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MICROBIAL BIOSURFACTANT PRODUCTION: THE EFFECT OF THE *BACILLUS* STRAIN JF-2 BIOSURFACTANT ON ANAEROBIC HYDROCARBON DEGRADATION, AND THE PRESENCE OF INDIGENOUS BIOSURFACTANT-PRODUCING BACTERIA IN SOILS

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MICROBIAL BIOSURFACTANT PRODUCTION: THE EFFECT OF THE *BACILLUS* STRAIN JF-2 BIOSURFACTANT ON ANAEROBIC HYDROCARBON DEGRADATION, AND THE PRESENCE OF INDIGENOUS BIOSURFACTANT-PRODUCING BACTERIA IN SOILS

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ABSTRACT

This manuscript includes a review of information regarding biosurfactants. A detailed description of the *Bacillus* species JF2 biosurfactant, including the genetics and physiology of biosurfactant production, is provided. Additionally, a comprehensive review of the literature regarding the use of biosurfactants in hydrocarbon degradation studies is included.

A series of experiments was designed utilizing the biosurfactant produced by *Bacillus* JF2. Specifically, these experiments were designed to determine if the presence of various levels of pre-purified JF2 biosurfactant would affect the degradation of a range of hydrocarbons under a variety of anaerobic conditions. A second goal of these experiments was to discern if *in situ* production of the JF2 biosurfactant would increase the degradation of these hydrocarbons to a greater degree than if the biosurfactant was added in its pre-purified form. These experiments have indicated how future work should proceed. This work also suggests that it may be more practical to stimulate indigenous biosurfactant producing bacteria within a soil than to add either pre-purified compound or to attemp *in situ* production.

The second portion of this dissertation investigates the presence of biosurfactant producing bacteria in uncontaminated and hydrocarbon contaminated soils. Prior research indicated that the number of bacterial biosurfactant producers in soil may be influenced by the presence of hydrocarbon contamination as well as levels of organic matter and fungi. The purpose of this

study was to determine the relationship between soil organic matter levels, hydrocarbon contamination, numbers of fungi, and numbers of indigenous biosurfactant producers. Six soils were used: two hydrocarbon impacted soils, each paired with uncontaminated soil, and two pristine soils with different levels of organic matter. Gross numbers of fungi were higher in soils with higher levels of organic matter. Soils containing higher numbers of fungi contained a greater percentage of biosurfactant-producing aerobic heterotrophs, but only in the absence of a hydrocarbon. The percentage of biosurfactant producers was greater when hydrocarbon contamination was present. Additionally, the presence of the *Bacillus subtilis srf*A gene, which encodes a highly conserved region of the surfactant synthetase complex, was monitored directly in soils for the first time by PCR amplification and Southern hybridization.

CHAPTER ONE

Background Information

GENERAL INFORMATION ON BIOSURFACTANTS

Introduction

Biosurfactants are defined as microbially produced surface-active compounds. They are amphiphilic molecules with both hydrophilic and hydrophobic regions allowing them to aggregate at interfaces between fluids with different polarities such as water and hydrocarbons (Banat, 1995a; Fiechter, 1992; Georgiou, 1992; Karanth et al., 1999; Kosaric, 1993). This bi-polar characteristic makes it energetically favorable for biological and synthetic surfactants to aggregate at fluid-fluid, fluid-air, and fluid-solid interfaces. Such aggregation determines the arrangement of liquid molecules at an interface. This subsequently influences interactions between hydrophilic and hydrophobic entities within the system. The overall result is a reduction of surface and interfacial tensions (Fiechter, 1992; Jones, 1997; Rouse et al., 1994; Shafi and Khanna, 1995).

Each surfactant, either natural or synthetic, has a concentration called the critical micelle concentration (CMC) where surfactant monomers will aggregate into a three-dimensional supramolecular structure called a micelle (Fiechter, et al., 1992; Georgiou et al., 1992). Below this concentration, the biosurfactant monomers remain as individual units. At and above the CMC concentration, monomer concentrations are high enough to favor the formation of micelles. Surface tension will reach a maximum reduction when a surfactant is at the CMC concentration.

Within the micelle structures, the individual biosurfactant monomers are held together with forces including hydrophobic, van der Waals, electrostatic, and hydrogen bonding interactions (Georgiou et al., 1992). No true chemical bonds are formed between biosurfactant monomers and thus they are free to return to the singular, monomer state if environmental changes occur (Georgiou et al., 1992). Therefore, it is more accurate to say that individual monomers are in a state of equilibrium with those in the micelle structure since a monomer leaving a micelle may be easily replaced by a new monomer unit (Fiechter et al., 1992; Jones, 1997).

The three-dimensional shape of a micelle can vary widely (Fiechter, 1992; Maier, 2003). Shapes include flat bilayers, monolayers stacked into organized multiple sheets, and completely encapsulated monolayer spheres. Biosurfactant monolayers may also form long, narrow, tube-like structures. Biosurfactants may sometimes form spherical, closed structures known as vesicles. These bilayer structures can have both a hydrophilic exterior and interior separated by a hydrophobic region created by the two layers of surfactant monomers. The name "liposomes" has been given to vesicles formed by phospholipids, in reference to the similarity to structures produced by many living cells (Maier, 2003).

In an aqueous medium, monomers within micelle structures will have their hydrophobic, hydrocarbon tails pointing to the interior and the hydrophilic moiety on the exterior. This creates a hydrophobic "micro-environment" on the interior of what is externally a hydrophilic structure. If these micelle structures exist at an interface between an aqueous and non-aqueous liquid, the non-aqueous substance

may be drawn into the center of the micelle, resulting in the emulsification of the non-aqueous liquid into the aqueous phase (Desai and Banat, 1997; Lin, 1996; Shafi and Khanna, 1995). The formation of micelles therefore allows for the concentration of a compound, such as a hydrocarbon, to be in solution above its normal aqueous solubility. Thus, these molecules have important tertiary oil recovery and bioremediation applications (Fiechter, 1992; Georgiou et al., 1992; Karanth et al., 1999; Lin, 1996; McInerney et al., 1990; Rouse et al., 1994; Shafi and Khanna, 1995; Volkering et al., 1998).

Microbial biosurfactants are classified as a secondary metabolite because they are typically thought of as nonessential for the survival or proliferation of the producing organism (Bu'lock, 1961; Zuber et al., 1993). Although not mandatory for survival, these molecules have the potential to benefit microorganisms in a number of ways. Many biosurfactants possess antibacterial, antifungal, and even anti-viral activities (Banat, 1995a; Banat, 1995b; Lin, 1996; Ron and Rosenburg, 2001; Vollenbroich et al., 1997). Some biosurfactants can be utilized in bacterial motility or act in a variety of host-microbe interactions, including pathogenesis (Banat, 1995a; Banat, 1995b; Glessner et al., 1999; Haferburg et al., 1986; Kinsinger et al., 2003; Lin, 1996; Maier, 2003; Rumbaugh et al., 1999; Toguchi et al., 2000). Biosurfactants may also enhance the growth of the producing organism on hydrophobic substrates that would otherwise be unavailable, such as hydrocarbons (Haferburg et al., 1986; Ron and Rosenberg, 2001; Rouse et al., 1994). This occurs through three primary actions of biosurfactants: increasing the surface area of the compound via emulsification, desorbing the compound from

surfaces and thus increasing the concentration in the aqueous phase, and increasing the solubility of the compound as a result of micelle formation (Cooper and Zajic, 1980; Fiechter, 1992; Jones, 1997; Ron and Rosenberg, 2001).

Classes of Biosurfactants

Biosurfactants are produced by a wide range of microorganisms. These include both Gram-positive and Gram-negative bacteria as well as fungi (Haferburg et al., 1986; Healy et al., 1996; Ron and Rosenberg, 2001). The most commonly studied biosurfactants are from bacteria, particularly those produced by members of the *Pseudomonas* and *Bacillus* genera (Desai and Banat, 1997; Katz and Demain, 1997; Ron and Rosenberg, 2001).

Microbially produced surfactants are classified on the basis of microbial origin as well as chemical composition (Desai and Banat, 1997). There are between four and six general classes of biosurfactants, depending on the review (Cooper and Zajic, 1980; Desai and Banat, 1997; Haferburg et al., 1986; Healy et al., 1996, Maier, 2003; Shafi and Khanna, 1995). This summary will organize biosurfactants into five classes: glycolipids, lipopeptides, polymeric biosurfactants, particulate biosurfactants, and a fifth class which contains the fatty acids, neutral lipids, and phospholipids (Desai and Banat, 1997).

