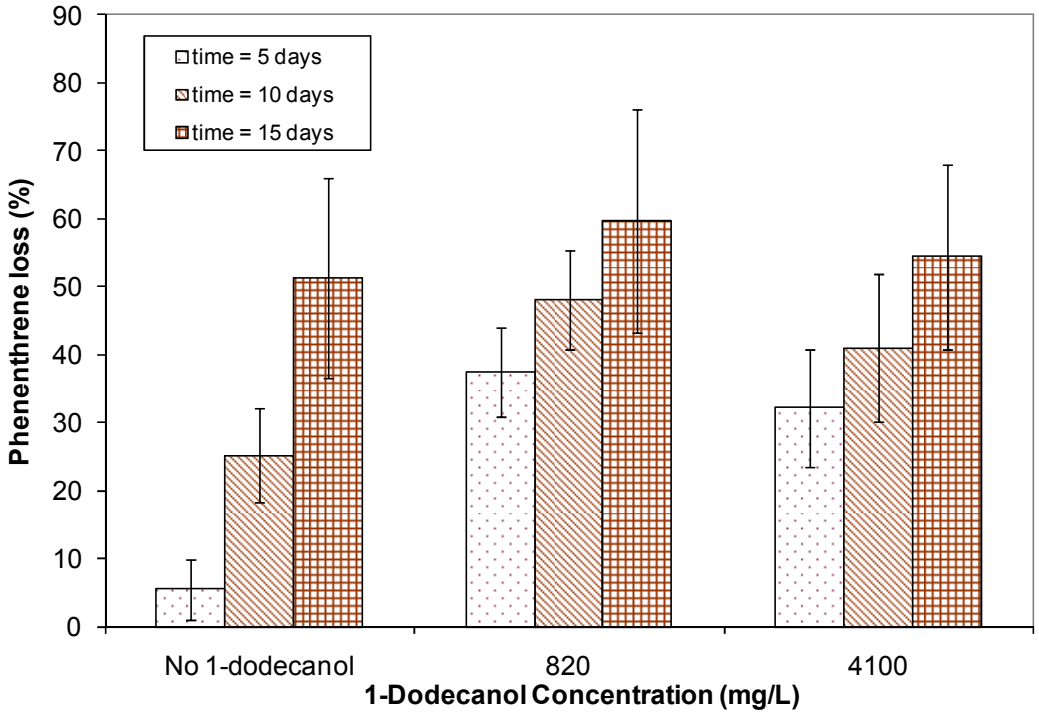

Figure 4.3: Effect of different 1-dodecanol concentrations on phenanthrene degradation after 120, 240 or 360 h incubation with uninduced LP6a. Each column represents the mean value of three replicates and error bars show the standard deviation. Initial phenanthrene concentration was 891 mg/L. The volume of HMN was constant in all experiments at 1mL.

4.3.4 Mineralization experiments with induced cultures

A series of experiments was conducted using the induced *P. fluorescens* LP6a cultures to determine the effect of inoculum size and 1-dodecanol concentration on mineralization of ^{14}C-phenanthrene dissolved in HMN. The use of radiolabeled phenanthrene and sampling the produced $^{14}CO_2$ allowed frequent monitoring of biodegradation without sacrificing the whole culture. These experiments were designed to obtain data on shorter time scales and to achieve a better observation of phenanthrene conversion in a two phase system. The data in Figure 4.4 show no significant effect of biomass density on mineralization of phenanthrene by LP6a, given an average standard deviation for these samples of ±0.51. These data suggest that initial inoculum size does not control the mineralization of phenanthrene in this system.

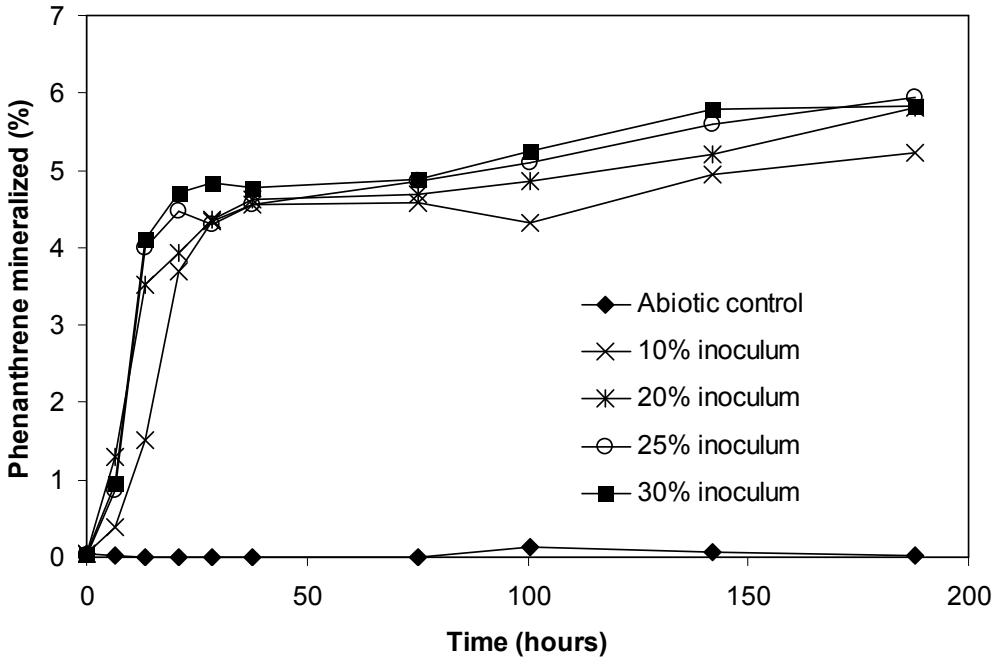

Figure 4.4: Effect of the inoculum size on the biodegradation of phenanthrene by *P. fluorescens* LP6a. Means of triplicate cultures are shown. Error bars are omitted for clarity.

The data in Figure 4.5 reveal that addition of 120 mg/L or 160 mg/L of 1-dodecanol enhanced the mineralization of phenanthrene present in HMN at an initial concentration of 178 mg/L. Addition of 60 mg/L 1-dodecanol did not change the mineralization significantly. 2-Dodecanol, however, was effective at this concentration (60 mg/L). The effect of 2-dodecanol was similar to that of 1-dodecanol at higher concentrations. Initial rates of $^{14}CO_2$ production were comparable for all cultures. After 40 h the rate of mineralization slowed in all cultures. By 100 h incubation, cultures initially containing 120 or 160 mg 1-dodecanol/L or 60 mg/L 2-dodecanol achieved above 10% mineralization, versus the culture with 0 or 60mg/L 1-dodecanol which reached a plateau at just under 5% mineralization. These results clearly demonstrate the positive effect of 1-dodecanol at concentrations above 120 mg/L and for 2-dodecanol at concentrations above 60 mg/L.

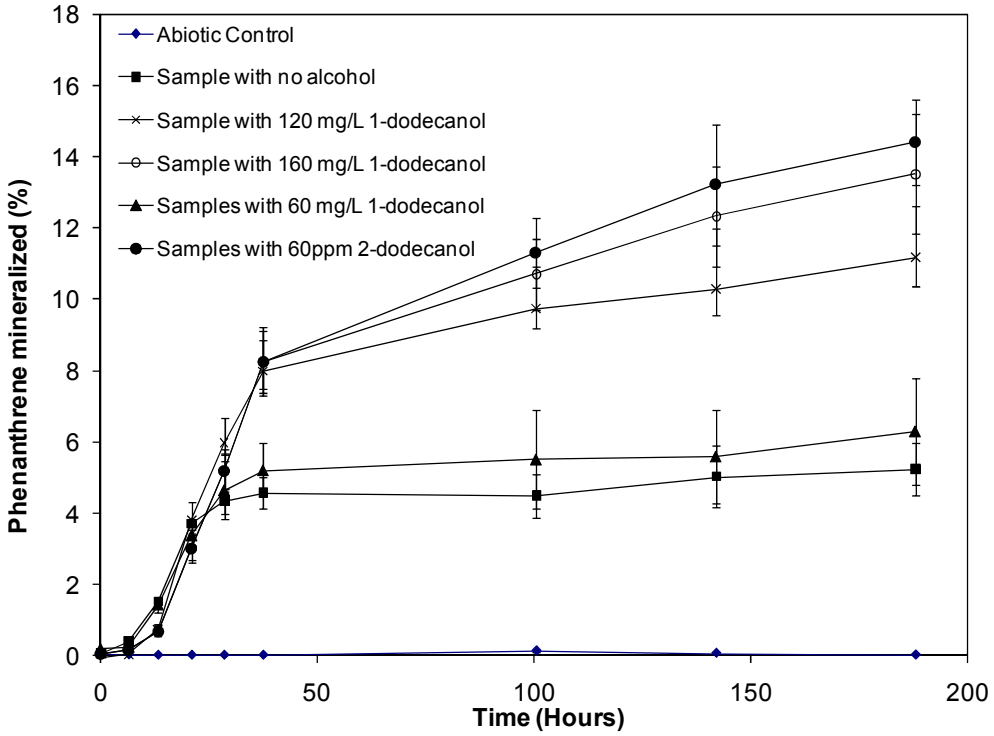

Figure 4.5: Effect of different concentrations of 1-dodecanol on phenanthrene mineralization. Data points represent the mean of three replicates and error bars show the standard deviation. Mineralization is calculated based on the percentage of ^{14}C recovered as $^{14}CO_2$.