Glycolipid biosurfactants contain a carbohydrate moiety in combination with a long hydrocarbon chain that may or may not branch (Desai and Banat, 1997). The most studied are the rhamnolipid biosurfactants produced by many *Pseudomonas* species. These compounds are composed of one or two molecules

of rhamnose linked to one or two molecules of β- hydroxydecanoic acid. The resulting compound is capable of significantly decreasing both surface and interfacial tensions, and has been demonstrated to increase the degradation of various hydrocarbons (Desai and Banat, 1997, Rouse et al., 1994).

Lipopeptide biosurfactants compose the second class of biosurfactants. These molecules contain a hydrophilic region composed of various amino acids, most commonly in a cyclical structure. The hydrophobic region is primarily composed of hydrocarbon tails of varying lengths and degrees of branching (Desai and Banat, 1997; Katz and Demain, 1977). Biosurfactants produced by many members of the *Bacillus* genus, including *B. subtilis* and *B. licheniformis* are in this class, and many of these compounds possess antibacterial or antifungal properties. The amino acid moiety of such molecules may contain amino acids not commonly found in cellular proteins, including D-amino acids, basic amino acids, and sulfur containing amino acids (Katz and Demain, 1977; Bodanszky and Perlman, 1964; Bodanszky and Perlman, 1969). There is a link between the production of these biosurfactant compounds and growth in *Bacillus* cells. Most biosurfactants are produced in the exponential stage of growth (Katz and Demain, 1977; McInerney et al, 1990) and the genes involved are linked to bacterial competence and spore formation (Javaheri et al., 1985; Marahiel et al., 1993; McInerney et al., 2001).

Polymeric biosurfactants are a class of surfactants with highly variable characteristics. Members of the *Acinetobacter* genus can produce a surfactant called emulsan that is composed of carbohydrates, amino acids, and fatty acids

(Rosenberg et al., 1979; Zukerberg et al., 1979). A second, widely studied, biosurfactant produced by *Saccharomyces cervisiae* is called mannoprotein (Cameron et al, 1988). This compound contains sugar and protein components. Derivatives of mannoprotein are also produced by members of the *Candida* genus (Kappeli et al., 1978; Kappeli et al., 1984). A third polymeric biosurfactant is liposan. This carbohydrate-protein complex is produced by *Candida lipolytica*, and is a water-soluble emulsifier (Cirigliano and Carman, 1984; Kappeli and Fiechter, 1977).

The particulate class of biosurfactants is produced by members of the *Acinetobacter, Aeromonas, Serratia, Bacillus,* and *Staphylococcus* genera (Desai, 1987; Fattom and Shilo, 1985; Lang and Wagner, 1987; Rosenberg, 1986; Wilkinson and Galbraith, 1975). These extracellular membrane vesicles are typically involved in partitioning hydrocarbons into microemulsions, facilitating hydrocarbon uptake and subsequent degradation. These vesicles are composed of various amounts of phospholipids, protein, and lipopolysaccharide (Kappeli and Finnerty, 1979).

The final class of biosurfactants is those composed of various fatty acids, phospholipids, and neutral acids. Such compounds are produced by both bacteria and fungi, including members of the *Acinetobacter, Thiobacillus, Pseudomonas, Arthrobacter,* and *Aspergillus* genera (Beeba and Umbreit, 1971; Kappeli and Finnerty, 1979; Robert et al., 1989; Wayman et al., 1984). Production is most commonly detected when the organism is grown on in on *n*-alkanes and may

increase hydrocarbon degradation (Asselineau et al., 1978; Cirigliano and Carman, 1985; Cooper et al., 1978; Robert et al., 1989).

Comparison Between Biological and Synthetic Surfactants

Unlike biosurfactants, chemically manufactured surfactants are classified according to the composition of their polar group (Camp et al., 1985; Desai and Banat, 1997). Such chemicals were designed to perform specific tasks and include detergents, emulsifying agents, and foaming agents (Cameotra and Makkar, 1998; Camp et al., 1985; Desai and Banat, 1997). The majority of these synthetic surfactants are derivatives of petroleum and petroleum by-products (Banat et al., 2000; Cameotra and Makkar, 1998; Desai and Banat, 1997). This makes the financial cost of production partially dependent on the highly volatile world petroleum markets. In the 1990's worldwide synthetic surfactant production was a $9.4 billion per year business, with up to 106 tons used per year (Banat, 1995a; Desai and Banat, 1997; Maier, 2003; Shaw, 1994).

There are similarities between many of the synthetic surfactants and their biologically produced counterparts. Both are highly stable through a wide range of temperature and pH conditions (Banat, 1995a; Cameotra and Makkar, 1998; Georgiou et al., 1992). Synthetic surfactants have been shown to be highly effective in hydrocarbon removal from various soil media (such as sand or clay cores) and can successfully decrease surface tension (Banat, 1995a; Rouse et al., 1995). Like biosurfactants, synthetic surfactants can also have a highly variable effect on hydrocarbon degradation, ranging from enhancing the biological

removal of a contaminant to inhibiting degradation (Atlas, 1991; Jennings and Tanner, 2004; Rouse et al., 1994).

Despite these similarities, biosurfactants have a number of advantages over synthetic surfactants. Unlike synthetics, biosurfactants are biodegradable and non-toxic to the environment (Banat, 1995a; Banat, 1995b; Fiechter, 1992; Georgiou et al., 1992; Rouse et al., 1994). Biosurfactants are typically nonirritating to skin and therefore may be more suited for use in cosmetic and health care industry products (Kleckner and Kosaric, 1993; Maier, 2003). Perhaps most importantly, biosurfactants are produced through the metabolism of renewable resources and therefore production is not as intimately influenced by world oil prices and is more environmentally acceptable (Banat, 1995a; Fiechter, 1992; Makkar and Cameotra, 2002). An additional advantage of biosurfactants over synthetic surfactants is that the final biosurfactant product can be modified through genetic manipulation and biochemical treatments, allowing for an expansion of applications (Banat et al., 2000; Cameotra and Makkar, 1998; Fiechter, 1992).

Despite these advantages, there are still issues for the biologically produced surfactant market to reconcile before competition with synthetics can be successful. First, biosurfactants are currently more expensive to produce than synthetic surfactants due to high production costs associated with inefficient bioprocessing and comparatively low strain productivity (Cameotra and Makkar, 1998; Fiechter, 1992). The cost of raw material for biosurfactant production accounts for approximately 10-30% of the overall production costs. Currently

there is great interest in producing biosurfactants from materials once thought to be waste products of the distillery and dairy industries, thus lowering the cost of the raw materials used in the production process (Makkar and Cameotra, 2002). Second, in order to gain approvals for use in foods and pharmaceuticals, the structure of a biosurfactant must be elucidated – an often difficult process (Maier, 2003). The structure of the biosurfactant must also be constant and predictable during production, and this is often times not the case (Maier, 2003; Robert et al., 1989). Third, a characteristic of biosurfactants that makes them appealing also poses a problem – their biodegradability. In the environment, biosurfactants may degrade before enough time has passed to demonstrate significant hydrocarbon degradation or recovery (Jenneman et al., 1983; Lin, 1996; Lin et al., 1998).

The latest surfactant research appears to involve the production of surfactants that incorporate the beneficial characteristics of both biological and synthetic surfactants into the product. These compounds mimic the structure of various natural compounds, such as lipopeptide biosurfactants, but are produced artificially through the use of renewable raw materials (Clapés and Infante, 2002). Bio-based surfactants include lipoamino acid/ peptide and lipoamino acid/glycolipid analogues of various bacterial biosurfactants (Infante et al., 1997). These compounds can form micelle structures resulting in decreased surface tension while demonstrating low environmental toxicity (Clapés and Infante, 2002).

CHARACTERISTICS OF THE JF2 BIOSURFACTANT

General Characteristics of JF2 and Mutant

Bacillus strain JF2 (ATCC 39307) is a Gram-positive, spore-forming rod isolated from oil well injection brine from a site in Carter County, Oklahoma (Javaheri et al., 1985). This organism is capable of growth in a wide range of temperatures (up to 50 ^{o}C), NaCl concentrations (up to 10%), and pH values (4.6 to 9.0), and is capable of biosurfactant production under aerobic, microaerophilic, and anaerobic conditions (Javaheri et al., 1985; Jenneman, 1983; Lin et al., 1990; Lin et al., 1994a; Lin et al., 1994b; McInerney et al., 1990). Biosurfactant recovery is maximized from mid to late exponential growth phase cultures grown on minimal medium containing 5% NaCl at 37-40^{o}C (Javaheri, 1985; Javaheri et al., 1985). Production of the biosurfactant is non-ribosomal, and the compound is classified as a secondary metabolite (Javaheri et al., 1985; Konz et al., 1999).

Bacillus strain JF2 was originally classified as a member of the *B. licheniformis* species (Javaheri et al., 1985; Lin et al., 1993; Lin et al., 1994a; Lin et al., 1994b; McInerney et al., 1990). However, later studies determined that JF2 is a strain of *B. mojavensis* (Han et al., 2001; Roberts et al., 1994) . A mutant form of JF2 was obtained by repeated culture in medium containing no NaCl (Cooper et al., 1980; Javaheri et al., 1985; Marsh et al., 1995). This mutant does not produce biosurfactant.

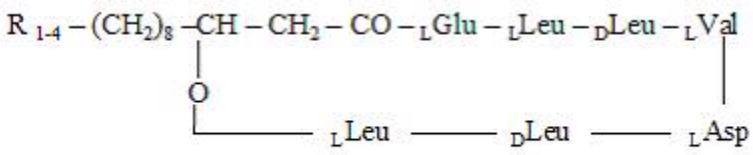
Physical Description of JF2 Biosurfactant, Monomer and Micelle

The *Bacillus* JF2 biosurfactant contains a hydrophilic moiety composed of amino acids as well as a hydrophobic hydrocarbon chain region (Desai and Banat, 1997; Katz and Demain, 1977). Under optimal conditions, the JF2 biosurfactant exhibits a critical micelle concentration (CMC) of 10 mg/L (Lin et al., 1994a) and can lower the surface tension of water from 74 to 27 mN/m (Jenneman et al., 1983).

Production of this secondary metabolite is non-ribosomal, and occurs within the cell cytoplasm. The structure of the JF2 biosurfactant puts the molecule into a special category of lipopeptides known as lichenysins (Cosmina et al., 1993; Marahiel et al., 1997; Ullrich et al., 1991; Yakimov et al., 1995) (Figure 1.1).

The biosurfactant contains a ring of amino acids with a sequence of: $_{L}$-Glu – $_{L}$-Leu – $_{D}$-Leu – $_{L}$-Val – $_{L}$-Asp – $_{D}$–Leu – $_{L}$–Leu (Konz et al., 1999). This ring is connected to a heterogeneous β–hydroxyl fatty acid via a lactone linkage (Desai, 1987; Haferburg, 1986; Peypoux et al., 1999). There are four different fatty acid tails commonly associated with the JF2 biosurfactant monomer (Fiechter, 1992). The three most predominant fatty acids are (36.4%) anteiso-C_{15}, (30.5%) iso-C_{14}, and (18%) iso-C_{15} structures (Yakimov et al., 1995). The biosurfactant molecule has a molecular mass of approximately 1,035 Da (Lin et al., 1994a).

The structure of the JF2 biosurfactant has been shown to be remarkably similar to the surfactin biosurfactant produced by *B. subtilis* (McInerney et al., 1990; Fiechter, 1992; Jenny et al., 1991; Lin et al., 1994a). One difference



$R_1 = (CH_3)_2 – CH –$

$R_2 = CH_3 – CH_2 – CH_2 –$

$R_3 = (CH_3)_2 – CH – CH_2 –$

$R_4 = CH_3 – CH_2 – CH(CH_3) –$

Figure 1.1: Structure of *Bacillus* JF2 Biosurfactant (Konz et al., 1999; Fiechter et al. 1992; Yakimov et al., 1995).

between the *B. licheniformis* biosurfactant and surfactin was thought by some to be the substitution of the C-terminal amino acid Iso for Leu by *B. subtilis* (Jenny et al., 1991). However, further analysis of a variety of biosurfactants produced by various strains of *B. licheniformis* found that although the most common C-terminal amino acid was Iso, the surfactant produced by JF2 (lichenysin B) has Leu in the C-terminal position (Konz et al., 1999, Yakimov et al., 1995). Thus, the amino acid sequence of the JF2 biosurfactant and surfactin molecules is identical.

This chemical similarity has been reinforced in a number of ways, including fast atom mass and infrared spectroscopy (Lin et al., 1994a). Polyclonal antibodies raised against surfactin have had identical reactivity to the JF2 biosurfactant in enzyme linked immunosorbent assays (Lin et al., 1994a). Furthermore, a molecular probe designed from the *sfrA* gene of *B. subtilis* positively reacts with DNA isolated from JF2 in both PCR and Southern hybridization procedures (Jennings et al., 2005; McInerney et al., 2001, Youssef et al., 2004).

As a result of the chemical similarity between the *B. subtilis* surfactin and *Bacillus* strain JF2 biosurfactant monomers, the three dimensional structures are assumed to be similar (Horowitz and Griffin, 1991; Jenny et al., 1991; Lin et al., 1994a, Lin et al., 1998; Sullivan, 1998). Overall, the surfactin molecule is described as having a "horse saddle" topology, reflecting the β–sheet structure of the compound. One side of the molecule contains amino acids 2 ($_{L}$Leu) and 6 ($_{D}$Leu) facing each other while nearby acidic residues from amino acids 1 ($_{L}$Glu)

and 5 ($_{L}$Asp) form a mildly polar domain. On the opposite side of the molecule, amino acid 4 ($_{L}$Val) faces the connection point of the fatty acid chain. This, in addition to the side chains of amino acids 3 ($_{D}$Leu) and 7($_{L}$Leu), creates a significant hydrophobic region (Bonmatin et al., 1994; Ishigami et al., 1995; Peypoux et al., 1999).

Once formed, the surfactin molecule is quite compact due to the cyclization of the protein moiety (Bonmatin et al., 1994; Ishigami et al., 1995). The hydrocarbon lipidic-chain moiety of the biosurfactant will extend freely from the protein region when the biosurfactant is present in concentrations less than the CMC value. However, when a micelle is formed, the interaction of these tails provides the hydrophobic microenvironment required for interfacial / surface tension reduction (Ishigami et al., 1995).

In regards to the protein region of the biosurfactant, the arrangement of amino acids 1 and 5 allows the biosurfactant molecule to move cations, particularly divalent ones, through polar solvents with great efficiency. This is because these two amino acid residues form what has been described as a “claw”, which has the ability to stabilize cations. The calcium cation is the most favorable cation for this interaction (Osman et al., 1998). The stabilization of calcium subsequently acts as a stabilizing force on the overall surfactin molecule. This may be due to the resulting neutralization of the acidic amino acid residues (residues 1 and 5) at an air/water interface (Maget-Dana and Ptak, 1992). Once stabilized, this surfactin-calcium unit can act as a template for micelle assembly (Osman et al., 1998).

The surfactin molecule has a lower affinity for magnesium cations, demonstrating a high degree of discrimination by the biosurfactant. Certain mono-valent cations, such as potassium and sodium, only partially neutralize the acidic amino acids, resulting in only partial stabilization of the overall biosurfactant molecule (Maget-Dana and Ptak, 1992; Osman et al., 1998). A less stable overall biosurfactant molecule results in a less stable backbone to act as a seed for micelle formation.

Review of the Genetics and Physiology of JF2 Biosurfactant Production

The genetics specifically responsible for JF2 biosurfactant production have not been studied in detail. Most of what is known is based on biosurfactant production by a variety of *Bacillus licheniformis* strains. Most of this knowledge, in turn, is based on extensive research on biosurfactant production by *Bacillus subtilis*.

Berdy (1974) composed a review of the then current antibiotic literature, and made a detailed classification of antibiotics according to their chemical structure. A portion of this work was later expanded as a detailed summary of the peptide antibiotics produced by the genus *Bacillus* (Katz and Demain, 1977). This report detailed the production of the various *Bacillus* antibiotics during late exponential growth phase / early stationary phase, and reviewed what was known about the temporal association between spore formation and antibiotic production. The Katz and Demain (1977) work was subsequently updated by Zuber et al. (1993) and Peypoux et al. (1999). By taking advantage of advances in both

analytical and genetic techniques, a more precise picture of how *Bacillus* species produce peptide antibiotics was formed. For the first time, a detailed summary of the genetics behind lipopeptide biosurfactant formation was presented.

However, a simple discussion of the genes immediately and intimately involved with the formation of a biosurfactant is insufficient when dealing with the biosurfactants produced by *B. subtilis* and *B. licheniformis*. It has been well established that these biosurfactant systems are highly influenced by additional gene products in a complex series of regulatory steps. These outside influences have been reviewed in detail by Marahiel et al. (1993) and Sullivan (1998).

These reviews, along with individual research publications, are the sources for the following summary of *Bacillus* JF2 biosurfactant production. Much of this information is based on the *B. subtilis* biosurfactant formation system. However, it has been well established that the mechanisms of biosurfactant formation by *B. subtilis* and *B. licheniformis* are quite similar and there is strong precedence in the literature for making such comparisons (Horowitz and Griffin, 1991; Jenny et al., 1991; Konz et al., 1999; Lin et al., 1994a, Lin et al., 1998; Peypoux et al., 1999; Sullivan, 1998). It will be noted when differences occur between the two systems. The following summary begins with a discussion of the general mechanism by which the peptide and fatty acid moieties of a lipopeptide biosurfactant are formed. This will be followed by a description of the mechanism as it applies specifically to surfactin production. Next will be a description of the known, specific details involved with the formation of the JF2 biosurfactant.