The effect of different concentrations of phenanthrene and 1-dodecanol on mineralization was examined by conducting experiments with phenanthrene concentrations of 178, 214, 356 or 445 mg/L and initial 1-dodecanol concentrations of 120 mg/L or 180 mg/L (Table 4.1). The different concentrations of 1-dodecanol were used to avoid large changes in the ratio of phenanthrene to 1-dodecanol. A control culture lacking 1-dodecanol at each concentration of phenanthrene provided a comparison. Only the final extent of mineralization of phenanthrene after 150 h is shown on Table 4.1. In all these samples the presence of 1-dodecanol enhanced mineralization of phenanthrene when compared to the sample with no 1-dodecanol, however, the effect of 1-dodecanol on these cultures was more pronounced at higher phenanthrene concentration (Table 4.1). After 150 h all cultures had reached a plateau, similar to the data in Figure 4.5. The data in Table 4.1 indicate that, while presence of 1-dodecanol improves the

mineralization of phenanthrene about two-fold in the culture containing 178 mg/L phenanthrene, it is almost twice as effective when the phenanthrene concentration is 445 mg/L.

Table 4.1: Mineralization by *P. fluorescens* LP6a of phenanthrene dissolved in HMN after 150 h incubation. Control culture was setup without 1-dodecanol.

phenanthrene initial concentration (mg/L)	1-dodecanol concentration (mg/L)	Mineralization (%) (No 1-dodecanol control)	Mineralization (%) (in presence of 1-dodecanol)
445	180	1.0	3.8
356	180	2.2	5.8
214	120	4.9	10.8
178	120	5.0	10.3

Because the extent of mineralization was low in all cultures, we performed a partial radiochemical balance to determine the fate of ^{14}C phenanthrene that had not been mineralized. To do this we removed samples of the aqueous phase, KOH containing trapped $^{14}CO_2$, and the cell pellet. The initial concentration of phenanthrene was 445 mg/L and that of 1-dodecanol was 180 mg/L. The ^{14}C measured in the aqueous phase followed the same trend as the ^{14}C mineralized (Figure 4.6). With addition of 1-dodecanol, 6% of phenanthrene was mineralized after 140 hours, and 85% of the total ^{14}C was recovered in the aqueous phase. In contrast, the sample without 1-dodecanol showed mineralization of 2.3% in the same time period, and only 40% of radioactive activity was recovered at the aqueous phase. The ^{14}C content of the biomass was under 10% in both cases after 140 hours (Table 4.2). Consequently, with addition of 1-dodecanol, essentially all of the added phenanthrene was either mineralized, converted to water-soluble metabolites, or incorporated into biomass. In the sample without alcohol, almost 50% of ^{14}C remained in the HMN phase, as determined by difference. These data indicate that most of non-mineralized ^{14}C end up in the aqueous phase as water soluble metabolites of phenanthrene biodegradation. Table 4.2 summarizes these data at the end of the experiment period (after 140 hours).

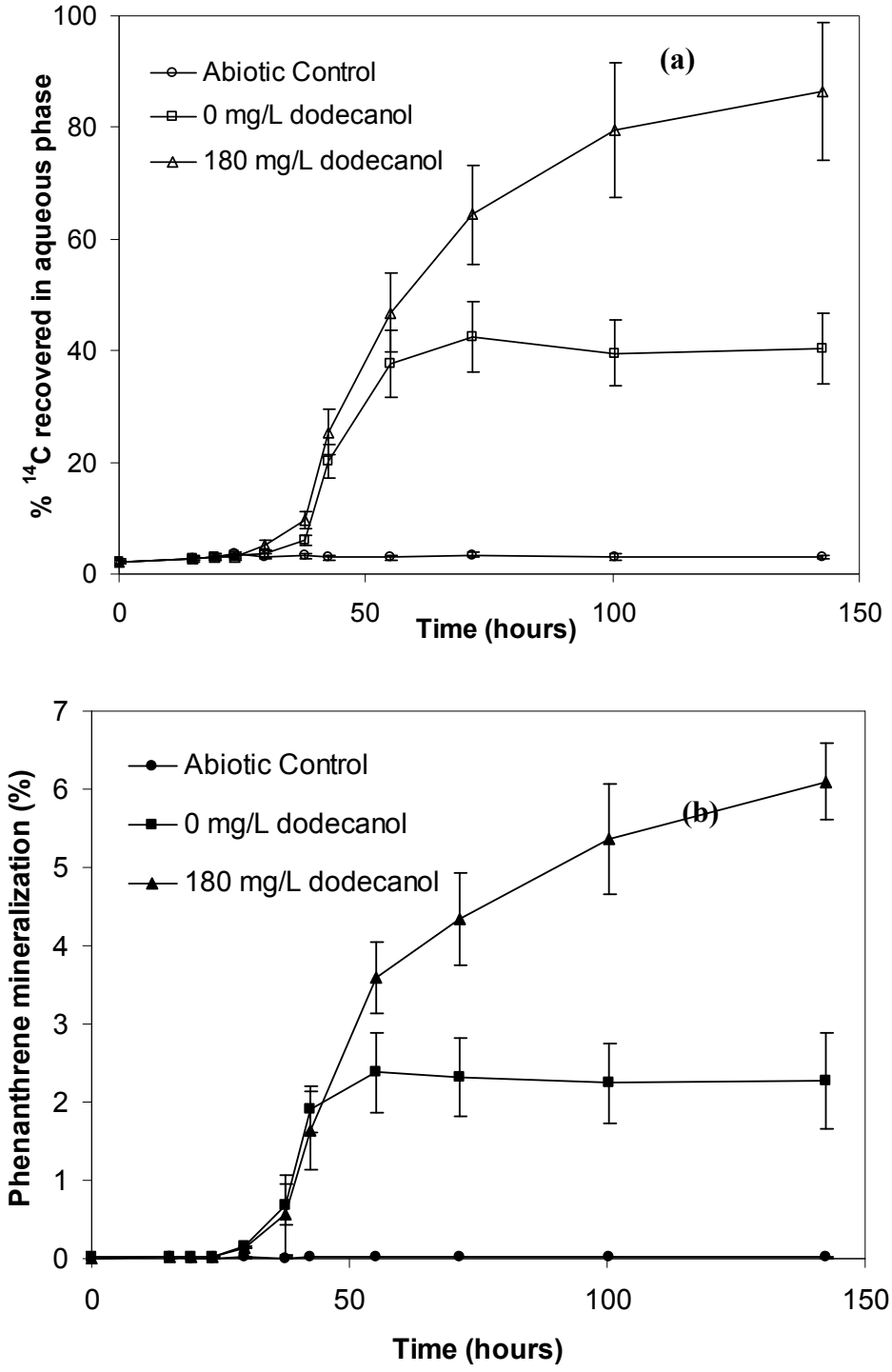

Figure 4.6: ^{14}C recovered in aqueous phase (a) and as $^{14}CO_2$ (b). Initial concentration of phenanthrene was 356 mg/L and of 1-dodecanol (when present) was 180 mg/L.

Table 4.2: Distribution of ^{14}C after 150 h incubation of *P. fluorescens* LP6a with HMN-dissolved phenanthrene with or without 1-dodecanol

Culture	% recovery of added ^{14}C from various culture fractions [a]				
	$^{14}CO_2$	aqueous phase	pellet	total measured	HMN phase (by difference)
LP6a with no 1-dodecanol	2.3 ± 0.6	40.3 ± 6.3	9.6 ± 1.7	52.2	47.8
LP6a with 180 mg/L 1-dodecanol	6.1 ± 0.5	86.4 ± 12.4	7.2 ± 1.2	99.7	0.3
abiotic control [b]	0.0 ± 0.0	3.0 ± 0.4	NA	3.0	97

[a] Mean values of percentage of recovery for 150 h cultures. Mean ± standard deviation (n=3), where applicable

[b] Abiotic control was set up with both phenanthrene and 1-dodecanol but no LP6a.