General mechanism for the formation of the peptide and fatty acid moieties of a lipopeptide biosurfactant

The protein portion of a *Bacillus* lipopeptide biosurfactant is produced by a multienzyme thiotemplate mechanism first described by Kluge et al. (1988). A general description of the model is as follows: a large, multi-subunit enzyme complex called a peptide synthetase catalyses the synthesis of the hydrophilic ring from amino acids. Each subunit or domain within the enzyme complex codes for one amino acid. These domains (and hence the amino acids) are aligned on the template such that they are in order of their addition to the peptide chain. For each domain, the appropriate amino acid is covalently bonded to the domain site by a thioester linkage. Elongation of the peptide (transpeptidation) is facilitated by a 4'-phosphopantetheine cofactor that is attached to an enzyme subunit. This cofactor transfers the growing protein from one thiotemplate position to the next (Kluge et al., 1988; Konz et al., 1999; Peypoux et al.1999; Zuber et al., 1993).

In addition to the seven amino acids, there is a fatty acid moiety linked to the amino acid ring. The production of this hydrophobic portion of the biosurfactant proceeds via standard fatty acid synthesis pathways (Hommel and Ratledge, 1993). Biosynthesis of these fatty acids can proceed from a wide variety of starting-compounds including acetyl-coenzyme A, alcohols, hydrocarbons, and carbohydrates (Boulton and Ratledge, 1987; Hisatsuka et al., 1971; Hommel and Ratledge, 1993; Shafi and Khanna, 1995; White, 2000). The

process by which this hydrocarbon tail is linked onto the lipopeptide is not fully understood (Cosmina et al., 1993; Zuber et al., 1993).

Description of surfactin production

Specifically regarding surfactin production by *B. subtilis*, the peptide sequence of the initial surfactin synthetase molecule is coded for by three of the four open reading frames (ORF) in the *srfA* operon: *srfA*-A, *srfA*-B, and *srfA*-C, respectively (Cosmina et al., 1993; Vollenbroich et al., 1994, Zuber et al., 1993). The first ORF, *srf*A-A, encodes the peptide synthetase subunit that functions in the incorporation of the first three amino acids of the peptide chain: $_{L}$-Glu – $_{L}$-Leu – $_{D}$-Leu (Marahiel et al., 1993). The second ORF, *srf*A-B, encodes the peptide synthetase subunit for the incorporation of the next three amino acids: $_{L}$-Val – $_{L}$-Asp – $_{D}$–Leu. The third ORF, *srf*A-C encodes the peptide synthetase for the incorporation of the final amino acid, $_{L}$–Leu (Marahiel et al., 1993). The fourth ORF, *srf*A-D, is not necessary for surfactin production (Cosmina et al., 1993; Sullivan, 1998).

It is interesting that the first two ORFs incorporate the third amino acid (Leu) in the D configuration, since surfactin synthetase does not accept $_{D}$-Leu in the *in vitro* synthesis of surfactin (Ullrich et al., 1991). It was therefore believed that these regions accept $_{L}$-Leu, and through a racemization reaction, convert the molecule into the D form (Cosmina et al., 1993; Ullrich et al., 1991). The presence of racemases for various amino acids has been observed in the production of wide range of antibiotics (Katz and Demain, 1977). However, work

by Menkhaus et al. (1993) has shown that the conversion of Leu from the L to D form does not occur during the process of Leu activation as if a racemase enzyme was present. Instead, it is believed that this conversion occurs at a later, undetermined stage in peptide elongation (Menkhaus et al., 1993).

It should also be noted that peptide synthetase enzymes show a lower specificity for their substrate (the amino acids) than do other synthetases (Galli et al., 1994; Katz and Demain, 1977; Kleinkauf and von Dohren, 1988). This can explain the slight variations in amino acid sequences that occur in the biosurfactants of some strains of both *B. subtilis* and *B. licheniformis*. For example, the seventh amino acid in the lichenysin molecules produced by various strains of *B. licheniformis* can be $_{L}$–Ile or $_{L}$-Val, instead of the $_{L}$-Leu as found in the lichenysin B of JF2 (Galli et al., 1994; Konz et al., 1999; Yakimov et al., 1998; Zuber et al., 1993). Such variations are common in microbially-produced antibiotics (Katz and Demain, 1977).

In some peptide antibiotics, such as polymyxin, the fatty acid tail is incorporated into the lipopeptide molecule in the early stages of synthesis (Zuber et al., 1993). This is similar to the process of surfactin synthesis where an acyltransferase is responsible for the transfer of the fatty acid moiety to the first amino acid product of *srf*A-A (Menkhaus et al., 1993). It is believed that surfactin synthesis begins when the β–hydroxy fatty acid substrate is transferred from the acyltransferase to the first amino acid of the peptide chain ($_{L}$-Glu), forming a β–hydroxy-acylglutamate intermediate (Menkhaus et al., 1993). Additional amino acids are then added to this structure. A thioesterase known as

srfA-TE then cleaves the cytoplasmic, growing peptide chain (Peypoux et al., 1999). The peptide chain folds as the carboxy-terminal amino acid forms a lactone ring to the β–OH group of the lipophilic molecule (Konz et al., 1999). The molecule is then transported to the exterior of the cell.

This transport mechanism has not been fully investigated in either the *B. subtilis* or JF2 system. However, a genetic analysis of *B. subtilis* and a lichenysin-producing *B. licheniformis* strain revealed a region outside of the main, biosurfactant-production region that was homologous to sequences for certain *Bacillus* transmembrane proteins and had a high similarity to *E.coli* antibiotic transporter genes (Cosmina et al., 1993; Yakimov et al., 1998). These gene products may be responsible for the transport of the biosurfactant to the exterior of the cell after synthesis. It is also hypothesized that they are involved in providing resistance to the antibiotic properties of other biosurfactants by secreting any of such compounds that may have entered the cell (Yakimov et al., 1998). Thus, the exact function of these *Bacillus* gene products has not yet been established.

Differences in surfactin production to the production of the JF2 biosurfactant

As stated earlier, most of what is known about the genetics of *Bacillus* JF2 biosurfactant formation is due to the similarities between it and surfactin. Whereas surfactin is produced by surfactin synthetase encoded by the *srf*A operon, lichenysin B production is governed by a similar lichenysin synthetase

encoded by the *lic*A-C operon (Konz et al., 1999; Yakimov et al., 1998). Similar to the *srf*A operon, the fourth ORF of the *lic* operon is not essential for biosurfactant production (Sullivan, 1998). Surfactin production involves a thioesterase known as srf*A*-TE, which cleaves the cytoplasmic, growing peptide chain (Peypoux et al., 1999). Until recently, it was assumed that there was an analogous structure in lichenysin production (Sullivan, 1998). This structure has in fact been identified as the product of the *lic*TE gene (Konz et al., 1999; Yakimov et al., 1998). The fatty acid tails of surfactin are primarily (40%) *n*-C_{15} tails while the fatty acid moiety of lichenysin B (that produced by JF2) is made up of approximately 30% *iso*-C_{14} and 36% *anteiso*-C_{15} tails (Yakimov et al., 1995).

Regulation of the *srf*A Operon

The *srf*A operon is a highly complex system tightly integrated with the competence, sporulation, and quorum sensing pathways of *Bacillus* (Zuber et al., 1993). This is not surprising when it is remembered that all four pathways are affected by environmental conditions associated with late logarithmic and early stationary growth phases. The full extent of the intimate relationship between these events, however, has yet to be determined.

I. Influence of competence genes

The presence of a functional *srfA* operon is necessary for the expression of late competence genes (Dubnau, 1991). However, expression of the *srf*A operon is dependent on *com*A, an early competence gene (also called *srf*B) (Dubnau, 1989; Dubnau, 1991; Nakano and Zuber, 1989; Peypoux et al., 1999). Therefore,

the *srfA* operon can both influence and be influenced by genetic competence genes.

ComA regulation is complex, and involves a second competence gene product known as ComP. The *comP* gene has a high sequence similarity to a histidine protein kinase class of two-component regulatory proteins (Marahiel et al., 1993; Nakano et al., 1991; Weinrauch et al., 1989). Additionally, ComP contains a membrane-spanning domain that may allow for the detection of glucose and glutamine levels in the environment (Weinrauch et al., 1990).