To evaluate the possibility of any impact on biodegradation due to surface active properties of 1-dodecanol, the surface and interfacial tension of culture medium incubated in the presence and absence of 100 and 200 mg/L 1-dodecanol was measured. The results in Table 4.3 for the surface and interfacial tension measurements for deionized water, fresh TSB medium and cell-free TSB spent medium of *P. fluorescens* LP6a cultures show only a small decrease in surface tension and interfacial tension of the cell free culture medium in the presence of 1-dodecanol. Addition of 1-dodecanol at 100 or 200 mg/L reduced the surface tension by 5.2% and 10.5% respectively. Interfacial tension measurements for the same samples revealed declines of 11.8% and 26.6% accordingly. The biggest effect was on the interfacial tension of the spent medium from which cells had been removed by filtration, which dropped from 44.5 mN/m without 1-dodecanol to 32.6 mN/m with 200 mg/L of 1-dodecanol. These results show that 1-

dodecanol reduces the interfacial tension by at most 27% at a concentration of 200 mg/L.

Table 4.3: Effect of 1-dodecanol on surface and interfacial tension of deionized water, fresh TSB medium and TSB spent medium of *P. fluorescens* LP6a culture at 1-dodecanol concentrations of 0, 100 or 200 mg/L.

Solution	1-dodecanol (mg/L)	Surface tension (mN/m)	Interfacial tension (mN/m)
Deionized water	0	72.0 ± 0.41	48.9 ± 0.46
Fresh TSB medium	0	70.3 ± 0.50	46.5 ± 0.67
Spent TSB medium (cell free)	0	64.7 ± 0.70	44.5 ± 0.77
	100	61.3 ± 0.76	39.2 ± 0.46
	200	57.9 ± 0.72	32.6 ± 0.66

4.4 Discussion

The data of Figure 4.1 confirm that the addition of 1-dodecanol prior to the biodegradation of phenanthrene increased the ability of growing LP6a cells to adhere to the oil-water interface as measured by MATH test during incubation up to 60 h. In contrast, adhesion of cells to the oil-water interface gradually increased with time in the 1-dodecanol-free control. This observation is consistent with the ability of 1-dodecanol to enhance bacterial adhesion by increasing the three phase contact angle between cell surface, oil and water, as mentioned in Chapter 3. In fact the MATH test performed on cells grown on phenanthrene in the two phase system gave similar results to those observed with the cells grown on TSB (Figure 3.3). This is also consistent with the fact that the effect of dodecanol on cells adhesion to the oil-water interface does not depend on the metabolic activity of cells. The gradual increase in adhesion of LP6a to the oil-water interface during biodegradation is in agreement with several other reports that hydrocarbon-degrading bacteria exhibit an increase in cell surface hydrophobicity and adhesion to oil during growth (Al-Tahhan, et al. 2000; Norman, et al. 2002; Prabhu and Phale 2003; Wick, et al. 2003). The change in adhesion with time led us to expect

that any influence of 1-dodecanol on biodegradation due to enhancement in cell surface hydrophobicity will be mostly noticed over short times, diminishing with time as the bacteria became more hydrophobic even without 1-dodecanol.

The results of our study demonstrate that enhancement of bacterial attachment to the oil-water interface can increase the biodegradation of phenanthrene by *P. fluorescens* LP6a. Increases in 1-dodecanol concentration from 217 mg/L up to 4100 mg/L did not further enhance biodegradation (Figure 4.2a). Since 1-dodecanol is also degradable by LP6a, these results, suggest that catabolism of 1-dodecanol and the resulted increase in biomass because of that was not an important factor in biodegradation of phenanthrene. In fact, later experiments with 10, 20, 25 or 30% initial inoculum sizes (Figure 4.4) showed that phenanthrene mineralization in this system does not change significantly with the biomass density. At constant initial 1-dodecanol concentration, the rate of biodegradation increased with the initial concentration of phenanthrene (Figure 4.2b), consistent with an enhancement in the uptake of phenanthrene by attached cells.

As demonstrated in Figure 4.1, at longer incubation times, the cells showed the same level of adhesion to the oil-water interface regardless of 1-dodecanol presence. A similar trend was observed in biodegradation experiments, where cultures eventually achieved comparable levels of phenanthrene degradation in longer time scales (55 ± 15% after 360 h; Figure 4.3). Since 50% of initial 1-dodecanol remained in the sample containing 4100 mg/L of 1-dodecanol, the disappearance of 1-dodecanol cannot account for this observation. Rather, the bacteria likely adapted to attach to the HMN more effectively (Figure 4.1) giving a delayed increase in the rate of biodegradation in the absence of 1-dodecanol.

The data on mineralization offer additional insight into phenanthrene degradation in these cultures. As illustrated in Figure 4.5, the extent of mineralization by cultures grown in the presence of 120 or 160 mg/L 1-dodecanol or 60 mg/L 2-dodecanol was more than two times higher than mineralization by cells grown without any alcohol. According to the MATH test results reported in section 3.3,

2-dodecanol resulted in slightly higher adhesion of LP6a, however, these differences were not significant and both alcohols resulted in a comparable increase in LP6a adhesion to an oil-water interface (Figure 3.3). According to data on the two alcohols, degradability by LP6a cultures (Appendix A), the 2-dodecanol degradation rate is slightly lower than that of 1-dodecanol and this might be the reason why 2-dodecanol is more effective at lower concentrations. At higher concentration of these alcohols, both of the compounds had a similar influence on mineralization of phenanthrene and cultures achieved more than two-fold increase in mineralization of phenanthrene dissolved in HMN (Figure 4.5). The advantage offered by the alcohols (tested for 1-dodecanol only), was even higher when the phenanthrene: 1-dodecanol ratio was higher (Table 4.1).

In all mineralization experiments conducted, the percentage of ^{14}C recovered as CO_2 did not exceed 15%. Further experiments, conducted to determine distribution of the remaining ^{14}C, revealed that the most of phenanthrene has been converted to other water soluble metabolites in cultures incubated with 1-dodecanol (Table 4.2). The data of Table 4.2 indicated that phenanthrene was completely degraded after 50 h, to carbon dioxide, biomass and water-soluble metabolites in the presence of 1-dodecanol. When cultures were incubated without any 1-dodecanol present in the media, about 50% of the initially added ^{14}C was not recovered anywhere in pellets, aqueous phase or as $^{14}CO_2$. In other words, it can be concluded that in absence of 1-dodecanol, 50% of labeled carbon still remained in organic phase most likely as phenanthrene, while cells incubated with 1-dodecanol converted all phenanthrene initially present in organic phase.

Figure 4.6 clearly shows that the increase in the content of labeled carbon in the aqueous phase follows the same trend as the ^{14}C that is mineralized and recovered as $^{14}CO_2$ in all cultures. Once the HMN was cleared of phenanthrene, the advantage of higher adhesion (in the presence of 1-dodecanol) is expected to disappear and have no further influence. With increases in the initial phenanthrene concentration, there was more phenanthrene present in the organic phase and for a longer time, therefore the uptake by the cells would be enhanced by 1-dodecanol-

mediated adhesion for a longer period of time and this could explain the data in Table 4.1. The lack of conversion of the aqueous metabolites (Figure 4.6) was likely due to inhibition. The accumulation of water soluble metabolites and inhibition of PAHs mineralization by them has been reported previously (Bouchez, et al. 1996; Heitkamp and Cerniglia 1988; Kazunga and Aitken 2000). In this study the metabolites were not characterized and further research is required to clarify the details of this phenomenon.

Several mechanisms could explain the positive influence of 1-dodecanol on the biodegradation. One mechanism could be that 1-dodecanol served as an extra carbon source and increased the biomass population. Since 1-dodecanol is biodegradable by LP6a it is possible this could be the case, but the data of Figure 4.4 showed that biomass population was not a controlling factor in mineralization of phenanthrene. An unlikely explanation would be induction of the phenanthrene biodegradation pathway by the addition of 1-dodecanol. Given that the pathways for degradation of PAHs and long-chain alcohols share no common enzymes (Ludwig, et al. 1995; Peng, et al. 2008), this mechanism is unlikely.