ComP forms a protein complex with ComA. In response to glucose and glutamine levels, ComP will autophosphorylate. The phosphate group on ComP then transfers to ComA, which contains a helix-turn-helix motif characteristic of DNA-binding proteins (Marahiel et al., 1993). ComA subsequently binds to the *srfA* promoter and acts as a positive regulator of *srfA* operon transcription (Marahiel et al., 1993, Nakano et al., 1991; Peypoux et al., 1999; Roggiani and Dubnau, 1993). In this way, competence genes can regulate biosurfactant formation.

Although competence genes can regulate *srfA* transcription, *srfA* can influence competence. It was determined that the first 20,535 base pairs of the 5'end of the *srfA* operon are required for successful competence in *B. subtilis* (Dubnau, 1991; Fuma et al., 1993). This region encodes all of *srfAA* (which codes for the first three amino acid of the surfactin molecule) and the first amino-acid activating domain of *srfAB* (coding for the fourth amino acid in the surfactin molecule). The specific location of competence-dependence was then narrowed

down to the first amino-acid activating domain of *srfAB* (Van Sinderen et al., 1993). The gene for this location was named *srfA-d4*. The location of competence regulation was still further refined by Hamoen et al. (1995). This study determined that a small, out-of-frame region of*srfA -d4,* called *comS,* was in fact the portion of the 5'end of *srfA* that was necessary for competence (Hamoen et al., 1995). Expression of *comS* is required for competence to fully develop and results in the uptake of exogenous DNA (D'Souza et al., 1994; D'Souza et al., 1995; Kleerebezum et al., 1997). The expression of *comS* is not required for biosurfactant formation. However, the *srfA* operon must be functional for the successful expression of this out-of-frame competence gene. Therefore, the condition of biosurfactant genes can affect competence.

II. Influence of sporulation genes

Just as there is a relationship between genetic competence and biosurfactant formation, there is also a relationship between sporulation and biosurfactant formation. Sporulation genes are typically named as variations of *spoO* (*spoOA, spoOB,* etc.). The SpoOA protein can phosphorylate ComA through an intermediate (Grossman, 1995; Marahiel et al., 1993). ComA can then bind to the *srfA* promoter, initiating transcription. A second sporulation gene product, SpoOK, is thought to somehow affect ComP. Although the mechanism is unknown, it has been determined that SpoOK is necessary for biosurfactant production by *B. subtilis* when glucose and glutamine are present in an environment (Cosby et al., 1998). Therefore, under certain environmental

conditions, SpoOK can (through intermediates) phosphorylate ComP, which in turn results in *srfA* transcription.

III. Influence of quorum sensing

Quorum sensing is also known to regulate the expression of the *srfA* operon (Ren et al., 2004; Sullivan, 1998). In Gram-positive bacteria, quorum sensing occurs when extracellular peptides known as N-acyl homosterine lactones accumulate in the environment to a concentration sufficient to stimulate a multi-component regulatory system (Dunny and Leonard, 1997; Kleerebezum et al., 1997; Sullivan, 1998). These compounds may (through a number of intermediates) trigger the activation of the *spoOA* gene (Grossman, 1995). In regards to spore formation, the SpoOA protein is responsible for axial filament formation and asymmetric division (Frisby and Zuber, 1991; Hilbert and Piggot, 2004). However, in regards to biosurfactant formation, the activation of the *spoOA* gene can indirectly cause the phosphorylation of ComA, which then initiates the transcription of *srfA*. Thus, quorum sensing compounds may indirectly trigger biosurfactant formation.

In summary, the *srf*A operon is a highly complex system highly influenced by the competence, sporulation, and quorum sensing pathways of *Bacillus.*

BIOSURFACTANTS IN EXPERIMENTAL SYSTEMS - LITERATURE REVIEW

Most compounds, organic or synthetic, are (in theory) potentially biodegradable (Jones, 1997). The key to biodegradation is the presence of both

an appropriate microbial population and appropriate physical conditions to stimulate degradation. However, the low solubility of a compound in water often becomes a limiting factor in a biodegradation process. Low solubility may result in poor desorption from surfaces (such as soil) as well as low amounts of the compound coming into contact with the degrading microbes which live in primarily aqueous environments. In summary, low solubility can result in low bioavailability (Fiechter, 1992; Jones, 1997; Van Hamme et al., 2003).

The addition of surfactants is supposed to increase the contact between the hydrophobic target compounds and the degrading microbial population (Fiechter, 1992; Jones, 1997). Sometimes this is highly effective. Other times, degradation is negligible or even inhibited. Unless noted, the below studies were performed under aerobic conditions.

Hexadecane

Because of the insoluble nature of hexadecane, it is difficult to partition the hydrocarbon into the aqueous phase of a system. This limitation decreases the bioavailability of the hydrocarbon to microorganisms and thus degradation is limited. As a result, a significant amount of attention has been paid to the potential impact that natural and synthetic surfactants can have on degradation rates of this and similar hydrocarbons.

MacDonald et al. (1981) and Whyte et al. (1999) observed biosurfactant production in a *Rhodococcus*, and found that production positively increased hexadecane solubility. One of the several biosurfactants produced by *Rhodococcus* includes a lipopeptide (Whyte et al., 1999). Bruheim et al. (1999)

compared hexadecane (as a component of crude oil) degradation rates in the presence of two different surfactants. One of these surfactants increased the degradation rate significantly. The second surfactant, under identical test conditions, inhibited degradation (Bruheim et al., 1999). It was not mentioned if the surfactants for this study were added below, at, or above their CMC amount.

Foght et al. (1989) reported a decrease in hexadecane degradation in the presence of an *Actinobacter* biosurfactant. However the biosurfactant was reported in terms of μg/ml with no information with which to convert the amounts into a CMC value for comparison. A study by Zhang and Miller (1995) reported that degradation increased in the presence of a *Pseudomonas* biosurfactant below its CMC concentration. A preparation of pre-purified surfactin, produced by *Bacillus subtilis*, was capable of enhancing the degradation of both aliphatic and aromatic hydrocarbons, but only when the biosurfactant was added above CMC levels (Moran et al., 2000). Stimulation of degradation was not observed at biosurfactant levels lower than this. The greatest benefits were seen in regards to the long-chain alkane hydrocarbons, likely due to their strong hydrophobicity (Moran et al., 2000).

Studies suggest that in addition to the type of alkane being studied, experimental results may depend on a number of other variables including environmental chemistry, concentrations of surfactants, and hydrocarbon delivery methods. A study by Bai et al. (1998) determined that the levels of sodium, magnesium, and small amounts of calcium could increase hexadecane solubility by a rhamnolipid. The study also found that the addition of calcium above certain

levels began to inhibit hexadecane uptake by the rhamnolipid micelles. An investigation by Herman et al. (1997a) determined that hexadecane degradation rates varied with different biosurfactants. Hexadecane degradation also varied as concentrations of a given biosurfactant were changed. The same study revealed that some biosurfactants, added at a constant amount, increased hexadecane degradation under soil slurry conditions but inhibited degradation in a sand-packed column system. Efroymson and Alexander (1991) found that hexadecane degradation by bacterial cultures simultaneously producing biosurfactant was suppressed when the alkane was delivered in a NAPL (non-aqueous phase liquid) overlayer but did occur if the hydrocarbon was provided in neat form.

A second study by Herman et al. (1997b) examined the ability for a pre-isolated biosurfactant to dislodge hexadecane bound to sand in flooded soil columns. It found that 10 CMC of a rhamnolipid biosurfactant significantly increased degradation over a similar core with only the CMC concentration of biosurfactant added. This was despite the fact that both cores exhibited similar hexadecane mobility after treatment with pre-purified biosurfactant. Thus, hydrocarbon mobilization occurred when both at and above (10 X) CMC levels of biosurfactant were added but degradation only occurred in the later condition (Herman et al., 1997b). This contradicts the results of an earlier study (Herman et al., 1997a) in which ten times the CMC value of a rhamnolipid resulted primarily in the mobilization of hexadecane while addition of less than the CMC value resulted primarily in mineralization of the hydrocarbon.

A study by Bruheim and Eimhjellen (1998) found that the physiological state of the degrading microorganism (i.e. exponential growth phase versus stationary growth phase) at the time of pre-purified biosurfactant addition was a key factor in determining how degradation would be effected. This confirmed earlier work (Bruheim et al., 1997) in which it was noticed that hexadecane (as a component of crude oil) degradation was negatively affected if a surfactant was added when hydrocarbon degrading cells were in exponential growth phase. However, degradation was stimulated if surfactant was added to stationary growth phase cells.