A third mechanism could be an increase in emulsification of the HMN phase due to presence of 1-dodecanol, giving higher surface area for dissolution of phenanthrene from smaller droplets. According to Bancroft's rule (Langevin 2006), oil-soluble surfactants, such as 1-dodecanol are not expected to stabilize oil-in-water emulsions. The data in Table 4.2 confirmed that 1-dodecanol reduced the interfacial tension by at most 27% at a concentration of 200 mg/L, and still gave a surface tension of 57 mN/m. The HMN was not visibly emulsified in the aqueous phase in the cultures, and other investigators have reported that this magnitude of reduction in surface and interfacial tension by surfactants could not result in any significant emulsification (Huang, et al. 2009; Willumsen and Karlson 1997; Youssef, et al. 2004). A study of oil-soluble surfactants in hexadecane-water emulsions (Lobo and Svereika 2003) showed that even larger reductions of interfacial tension had no significant effect on droplet size distribution. A study of the characteristics of surfactant-producing bacteria

(Huang, et al. 2009) observed that bacterial cultures with surface tensions above 50 mN/m did not give any emulsification of oil, as measured by a drop-collapse test.

The enhancement in phenanthrene biodegradation observed in this study was consistent with the positive influence of the alcohols on microbial adhesion to the oil-water interface, thereby providing better uptake by the bacteria. As mentioned above, aqueous metabolites accumulated during biodegradation of phenanthrene (Figure 4.6) and it is possible that these metabolites acted to inhibit the further degradation by bacteria. Higher adhesion of the bacterial cells to the oil-water interface could remove a great portion of cells from the aqueous phase that contains the inhibitors and hence contribute to the improved biodegradation via reducing the influence of inhibitors on bacteria. On the other hand, based on many studies, adhesion can also facilitate the mass transfer of substrate from organic phase to the cells (Ortega-Calvo and Alexander 1994; Rosenberg and Rosenberg 1981; Weisz 1973). In our experiments improved adhesion of LP6a to the oil-water interface could have helped the biodegradation in both of these ways. Regardless of the exact mechanism, our data on adhesion, biodegradation, and mineralization illustrate that adhesion of LP6a to the oil-water interface correlates with a higher degree of biodegradation and mineralization.

The importance of adhesion in biodegradation processes may be underestimated in many studies. Most studies in research laboratories have focused on the role of surface active compounds to increase mass transfer, without considering their counter effect on spreading the contaminant and the possibility of detaching cells from the interface (Churchill, et al. 1995; Singh, et al. 2007; Volkering, et al. 1997). Laboratory experiments are usually conducted at a small scale where sufficient mixing is available. In large scale bioremediation applications this is rarely the case. Studies determining the effectiveness of full scale bioremediation projects are usually focused on the role of factors such as nutrients, temperature and surfactants (Bragg, et al. 1994; Gallego, et al. 2007). The role of adhesion has never been considered in field studies, mostly because there is not a well

established connection between adhesion and biodegradation in laboratory studies. When there is a contamination incident, such as the Exxon Valdez oil-spill (Shaw 1992), the contaminant is usually spread over an extended area and economic considerations prevent using any artificial mixing facilities. These conditions intensify the role of adhesion to the oil-water interface in the biodegradation of contaminants. In general, we expect the role of diffusion of substrate to the cells over short distances to become more and more dominant as the aqueous concentration of the substrate of interest decreases (Weisz 1973). The aqueous concentration of target compounds is further reduced by dilution in a non-aqueous phase, as in this study. Consequently, microbial adhesion may offer significant advantages in large scale projects for hydrocarbon bioremediation, in order to overcome bioavailability limitations and play a much more important role in increasing the overall mass transfer of hydrocarbons to the microorganisms. Development of bioremediation approaches that use adhesion as an asset to benefit process performance may be an attractive strategy.

Research has shown that the ability of different variants of a single hydrocarbon-degrading bacterium to take up poorly water soluble compounds correlates with the cell surface hydrophobicity (Obuekwe, et al. 2007a; Obuekwe, et al. 2008; Rosenberg and Rosenberg 1981). Other studies on bacterial populations isolated from various hydrocarbon-contaminated sites demonstrated that the ability of bacteria to degrade hydrocarbons correlates with their adhesion to hydrophobic surfaces (Bouchez-Naïtali, et al. 1999; Obuekwe, et al. 2009). In addition there have been few studies that discussed possible on the role of bacterial cell adhesion and its promotion in biodegradation (Deschênes, et al. 1996; Fu, et al.; Jirku, et al. 2001; Ortega-Calvo and Alexander 1994; Suchanek, et al. 2000), however, most of these studies were conducted in the presence of surfactants and involved an interplay of several complex factors such as emulsification, micellar solubilization and surfactant degradation which made it difficult to reach a conclusion on the role of adhesion on biodegradation experiments. In spite of these studies on the influence of bacterial adhesion on biodegradation, to the best of my knowledge, direct use of adhesion promoting agents to improve biodegradation of poorly

water soluble hydrocarbons such as alkanes and PAHs has not been reported. The present report is the first definitive example of an enhancement in biodegradation of a PAH using an additive to improve the adhesion of bacteria the oil-water interface. Due to the supreme importance of the oil-water interface in biodegradation and bioprocessing of hydrocarbons, my study can provide new designs for bioremediation and bioprocessing strategies and for optimizing existing processes. It can also help in developing some fundamental understanding of mechanisms and methods involved in such processes.

4.5 References

1. **Al-Tahhan RA, Sandrin TR, Bodour AA, Maier RM (2000).** Rhamnolipid-induced removal of lipopolysaccharide from pseudomonas aeruginosa: effect on cell surface properties and interaction with hydrophobic substrates. *Appl Environ Microbiol* 66:3262-3268.

2. **Bouchez M, Blanchet D, Vandecasteele JP (1996).** The microbiological fate of polycyclic aromatic hydrocarbons: Carbon and oxygen balances for bacterial degradation of model compounds. *Appl Microbiol Biotechnol* 45:556-561.

3. **Bouchez-Naïtali M, Rakatozafy H, Marchal R, Leveau JY, Vandecasteele JP (1999).** Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. *J Appl Microbiol* 86:421-428.

4. **Bragg JR, Prince RC, Harner EJ, Atlas RM (1994).** Effectiveness of Bioremediation for the Exxon-Valdez oil-spill. *Nature* 368:413-418.

5. **Bramwell DAP, Laha S (2000).** Effects of surfactant addition on the biomineralization and microbial toxicity of phenanthrene. *Biodegradation* 11:263-277.

6. **Breedveld GD, Karlsen DA (2000).** Estimating the availability of polycyclic aromatic hydrocarbons for bioremediation of creosote contaminated soils. *Appl Microbiol Biotechnol* 54:255-261.

7. **Chen P, Pickard MA, Gray MR (2000).** Surfactant inhibition of bacterial growth on solid anthracene. *Biodegradation* 11:341-347.

8. **Churchill PF, Dudley RJ, Churchill SA (1995).** Surfactant-enhanced bioremediation. *Waste Manage* 15:371-377(7).

9. **Deschênes L, Lafrance P, Villeneuve J, Samson R (1996).** Adding sodium dodecyl sulfate and *Pseudomonas aeruginosa* UG2 biosurfactants inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote-contaminated soil. *Appl Microbiol Biotechnol* 46:638-646.

10. **Douben, Peter E. T. (2003).** PAHs : An ecotoxicological perspective. NJ: Wiley & Sons, Inc., 392p.

11. **Edwards DA, Luthy RG, Liu Z (1991).** Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. *Environ Sci Technol* 25:127-133.

12. **EPA (2002).** National recommended water quality criteria: 2002 EPA-822-R-02-047:33.

13. **Foght JM, Westlake, D. W. S. (1996).** Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. *Biodegradation* 7:353-366.

14. **Foght JM, Westlake DWS (1991).** Cross hybridization of plasmid and genomic dna from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. *Can J Microbiol* 37:924-932.

15. Fu H, Zeng G, Zhong H, Yuan X, Wang W, Huang G, Li J (2007). Effects of rhamnolipid on degradation of granular organic substrate from kitchen waste by a *Pseudomonas aeruginosa* strain. *Colloids Surf B* 58:91-97.

16. **Gallego JR, Fernandez JR, Diez-Sanz F, Ordonez S, Sastre H, Gonzalez-Rojas E, Pelaez AI, Sanchez J (2007).** Bioremediation for shoreline cleanup: In situ vs. on-site treatments. *Environ Eng Sci* 24:493-504.

17. **Gillesby BE, Stanostefano M, Porter W, Safe S, Wu ZF, Zacharewski TR (1997).** Identification of a motif within the 5' regulatory region of pS2 which is responsible for AP-1 binding and TCDD-mediated suppression. *Biochemistry (N Y)* 36:6080-6089.