Pseudomonas rhamnolipid biosurfactant has been found to increase hexadecane degradation under a variety of environmental conditions (Hisatsuka et al., 1971; Jain et al., 1992; Koch et al., 1991). Early studies had indicated that hexadecane degradation by *Pseudomonas* was stimulated to a greater extent in the presence of a *Pseudomonas*-produced, pre-purified rhamnolipid than in the presence of synthetic surfactants (Nakahara et al., 1981) or biosurfactants produced by non-*Pseudomonas* organisms (Itoh and Suzuki, 1972). This was followed up by an interesting study by Noordman and Janssen (2002) who observed hexadecane degradation by another *Pseudomonas*. Hexadecane degradation was measured when the organism was in the presence of its own biosurfactant (pre-purified) as well as in the presence of pre-purified biosurfactant produced by four other microorganisms. The *Pseudomonas* culture only degraded hexadecane in the presence of its own biosurfactant. This *Pseudomonas* strain

clearly demonstrated specificity in regards to which biosurfactant it would interact with to degrade the hexadecane (Noordman and Janssen, 2002).

In conclusion, the rate of hexadecane degradation has been both stimulated and inhibited by a wide variety of conditions. These results often seem to conflict with one another. Furthermore, biosurfactants that have stimulated hexadecane degradation have had both stimulatory and inhibitory effects on the degradation of other alkanes and aromatics (Churchill et al., 1995; Jain et al., 1992; Lang and Wullbrandt, 1999; Tanaka and Fukui, 1971; Zhang and Miller, 1994). The difficulty of interpreting data regarding the effects of biosurfactants on hydrocarbon degradation is therefore not unique to hexadecane or to other alkanes.

Toluene

A similar difficulty in predicting the biodegradation effects of biosurfactants on hydrocarbons is found with toluene. However, fewer numbers of studies have been conducted to determine how the presence of a synthetic or biological surfactant will affect the degradation of the aromatic compound. This is likely due to the fact that most surfactant systems are designed to increase the solubility and therefore bioavailability of a relatively insoluble compound such as hexadecane (Rouse et al., 1994). Toluene is more soluble in water than hexadecane and therefore is more readily available for biodegradation. Despite this, there has been some work investigating the effect of surfactants and biosurfactants on the mineralization of toluene. The results of these studies are

similar to those of hexadecane in that the conclusions of one study may contradict the results of another.

Churchill et al. (1995) added approximately 10 times the CMC value of a rhamnolipid biosurfactant to their system and yet reported poor microbial degradation. They attributed their results to the already soluble, and therefore biologically available, nature of the targeted toluene. In contrast, Jain et al. (1992) reported that equal amounts of a similar rhamnolipid significantly increased toluene degradation. Another study found significant increases in toluene degradation, but with below CMC levels of both an artificial surfactant and a microbially produced biosurfactant (Strong-Gunderson and Palumbo, 1995). Finally, a study of six different surfactants by Wilson et al. (1995, anaerobic conditions) observed all possible effects on toluene degradation: two surfactants inhibited degradation, two enhanced degradation, and the last two had no effect.

In regards to the effects of synthetic surfactants, Woertz and Kinney (2004) reported that Tween 20, a nonionic surfactant, enhanced toluene degradation. They also noted a shorter lag-growth phase of the degrading microbial culture when in contact with the surfactant. Goudar et al. (1999) tested the degradation rates of toluene in the presence of four surfactants. Three surfactants failed to stimulate toluene degradation. The last surfactant, sodium dodecyl sulfate, was actually degraded preferentially to the hydrocarbon and therefore inhibited hydrocarbon removal. An investigation by Lee et al. (2001) investigated the use of two food-grade and two industrial surfactants. The study found that one of the food-grade surfactants was more effective in degrading

toluene in shaken batch experiments than the other three test surfactants. However, one of the industrial surfactants was the most effective at toluene degradation when used in sand-packed column studies (Lee et al., 2001). An interesting study by Lee et al. (2004) found that in soils with high organic matter content, anionic surfactants were more effective than cationic surfactants at removing toluene due to the cationic surfactant's tendency to adsorb to the soil surfaces. This cleverly demonstrated the need to select a surfactant of choice based, at least in part, on environmental factors such as soil classification.

Naphthalene

Naphthalene is highly soluble in water (relative to other hydrocarbons) and is extremely toxic to humans. Therefore, a lot of attention has been given to the potential stimulation of its biodegradation through the use of surfactants. However, as with other hydrocarbons, the results of such investigations have varied widely.

Production of a rhamnolipid by a *Pseudomonas* growing on naphthalene as the sole carbon source resulted in a significant increase in the aqueous concentration of hydrocarbon (Déziel et al., 1996). This was the first report of biosurfactant production resulting from polycyclic-aromatic hydrocarbon (PAH) degradation. In a later study, Arino et al. (1998) demonstrated that a biosurfactant, in the presence of a PAH – degrading *Pseudomonas,* accelerated the degradation of the hydrocarbon. Finally, a similar biosurfactant was shown to solubilize naphthalene to more than 30 times its normal aqueous concentration and to increase its final degradation by almost 30 mg/L (Vipulanandan and Ren,

2000). Under similar conditions, two synthetic surfactants failed to achieve similar naphthalene solubilization and at least one of the synthetic surfactants actually inhibited naphthalene degradation. A study by Garcia-Junco et al. (2003) found that a rhamnolipid biosurfactant enhanced the mobilization of a variety of PAHs, including phenanthrene, but was ineffective in regards to naphthalene. Additionally, significant stimulation of naphthalene degradation was not observed.

In regards to synthetic surfactants, a study by Volkering et al. (1995a) demonstrated that both naphthalene mobility and biodegradation were increased by the presence of two surfactants when the hydrocarbon was sorbed to an inert matrix. These results were upheld by additional investigations (Strong-Gunderson and Palumbo, 1995; Volkering et al., 1995b)). Another study attempted to quantify the amount of naphthalene available to degrading microorganisms in the presence of various surfactants, and found that mineralization rates were not affected (Liu et al., 1995).

Further studies on the effects of synthetic and natural surfactants on PAH degradation have been performed on phenanthrene. Two nonionic surfactants, Alfonic 810-60 and Novel II 1412-56, were found to increase biodegradation of phenanthrene in both mineral and organic soils (Aronstein et al., 1991). An investigation by Garcia-Junco et al. (2001) found that rhamnolipid biosurfactants added at above CMC levels increased the solubilization of phenanthrene, resulting in increased overall degradation of the hydrocarbon by microorganisms. Lower levels of biosurfactants did not stimulate phenanthrene loss. This result was

supported by findings by Zhang et al. (1997). Burd and Ward (1996) reported an increase in phenanthrene degradation in the presence of a *Pseudomonas* biosurfactant while the opposite results were found by Deschenes et al. (1995). Various other PAHs have been tested with a variety of surfactant types, and equally contradictory conclusions have been drawn (Aronstein and Alexander, 1993; Jain et al., 1992; Lantz et al., 1995; Roch and Alexander, 1995; Tiehm, 1994; Van Hamme and Ward, 1999; Van Hamme et al., 2000).

Literature Review -Summary

In summary, the effect of biological and synthetic surfactants on hydrocarbon degradation is somewhat unpredictable (Banat, 1995a; Desai, 1987; Ducreux et al., 1994; Gutnick and Minas, 1987; Karanth et al., 1999; Rouse et al., 1994; Volkering et al., 1998). Reports can be found suggesting that the use of surfactants will enhance degradation. Similarly, reports can also be found suggesting that stimulation is either non-existent or the biosurfactant is actually inhibitory to the degradation of the hydrocarbon.

An additional difficulty in assessing the published literature is a lack of standardized reporting of biosurfactant levels (Volkering et al., 1998). Many studies involving the addition of pre-purified surfactants (chemical or biological) only report the volume of compound added and not the added volume in relation to the CMC value of the surfactant, a value specific to each compound (Bruheim et al., 1997; Burd and Ward, 1996; Foght et al., 1989). Furthermore, studies that involve the addition of a biosurfactant-producing microorganism to a hydrocarbon degradation system typically do not measure the levels of biosurfactant being

produced. Therefore, it can be unclear how the particular biosurfactant is influencing the target hydrocarbon in comparison to if measured amounts of pre-purified biosurfactant were added (Arino et al., 1998; Burd and Ward, 1996; Jain et al., 1992). Accordingly, it is difficult to make clear comparisons among published studies and to draw conclusions about how a specific biosurfactant will interact with a target hydrocarbon in an environment.

PHYSICAL AND CHEMICAL EFFECT OF BIOSURFACTANTS ON HYDROCARBONS

General

One of the most common reasons investigators are interested in biosurfactants is the desire to remove a hydrocarbon contaminant from a system. Removal can mean simply mobilizing the hydrocarbon from one location to another so that the hydrocarbon can be more easily managed. Removal may also be defined in terms of degradation of the hydrocarbon. Both types of "removal", by physical relocation or mineralization, can theoretically benefit from the characteristics possessed by biosurfactants.