18. **Goldberg S, Konis Y, Rosenberg M (1990).** Effect of cetylpyridinium chloride on microbial adhesion to hexadecane and polystyrene. *Appl Environ Microbiol* 56:1678-1682.

19. **Heitkamp MA, Cerniglia CE (1988).** Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. *Appl Environ Microbiol* 54:1612-1614.

20. **Hori K, Matsuzaki Y, Tanji Y, Unno H (2002).** Effect of dispersing oil phase on the biodegradability of a solid alkane dissolved in non-biodegradable oil. *Appl Microbiol Biotechnol* 59:574-579.

21. **Huang X, Liu J, Lu L, Wen Y, Xu J, Yang D, Zhou Q (2009).** Evaluation of screening methods for demulsifying bacteria and characterization of lipopeptide bio-demulsifier produced by *Alcaligenes* sp. *Bioresour Technol* 100:1358-1365.

22. **Jirku V, Masak J, Cejkova A (2001).** Short communication - Significance of physical attachment of fungi for bio-treatment of water. *Microbiol Res* 156:383-386.

23. **Kazunga C, Aitken MD (2000).** Products from the incomplete metabolism of pyrene by polycyclic aromatic hydrocarbon-degrading bacteria. *Appl Environ Microbiol* 66:1917-1922.

24. **Laha S, Luthy RG (1991).** Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. *Environ Sci Technol* 25:1920-1930.

25. **Langevin D (2006).** Oil-water emulsions. encyclopedia of surface and colloid science. Taylor & Francis. p 4271-4287.

26. **Lobo L, Svereika A (2003).** Coalescence during emulsification: 2. Role of small molecule surfactants. *J Colloid Interface Sci* 261:498-507.

27. **Ludwig B, Akundi A, Kendall K (1995).** A long-chain secondary alcohol-dehydrogenase from *Rhodococcus-erythropolis* ATCC-4277. *Appl Environ Microbiol* 69:3729-3733.

28. **Marchesi JR, Owen SA, White GF, House WA, Russell NJ (1994a).** SDS-degrading bacteria attach to riverine sediment in response to the surfactant or its primary biodegradation product dodecan-1-ol. *Microbiology* 140:2999-3006.

29. **Marchesi JR, White GF, House WA, Russell NJ (1994b).** Bacterial cell hydrophobicity is modified during the biodegradation of anionic surfactants. *FEMS Microbiol Lett* 124:387-392.

30. **McInerney MJ, Javaheri M, Nagle Jr. DP (1990).** Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. *J Ind Microbiol* 5:95-102.

31. **Mulligan CN, Yong RN, Gibbs BF (2001).** Surfactant-enhanced remediation of contaminated soil: a review. *Eng Geol* 60:371-380.

32. **Norman RS, Frontera-Suau R, Morris PJ (2002).** Variability in *Pseudomonas aeruginosa* lipopolysaccharide expression during crude oil degradation. *Appl Environ Microbiol* 68:5096-5103.

33. **Obuekwe CO, Al-Jadi ZK, Al-Saleh E (2007a).** Insight into heterogeneity in cell-surface hydrophobicity and ability to degrade hydrocarbons among cells of two hydrocarbon-degrading bacterial populations. *Can J Microbiol* 53:252-260.

34. **Obuekwe CO, Al-Jadi ZK, Al-Saleh ES (2008).** Comparative hydrocarbon utilization by hydrophobic and hydrophilic variants of *Pseudomonas aeruginosa*. *J Appl Microbiol* 105:1876-1887.

35. **Obuekwe CO, Al-Jadi ZK, Al-Saleh ES (2007b).** Sequential hydrophobic partitioning of cells of *Pseudomonas aeruginosa* gives rise to variants of increasing cell-surface hydrophobicity. *FEMS Microbiol Lett* 270:214-219.

36. **Obuekwe CO, Al-Jadi ZK, Al-Saleh ES (2009).** Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. *Int Biodeterior Biodegrad* 63:273-279.

37. **Ortega-Calvo JJ, Alexander M (1994).** Roles of bacterial attachment and spontaneous partitioning in the biodegradation of naphthalene initially present in nonaqueous-phase liquids. *Appl Environ Microbiol* 60:2643-2646.

38. **Peng R, Xiong A, Xue Y, Fu X, Gao F, Zhao W, Tian Y, Yao Q (2008).** Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiol Rev* 32:927-955.

39. **Prabhu Y, Phale PS (2003).** Biodegradation of phenanthrene by *Psuedomonas* sp strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. *Appl Microbiol Biotechnol* 61:342-351.

40. **Rodrigues LR, Banat IM, van der Mei HC, Teixeira JA, Oliveira R (2006).** Interference in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicone rubber by rhamnolipid biosurfactants. *J Appl Microbiol* 100:470-480.

41. **Rosenberg M, Rosenberg E (1981).** Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *J Bacteriol* 148:51-57.

42. **Safe SH (1998).** Hazard and risk assessment of chemical mixtures using the toxic equivalency factor approach. *Environ Health Perspect* 106:1051-1058.

43. **Shaw DG (1992).** The Exxon-Valdez oil-spill: Ecological and social-consequences. *Environ Conserv* 19:253-258.

44. **Singh A, Van Hamme JD, Ward OP (2007).** Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnol Adv* 25:99-121.

45. **Skarpheoinsdottir H, Ericson G, Svavarsson J, Naes K (2007).** DNA adducts and polycyclic aromatic hydrocarbon (PAH) tissue levels in blue mussels (Mytilus spp.) from Nordic coastal sites. *Mar Environ Res* 64:479-491.

46. **Stelmack PL, Gray MR, Pickard MA (1999).** Bacterial adhesion to soil contaminants in the presence of surfactants. *Appl Environ Microbiol* 65:163-168.

47. **Suchanek M, Kostal J, Demnerova K, Kralova B (2000).** Use of sodium dodecyl sulphate for stimulation of biodegradation of n-alkanes without residual contamination by the surfactant. *Int Biodeterior Biodegrad* 45:27-33.

48. **Ulrich A, Guigard S, Foght J, Semple K, Pooley K, Armstrong J, Biggar K (2009).** Effect of salt on aerobic biodegradation of petroleum hydrocarbons in contaminated groundwater. *Biodegradation* 20:27-38.

49. **Vaneechoutte M, Tjernberg I, Baldi F, Pepi M, Fani R, Sullivan ER, van der Toorn J, Dijkshoorn L (1999).** Oil-degrading *Acinetobacter* strain RAG-1 and strains described as *Acinetobacter venetianus* sp. NOV belong to the same genomic species. *Res Microbiol* 150:69-73.

50. **Volkering F, Breure AM, Rulkens WH (1997).** Microbiological aspects of surfactant use for biological soil remediation. *Biodegradation* 8:401-417.

51. **Volkering F, Breure AM, Sterkenburg A, Andel JG (1992).** Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. *Appl Microbiol Biotechnol* 36:548-552.

52. **Weissenfels WD, Klewer HJ, Langhoff J (1992).** Adsorption of polycyclic aromatic-hydrocarbons (PAHs) by soil particles: Influence on biodegradability and biotoxicity. *Appl Microbiol Biotechnol* 36:689-696.

53. **Weisz PB (1973).** Diffusion and chemical transformation: An interdisciplinary excursion. *Science* 179:433-440.

54. **Wick LY, Pasche N, Bernasconi SM, Pelz O, Harms H (2003).** Characterization of multiple-substrate utilization by anthracene-degrading *Mycobacterium frederiksbergense* LB501T. *Appl Environ Microbiol* 69:6133-6142.

55. **Willumsen PA, Karlson U (1997).** Screening of bacteria, isolated from PAH-contaminated soils, for production of biosurfactants and bioemulsifiers. *Biodegradation* 7:415-423.

56. **Woo SH, Park JM (2004).** Microbial degradation and enhanced bioremediation of polycyclic aromatic hydrocarbons. *J Ind Eng Chem* 10:16-23.

57. **Youssef NH, Duncan KE, Nagle DP, Savage KN, Knapp RM, McInerney MJ (2004).** Comparison of methods to detect biosurfactant production by diverse microorganisms. *J Microbiol Methods* 56:339-347.