In regards to relocating a hydrocarbon, three major factors must be taken into account. These include the solubility of the hydrocarbon, the sorption of the hydrocarbon to another entity, and the location of the hydrocarbon (Bedient et al., 1999; Maier, 2000).

Each hydrocarbon has a degree of solubility in an aqueous medium, the most common environmental condition (i.e.: groundwater, soils, etc.). The solubility of hydrocarbons typically falls below 10 μg/l, whereas most carbohydrates have solubilities measured in terms of grams per liter (Bedient et al., 1999; Maier, 2000). Even among the hydrocarbons, however, there are degrees of solubility. Toluene and naphthalene are much more water soluble than hexadecane.

Sorption is the association of the target hydrocarbon with solid material (Bedient et al., 1999). This solid material may include soil particles. Adsorption is defined as the association of a compound (hydrocarbon) with the surface of a solid particle. Absorption is the association of a compound within the solid particle. The term sorption is used to describe the overall effect (Bedient et al., 1999).

The location of a hydrocarbon describes how easily accessible the hydrocarbon is to mobilization or degradation forces (Bedient et al., 1999). A hydrocarbon may be sequestered in a layer separate from the aqueous phase in what is known as a non-aqueous phase liquid (NAPL). Hydrocarbons may also be located between soil grains or within a micropore of a single grain. This location may be transient or semi-permanent. The location of the hydrocarbon may be dependent on solubility and sorption (Bedient et al., 1999).

As stated before, three major factors must be taken into account when trying to relocate a hydrocarbon. These include the solubility of the hydrocarbon, the sorption of the hydrocarbon to another entity, and the location of the

hydrocarbon (Bedient et al., 1999; Maier, 2000). The amphipathic nature of a biosurfactant can potentially:

1. Increase the amount of a hydrocarbon solubilized in an aqueous medium past its natural solubility point
2. Decrease the amount of hydrocarbon sorbed to surfaces
3. Alter the location of a hydrocarbon as a result of increased solubility and decreased sorption, or increase mineralization of the hydrocarbon.

Surfactants increase the solubility of hydrocarbons

One of the most common applications of a biosurfactant is for it to act as a solubilizing agent (Ron and Rosenberg, 2002; Rosenberg and Ron, 1999). As stated before, each biosurfactant has a concentration called the critical micelle concentration (CMC) where the biosurfactant monomers will aggregate into a three-dimensional supramolecular structure called a micelle (Fiechter, 1992; Georgiou et al., 1992;). If these micelle structures exist at an interface between an aqueous and non-aqueous liquid (NAPL), the non-aqueous substance may be drawn into the center of the micelle, resulting in the emulsification of the non-aqueous liquid into the aqueous phase (Desai and Banat, 1997; Lin, 1996; Salager, 1999; Shafi and Khanna, 1995). The formation of micelles can therefore significantly increase the concentration of a hydrophobic compound, such as a hydrocarbon, in solution to above normal solubility levels (Rouse et al., 1994;

Salager, 1999). These principles are true for both biologically produced biosurfactants as well as synthetic surfactants.

The effects of surfactants on hydrocarbon solubility have been investigated. Thangamani and Shreve (1994) found that a rhamnolipid biosurfactant increased hexadecane solubilization more than an artificial surfactant. The increased solubility of an alkylbenzene within a NAPL mixture was observed with the application of biosurfactants (McCray et al., 2001). The change in solubility of 12 hydrocarbons in the presence of a surfactant was investigated by Diallo et al. (1994). They found that the alkanes, including *n*-dodecane and *n*-hexane, demonstrated significant increases in solubility. The solubility of certain aromatics, including benzene and toluene, also increased but not to the same degree as the alkanes. These results were supported by soil column studies by Pennell et al.(1993). The solubilities of naphthalene, pyrene, and phenanthrene were all increased in the presence of a total of six different synthetic surfactant in a complex study by Edwards et al. (1991) and a separate investigation by Volkering et al. (1995b). A clever study by Miller and Bartha (1989) artificially created bilayer liposomes and then compared the effectiveness of these micelles in regards to encapsulating octadecane versus hexatriacontane (C_{36}). Uptake of octadecane rose from 1.3% to 23.5% one hour after the addition of liposomes. Growth of a *Pseudomonas* on the hydrocarbon was similarly stimulated. Similar results were found for hexatriacontane (Miller and Bartha; 1989).

An interesting study by Bernardez and Ghoshal (2004) studied the solubilization effects of four synthetic surfactants on phenanthrene when the hydrocarbon was or was not in the presence of a second compound, naphthalene. Both PAHs were delivered to the system within a hexadecane NAPL overlayer. Each of the four surfactants were shown to solubilize naphthalene, but not hexadecane. Two of the surfactants increased the solubility of phenanthrene when the hydrocarbon was in a mixture with naphthalene. Phenanthrene solubility did not increase when phenanthrene was alone. Two other surfactants had the opposite effect and demonstrated a decrease of phenanthrene uptake when the hydrocarbon was in a mixture with naphthalene (Bernardez and Ghoshal; 2004). Finally, Zhang et al. (1997) investigated the effect of differences in the chemical composition of rhamnolipid biosurfactants on phenanthrene solubilization. A monorhamnolipid was compared to a dirhamnolipid. The researchers found that although overall mineralization rates of the hydrocarbon were equal for the two biosurfactants, the monorhamnolipid form of the biosurfactant was significantly more effective at solubilizing the hydrocarbon than was the dirhamnolipid form (Zhang et al.; 1997).

By creating an emulsion, physical relocation of a hydrocarbon may occur when the micelle-encapsulated hydrocarbon is carried by the aqueous phase away from the original location. A study by Bouchez-Naitali et al. (1999) demonstrated that hexadecane mobilization was increased by a number of biosurfactants, predominantly those produced by the genus *Pseudomonas*. Harwell et al. (1999) published an extensive review of the use of surfactants as a successful method of

solubilizing and mobilizing fuels for the purpose of remediating ground water. This review included a number of field studies where such a process was used successfully. In a final study, a biosurfactant significantly solubilized a heavy crude oil, and the resulting microemulsion (particle sizes under 100 nm) was found to be significantly beneficial to mobilizing the crude oil as a method of oil recovery (Chiu and Kuo, 1999).

In addition to affecting mobilization of a hydrocarbon, an increase in solubility may increase the microbial degradation of the target hydrocarbon. A surfactant can move a hydrocarbon into the aqueous environment where degrading microbes have access to the compound (Maier, et al., 2000; Rosenberg and Ron, 1999, Ron and Rosenberg, 2001). Furthermore, the emulsification process increases the exposed surface area of the hydrocarbon (Rosenberg and Ron, 1999, Ron and Rosenberg, 2002). The result is an increase in hydrocarbon availability to degrading microorganisms.

Surfactants decrease sorption of a hydrocarbon to a matrix

In addition to increasing the solubility of a hydrocarbon, surfactants have the ability to desorb a hydrocarbon from a surface (Ron and Rosenberg, 2002; Rosenberg and Ron, 1999). It has been suggested that sorption is the phenomenon most likely to cause the persistence of hydrocarbon contaminants in soils (Bedient et al., 1999; Mihelcic et al., 1993). This is because soil organic matter is strongly hydrophobic in nature, and thus water tends to be excluded from and hydrocarbons are often attracted to this material. As a result, increasing

the ability to relocate a hydrocarbon from such a surface and into the aqueous phase would have important oil removal and remediation ramifications.

It was demonstrated that an *Acinetobacter* produced biosurfactant can increase desorption of crude oil, resulting in the biodegradation of various poly-cyclic components of the oil (Rosenberg and Ron, 1999). A review of multiple studies showed that multi-ring PAHs are biodegradable when in the aqueous phase (Mihelcic et al., 1993). However, desorption from the soil matrix becomes more difficult as the molecular weight of these PAHs increases. Compounds such as biosurfactants, capable of overcoming the mass transfer difficulties of initiating desorption, can stimulate biodegradation in these situations (Mihelcic et al., 1993).

Although some microorganisms have demonstrated the ability to degrade hydrocarbons sorbed to a matrix (Guerrin and Boyd, 1992; Shrimp and Young, 1988), more evidence seems to exist for the degradation of compounds released from their location. Shrimp and Young (1988) observed that dodecyltrimethylammonium chloride was degraded more rapidly when in the aqueous phase than when in contact with sediments as a result of sorption to the particles. However, the opposite result was found for phenol. As with the effects of biosurfactants on the degradation of compounds, the addition of a biosurfactant may not always have the desired results. One study by Ivshina et al. (1998) investigated the addition of a *Rhodococcus*-produced biosurfactant to four different sands and three samples of oil shale cuttings. The level of desorption of

hexadecane was inversely proportional to the amount of biosurfactant added in all cases.