5. Main Conclusions, Implications and Recommendations

5.1 Main Conclusions

This research examined the influence of adhesion-promoting compounds on biodegradation of phenanthrene by *P. fluorescens* LP6a. The main conclusions of this work can be summarized as follows:

1. Certain cationic compounds (CPC and CHX) and long chain alcohols (1-dodecanol, farnesol and 2-dodecanol) dramatically improved the adhesion of *P. fluorescens* LP6a to an oil-water interface.
2. Even though both types of compounds promoted cells adhesion to the oil-water interface, the mechanisms were different. Long chain alcohols acted through altering the cell surface hydrophobicity, whereas cationic surfactants changed the surface charge density.
3. The presence of the long chain alcohols, 1-dodecanol and 2-dodecanol, in the two phase biodegradation system consisting of phenanthrene dissolved in HMN and BH medium resulted in an immediate increase in the adhesion of LP6a cells to the oil-water interface and improved the biodegradation of phenanthrene by the bacteria.
4. Different concentrations of 1-dodecanol ranging from 217 mg/L up to 4100 mg/L resulted in a comparable increase in biodegradation of phenanthrene.
5. Investigation of the effect of various initial inoculum sizes of the bacteria and changes in surface and interfacial tension of the two phase system by 1-dodecanol demonstrated that neither biomass concentration nor the surface active properties of 1-dodecanol could explain the observed increase in biodegradation of phenanthrene in our experiments.
6. The data demonstrating biodegradation and mineralization of phenanthrene by LP6a illustrated that the enhancement in phenanthrene

biodegradation observed is consistent with the positive influence of the alcohols on microbial adhesion to the oil-water interface.

5.2 Implications

The finding that long chain alcohols can stimulate adhesion of *P. fluorescens* LP6a to an oil-water interface and hence can improve the biodegradation by this bacterium of phenanthrene in a two phase system has implications for designing effective bioremediation strategies. Various additives such as surfactants and fertilizers are employed in bioremediation projects to improve the efficiency of hydrocarbon removal from contaminated sites. These additives are used in order to improve the microbial activity or enhance the bioavailability of hydrocarbons (Edwards, et al. 1991; Pieper and Reineke 2000). Understanding the mechanism by which these additives influence the bioremediation process has a vital role in designing appropriate bioremediation methods. In the well studied case of Exxon Valdez bioremediation a fertilizer, Inipol EAP 22, was used as an oil-in-water microemulsion of nutrients in the clean-up of the oil spill from the aqueous environment (Pritchard and Costa 1991). This fertilizer contained nitrogen in the form of urea emulsified with oleic acid and phosphorus in the form of lauryl phosphate. Although initial reports of a positive effect of this additive was attributed to its ability to provide nitrogen and phosphorus in nutrient-limited environments (Glaser 1991), later studies questioned this argument (Churchill, et al. 1995a; Churchill, et al. 1995b) and emphasized the role of surface active property of compounds used in the formulation of the fertilizer as a possible reason for its positive influence on bioremediation. Other cases have been studied in which additives even acted to reduce the efficiency of bioremediation through some unexpected interactions with the system components (Laha and Luthy 1991; Mulkins-Phillips and Stewart 1974; Tsomides, et al. 1995). This shows the importance of various interactions that additives can have with biological and physico-chemical variables in the bioremediation process. Optimal design of bioremediation strategies requires sufficient knowledge of these interactions and their influence in outcome of process. One of the factors that has not received

adequate attention in this regard is the adhesion of oil degrading microbes to the oil-water interface. Our study demonstrated that improved adhesion of a PAH-degrading bacterium to an oil-water interface can stimulate biodegradation of phenanthrene in a two phase system. These results indicate that to design an efficient bioremediation strategy to remove poorly water soluble hydrocarbon contaminants, the influence of additives on microbial adhesion needs to be considered as an important factor. In an open environment such as oil spills in lakes, microbial adhesion can play an even more important role than that observed in this research due to lack of intense mixing in full scale applications. In fact, in one of the studies to assess the mechanism of biodegradation enhancement by Inipol EAP 22, it was evident that the positive effect of this fertilizer on mineralization of phenanthrene did not correlate with its ability to increase the solubility of phenanthrene when compared to Triton X-45, another surfactant tested in the study (Churchill, et al. 1995a). Although this matter was not discussed in the paper, it was evident that there are factors other than the surface active property of the Inipol EAP 22 that caused the very high mineralization rate by this fertilizer compared to other surfactants. Inipol EAP 22 contains lauryl phosphate ($C_{12}H_{27}PO_4$) in its formulation which is structurally very similar to 1-dodecanol ($C_{12}\ H_{26}OH$). It can be speculated that Inipol EAP 22 had a positive effect on microbial adhesion to the oil-water interface and this may have been the reason for better than expected results obtained with this additive in the mentioned study. My results combined with suggestive data available from other studies indicate the effect of microbial adhesion to the oil-water interface deserve a serious consideration.

Bacterial adhesion to hydrophobic surfaces has recently been considered in flotation and flocculation for mineral processing. The fact that some microorganisms can adhere to the surfaces of minerals creates potential applications for microorganisms as flotation collectors, flotation depressants and activators. Modification of surface hydrophobicity of microorganisms can make them more suitable and specific for this separation processes and can offer significant improvement in dealing with these issues. Separation of fine

hydrophobic materials from hydrophilic mineral particles in coal production (Raichur, et al. 1996) and removing pyrite from mixtures of sulfide minerals (Nagaoka, et al. 1999) are just some of the many examples that have been examined for potential use of microorganisms for this sort of application (Smith and Miettinen 2006). Results from my study can offer significant advancements in the efficiency of these separation processes by adjusting bacterial cell surface hydrophobicity to a desired level using either long chain alcohols or cationic compounds.

5.3 Recommendations

1. The experiments on the influences of cationic compounds on the cell surface properties of *P. fluorescens* LP6a provided some indications in support of a particular mechanism to explain the interaction of these compounds with the cell surface (Figure 3.5). More studies are required to confirm or refute this mechanism. Changes in outer membrane proteins and lipopolysaccharide of LP6a cells and microscopic examination of cell surface are some of the experiments that are recommended in order to clarify the observed changes in cell surface hydrophobicity due to treatment with CPC and other cationic compounds.

2. More studies are needed to explain the observed increase in cell surface hydrophobicity of LP6a cells in presence of the long chain alcohols tested. Our results demonstrated that these alcohols dramatically change the three phase contact angle between bacterial cells surface, water and hexadecane without any significant change in the charge distribution on the bacterial cell surface. Other studies demonstrated that changes in the fatty acid composition of bacteria can increase their adhesion to hydrophobic surfaces, in this case the oil-water interface (Valeur, et al. 1988). It is proposed by others (Marchesi, et al. 1994) that due to the hydrophobic nature of these alcohols it is likely that they would enter the bacterial membranes and so alter their hydrophobicity. Further clarification of these

interactions can help in selection of more suitable compounds for any particular application and can have a vital role for any advancement in application of these compounds for bioremediation purposes.

3. The focus of this project was to investigate effects of several compounds on adhesion of a single bacterial strain to oil-water interface. It is recommended to assess the influence of the tested long chain alcohols on various gram negative and gram positive bacteria. Such experiments can provide some correlations between bacterial cell surface structure and influence of these alcohols on the bacterial adhesion to oil-water interface.

4. The study of influences of enhanced adhesion of bacteria to oil-water interface, were focused only on biodegradation of phenanthrene dissolved in HMN. It is expected that bacterial adhesion plays an even more important role in biodegradation of PAHs such as anthracene, fluoranthene or pyrene which have lower water solubility than phenanthrene. Studies need to be conducted to examine this presumption.

5. The results reported here on mineralization of phenanthrene suggested that accumulation of water soluble metabolites acts to inhibit further degradation of phenanthrene and its metabolites. Experiments to characterize these metabolites and examine the effect of individual metabolites on the biodegradation and mineralization of phenanthrene by *P. fluorescens* LP6a should be conducted.

6. Certain process parameters have been found to amplify the importance of adhesion to the oil-water interface in biodegradation of poorly water soluble hydrocarbons. It is recommended to conduct some experiments with different mixing rates and under various nutrient limitations such as substrate starvation, oxygen limitation and lower temperatures. These experiments can provide useful data for future implication of results in industrial projects.

5.4 References

1. **Churchill PF, Dudley RJ, Churchill SA (1995a).** Surfactant-enhanced bioremediation. *Waste Manage* 15:371-377(7).

2. **Churchill SA, Griffin RA, Jones LP, Churchill PF (1995b).** Biodegradation rate enhancement of hydrocarbons by an oleophilic fertilizer and a rhamnolipid biosurfactant. *J Environ Qual* 24:19-28.

3. **Edwards DA, Luthy RG, Liu Z (1991).** Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. *Environ Sci Technol* 25:127-133.

4. **Glaser J (1991).** Nutrient-enhanced bioremediation of oil-contaminated shoreline: the Valdez experience. In: Hinchee, R. E. Olfenbuttel,R.F., editor. On-site bioreclamation: processes for xenobiotic and hydrocarbon treatment. Stoneham, Butterworth- Heinemann, MA. p 366-384.

5. **Laha S, Luthy RG (1991).** Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. *Environ Sci Technol* 25:1920-1930.

6. **Marchesi JR, White GF, House WA, Russell NJ (1994).** Bacterial cell hydrophobicity is modified during the biodegradation of anionic surfactants. *FEMS Microbiol Lett* 124:387-392.

7. **Mulkins-Phillips GJ, Stewart JE (1974).** Effect of four dispersants on biodegradation and growth of bacteria on crude oil. *Appl Microbiol* 28:547-552.

8. **Nagaoka T, Ohmura N, Saiki H (1999).** A Novel Mineral Flotation Process Using *Thiobacillus ferrooxidans*. *Appl Environ Microbiol* 65:3588-3593.

9. **Pieper DH, Reineke W (2000).** Engineering bacteria for bioremediation. *Curr Opin Biotechnol* 11:262-270.

10. **Pritchard PH, Costa CF (1991).** EPAs Alaska oil-spill bioremediation project. *Environ Sci Technol* 25:372-379.

11. **Raichur AM, Misra M, Bukka K, Smith RW (1996).** Flocculation and flotation of coal by adhesion of hydrophobic *Mycobacterium phlei*. *Colloids Surf B* 8:13-24.

12. **Smith RW, Miettinen M (2006).** Microorganisms in flotation and flocculation: Future technology or laboratory curiosity? *Miner Eng* 19:548-553.

13. **Tsomides HJ, Hughes JB, Thomas JM, Ward CH (1995).** Effect of surfactant addition on phenanthrene biodegradation in sediments. *Environ Toxicol Chem* 14:953-959.

14. **Valeur A, Tunlid A, Odham G (1988).** Differences in lipid-composition between free-living and initially adhered cells of a gram-negative bacterium. *Arch Microbiol* 149:521-526.

Appendix A. Degradation and toxicity measurements

One of the criteria applied for selection of an appropriate adhesion enhancer for biodegradation purposes was the effect of the additive on metabolic activity of *P. fluorescens* LP6a. Experiments were conducted using non-induced cultures of *P. fluorescens* LP6a to test for the toxicity and biodegradability of additives used for enhancing bacterial adhesion to the oil-water interface. In order to test the toxicity of cationic compounds, solutions of each compound in water was prepared individually and sterilized using 0.22 micrometer pore sized filters (Millipore Corp, Billerica, MA) before adding to the cultures. In the case of long chain alcohols, solutions of these compounds were prepared in DMF and 1 mL of these solutions added to the culture to obtain a concentration of 410 mg/L. To investigate the toxicity of additives, TSB medium was used with the additives serving as supplemental carbon and energy sources or toxicants. LP6a growth was then monitored by measuring the OD_{600} of the cultures. At each time point, a 150-µL sample of the liquid culture was removed from flasks (samples diluted in fresh TSB when necessary) and the OD_{600} was measured on a SPECTRAmax PLUS 384 microplate spectrophotometer (Molecular Devices Corporation, CA, USA). As can be seen in Figure A.1, none of the alcohols (up to 10 g/L) were toxic to *P. fluorescens* LP6a and no significant change in growth of the bacteria was observed during 74 h incubation time. DMF also did not show any toxicity to LP6a cells.

All cationic compounds tested completely inhibited the growth of LP6a and no increase in OD_{600} was observed during incubation of cells in TSB medium that contained 25 mg/L or higher concentrations of these compounds. After 1 h incubation with cationic compounds, a loopful of culture liquid from each sample was streaked on PCA plates and incubated at 30°C for 74 h. No growth was observed in any of plates streaked from cultures that were treated with 25 or 50 mg/L of CPC, CHX or poly-L-lysine.

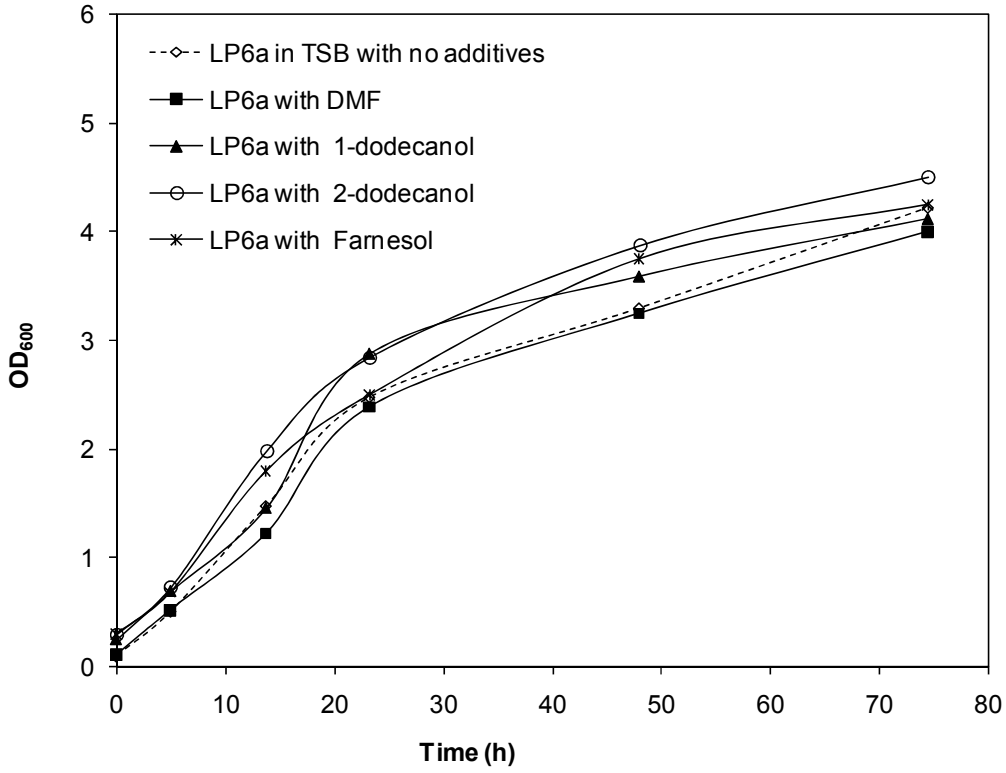

Figure A.1: Optical density at 600 nm for each of cultures incubated with 1-dodecanol, 2-dodecanol or DMF. Concentration of alcohols was 410 mg/L. DMF was added at 2% vol/vol concentration.

For degradability tests, two types of experiments were conducted. For preliminary degradability tests 1-dodecanol, 2-dodecanol or farnesol at concentration of 820 mg/L were added into TSB medium containing LP6a. After 14 d the cultures were acidified and analyzed by GC as described in section 3.2.3. GC results showed that after 14 days both 1-dodecanol and 2-dodecanol were completely degraded. The concentration of farnesol was reduced to 492 mg/L. The same experiment was repeated except that BH medium was used instead of TSB. After 14 days, 1-dodecanol and 2-dodecanol were completely consumed by LP6a. Farnesol degradation was not significant after 14 days.

The second type of degradability test was conducted in the presence of phenanthrene where both alcohols and phenanthrene were supplied into the culture medium (BH medium and LP6a inoculum). Only 1-dodecanol and 2-dodecanol were used in these experiments. Initial concentrations of 4100 mg/L and 820 mg/L were tested for both alcohols. Experiments were conducted in 500 mL Erlenmeyer flasks and the total volume of culture liquid was 100 mL. Alcohols were added directly to the culture liquid containing BH medium and LP6a in a volume corresponding to the desired concentration. Phenanthrene was dissolved in HMN and 2 mL of this solution was added to 100 mL of culture liquid containing alcohols and the inoculum. At each time point flasks were acidified, extracted by DCM and analyzed by GC as previously described (section 4.3.2). Figure A.2 demonstrates the results of these experiments. It can be seen in these figures that 2-dodecanol undergoes a slightly lower biodegradation, especially in the first 100 hours of incubation. When the initial concentration of alcohols was 4100 mg/L, the overall rate of biodegradation was 127 and 99 mg/d for 1-dodecanol and 2-dodecanol respectively. These values were 44 and 42 mg/d when the initial concentration of alcohols was 820 mg/L. According to data presented here, neither 1-dodecanol nor 2-dodecanol shows any inhibition of *P. fluorescens* LP6a growth on TSB. The two alcohols are degradable by the bacteria and 2-dodecanol degradation rate is slightly lower than that of 1-dodecanol.

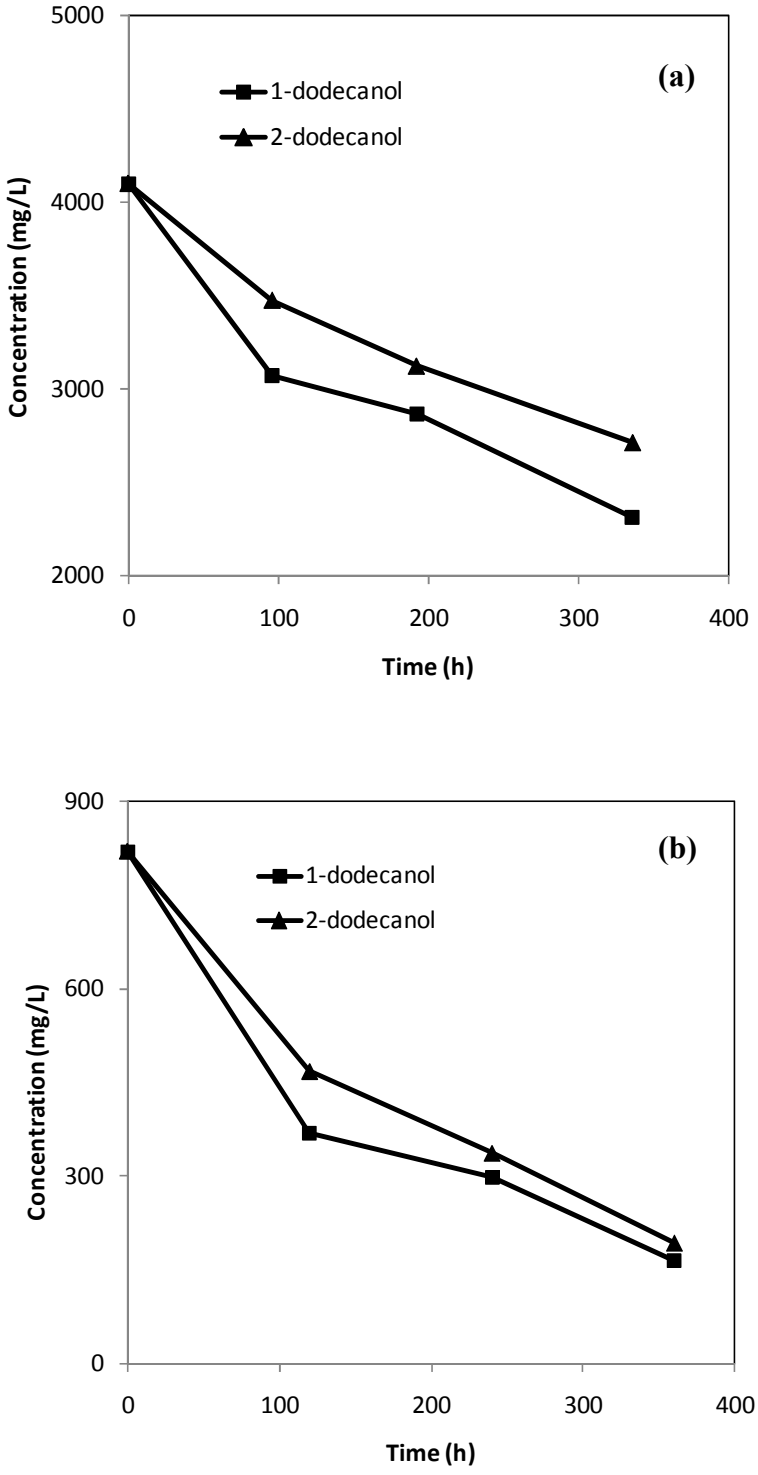

Figure A.2: Degradability of 1-dodecanol and 2-dodecanol in the presence of phenanthrene. Initial concentrations of alcohols were 820 and 4100 mg/L in (a) and (b) respectively. Initial concentration of phenanthrene was 890 mg/L in both cases.

Appendix B: Induction Experiments

One of the effective strategies to increase the degradation rate of PAHs is to add one or more known pathway inducers to the culture medium. Induction increases the production of pathway enzymes and results in the enhanced rate of degradation of the PAH. Three inducers were tested for their effect on phenanthrene mineralization by *P. fluorescens* LP6a. These included salicylate, naphthalene and 2-aminobenzoate. In all the induction experiments, phenanthrene was dissolved in HMN and added to culture flasks containing BH medium and the inoculum as described in section 4.2.6. A 0.5 M stock solution of each inducer was separately prepared in 95% ethanol and 125 μL of the stock solution was added at the desired time to 24.75 mL of culture liquid. This resulted in an inducer concentration of 2.5 mM at the time of addition into the culture flask. Figure B.1 demonstrates the results of induction tests for these three compounds. It can be observed that 2-aminobenzoate and naphthalene are very effective while salicylate has much less influence than other two inducers. That is most likely due to the fact that salicylate is an easily biodegradable substrate and disappears from the medium soon after addition into the flasks. Naphthalene is also biodegradable but it is effective in inducing phenanthrene mineralization most likely because naphthalene is expected to stay in the medium for a longer period of time than salicylate. 2-Aminobenzoate is not degraded by *P. fluorescens* LP6a and yet it is as effective as naphthalene. Since 2-aminobenzoate is not metabolized by LP6a, it has less interference with phenanthrene mineralization and is preferred for our induction experiments. For all mineralization experiments the culture were induced using 2.5 mM 2-aminobenzoate at the beginning of experiments.

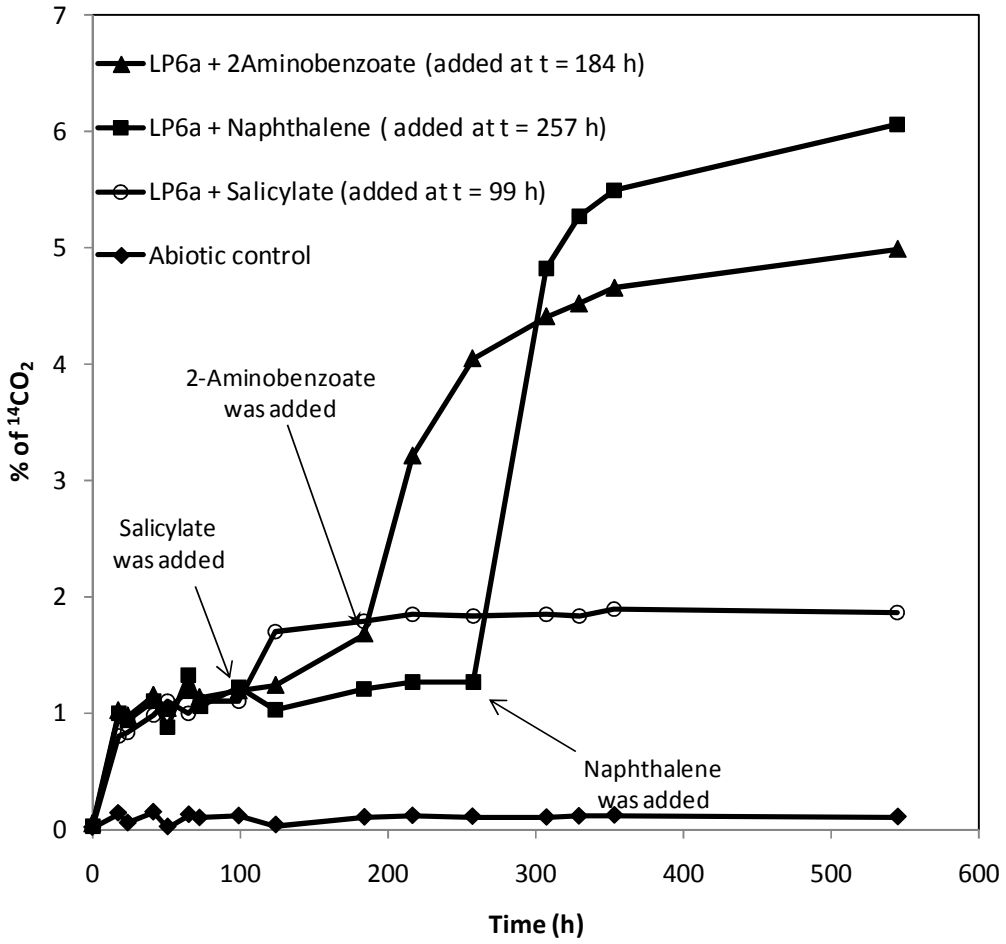

Figure B.1: Effect of salicylate, naphthalene and 2-aminobenzoate in inducing the phenanthrene mineralization by *P. fluorescens* LP6a cultures.