Thus, the understanding of the biosurfactant / hydrocarbon / solid matrix relationship is still rudimentary. More experiments are need to be conducted before a full understanding of this process is developed.

Surfactants alter location of hydrocarbons

The effect of natural and synthetic surfactants on hydrocarbon solubility and sorption to a surface, and how changes to these properties can lead to the mobilization of a hydrocarbon have already been discussed. A number of specific investigations into this effect and subsequent mobilization of alkanes, aromatics, and PAHs has also been discussed previously, including specific discussions of hexadecane, toluene, and naphthalene. The ultimate goal of most of these studies was the degradation of the particular target hydrocarbon.

However, this is not the only use of biosurfactants. One of the most common uses for mobilizing a hydrocarbon is for microbially-enhanced oil recovery (MEOR) (Bedient et al., 1999; Marsh et al., 1995). After an oil reservoir has been water flooded in an attempt to release more hydrocarbon from the subsurface, residual oil will remain entrapped in the pores of the reservoir matrix. The growth of microorganisms within a reservoir stimulates MEOR, taking advantage of natural metabolic by-products such as acids (to alter the reservoir matrix itself), gas (to increase pressure within the reservoir) and biosurfactants (to increase hydrocarbon mobility) (Bedient et al., 1999; Marsh et al., 1995).

The *Bacillus* strain JF2 biosurfactant has been successfully utilized in MEOR research (McInerney et al., 2001). An early study with JF2 demonstrated that the mobilization of crude oil was stimulated by the addition of pre-purified biosurfactant produced by the organism (Jenneman et al., 1983). These results were confirmed in a later study in which the pre-purified biosurfactant was injected into Berea sandstone cores, resulting in the increased release of four different crude oils (Thomas et al., 1991). Further studies investigating the addition of JF2 to unconsolidated limestone cores found that the addition of whole, biosurfactant-producing cells enhanced the recovery of oil by approximately 27% (Adkins et al., 1992). Marsh et al. (1995) further demonstrated the importance of JF2 biosurfactant production on the ability to stimulate hydrocarbon mobility. This study included a series of experiments where the wild-type JF2 strain (biosurfactant – producing) was injected into one series of Berea sandstone cores while the non-biosurfactant-producing mutant was injected into another series of cores. Oil recovery was almost four times higher in cores inoculated with the wild-type strain of JF2 than with the mutant (Marsh et al., 1995).

In conclusion, both sorption and solubility characteristics of hydrocarbons can be affected by surfactants. This effect can lead to an increased mobility of a hydrocarbon, resulting in important MEOR implications. Biodegradation of hydrocarbons may also be stimulated by increased mobilization.

Biosurfactants regulate the attachment / detachment of microorganisms to a matrix

Until this point, the effect of biosurfactants on hydrocarbon degradation has been discussed in terms of the direct relationship between the surfactant and the hydrocarbon. However, biosurfactants may indirectly affect hydrocarbon degradation by targeting the degrading microorganisms instead. In addition to influencing the desorption of hydrocarbons from a surface, surfactants may alter the adhesion patterns of microorganisms to a surface (Rosenberg et al., 1983; Rosenberg and Ron, 1999). In reference to medical microbiology, researchers have investigated the effect of biosurfactants on normal flora attachment within the human body (Velraeds et al., 1996). Research has also investigated the role of biosurfactants in infections caused by microbial attachment to and subsequent biofilm development on medical implant device (Busscher et al., 1997; Meylheuc et al., 2001). From these studies, it is apparent that biosurfactants play a role in the adhesion and detachment of medically important microbial cells to surfaces. The adhesion and detachment of microbes can also have important implications in regards to hydrocarbon degradation, however.

The production of a cell-bound biosurfactant may result in either increased hydrophobic or hydrophilic cell-surface characteristics. This subject was extensively reviewed in Neu (1996). For example, the production of a biosurfactant by *Pseudomonas* resulted in significantly increased cell-surface hydrophobicity while the opposite was true for a biosurfactant-producing *Acinetobacter* (Rosenberg et al., 1993; Zhang and Miller, 1994). In fact, a single

organism may alter their cell-surface properties specifically to attach or detach from a surface as needed. Such was the case with *Acinetobacter calcoaceticus* growing on crude oil (Rosenberg, 1993). After the utilization of the hydrocarbon substrate, starvation would induce the release of emulsan. This biosurfactant would surround the microorganism, allowing the cell to desorb from a surface. As a result, the cell was planktonic and remained so until fresh substrate was encountered and biosurfactant production ceased (Rosenberg, 1993). In a second study, Bruheim et al. (1997) reported that hexadecane oxidation was stimulated in the stationary growth phase of a *Rhodococcus* and not before. Biosurfactant production by the microorganism did not achieve significant levels before stationary growth phase. Once levels were high enough, the cell surface became hydrophobic, contact with the hydrocarbon increased, and hexadecane degradation was stimulated (Bruheim et al.,1997). This effect has been seen in other bacterial species as well (Bruheim and Eimhjellen, 1998).

An extensive set of studies has shown that high cell-surface hydrophobicity can stimulate the uptake of hydrocarbons by bacteria (Al-Tahhan et al., 2000; Bouchez-Naitali et al., 1999; Jones, 1997; Maier and Soberon-Chavez, 2000). This can have important effects on degradation rates when the target hydrocarbon is extremely hydrophobic, such as the long chain alkanes. These compounds typically are more difficult to remediate in soils due to their strong attachment to soil organic matter and resistance to flushing with water. By increasing the hydrophobicity of the cell-surface, microbial adherence to soil organic matter may increase. This may result in an increased likelihood of

contact to and mineralization of sorbed, long-chain alkane hydrocarbons (Harwell et al., 1999; Jones, 1997; Neu, 1996). Decreasing cell surface hydrophobicity, on the other hand, may enhance transport of the microorganism through the soil matrix (Brown and Jaffe, 2001).

Therefore, the ability to alter physical characteristics of microbial cell surfaces may enhance desirable traits. If mobility through high organic matter soil is desired, a decrease in surface hydrophobicity may be appropriate. In contrast, if an extremely insoluble hydrocarbon is sorbed to soils, degradation may be enhanced by increasing the physical contact between cell surfaces and the hydrophobic contaminant/soil matrix.

Summary of the physical and chemical effects of biosurfactants on hydrocarbons

In conclusion, biosurfactants can interact with hydrocarbons in three direct ways: by increasing the solubility of hydrocarbons into an aqueous medium, by increasing desorption from a matrix, and increasing the mobility of the hydrocarbon from the original site. Biosurfactants may also alter cell-surface characteristics, allowing for increased contact between a target hydrocarbon and a degrading microorganism. These traits of biosurfactants may have important effects on bioremediation. These characteristics may also have important tertiary oil recovery implications, as demonstrated by *Bacillus* strain JF2.

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CHAPTER TWO

The Effect of the *Bacillus* Strain JF2 Biosurfactant on the Anaerobic Degradation of Hydrocarbons

ABSTRACT

The current biosurfactant literature is deficient in regards to research involving biosurfactants produced by Gram-positive bacteria as well as the use of biosurfactants under anaerobic conditions. Furthermore, a mechanistic approach was needed in order for researchers to predict the impact of biosurfactants on anaerobic hydrocarbon degradation. In response to this, an extensive series of experiments was designed utilizing the biosurfactant produced by *Bacillus* species JF2. Specifically, these experiments were designed to determine if the presence of various levels of pre-purified JF2 biosurfactant would affect the degradation of a wide range of hydrocarbons under a variety of anaerobic conditions. A second goal of these experiments was to discern if *in situ* production of the JF2 biosurfactant would increase the degradation of these hydrocarbons to a greater degree than if the biosurfactant was added in its pre-purified form. It was hypothesized that as the concentration of pre-purified biosurfactant in a system increased, the degradation of the target hydrocarbon would increase as well. It was also hypothesized that *in situ* biosurfactant production would stimulate hydrocarbon degradation to levels that surpassed those achieved with the addition of pre-purified biosurfactant.

Toluene degradation under nitrate-reducing conditions was neither enhanced nor inhibited by the presence of various levels of the *Bacillus* species JF2 biosurfactant. Toluene degradation was inhibited under methanogenic conditions when CMC or above levels of biosurfactant were present. Degradation was stimulated under sulfate-reducing conditions when CMC or above levels of

biosurfactant were present. These results underscore the effect an environment on the interactions between biosurfactants and a hydrocarbon. Conditions favoring *in situ* production of the JF2 biosurfactant did not enhance toluene degradation. The presence of sediments slowed the degradation of toluene.

In the presence of a sediment matrix, the degradation of NAPL hexadecane was greater than that of neat hexadecane under both sulfate-reducing and methanogenic conditions. In the presence of hydrocarbon-contaminated sediments, the presence of biosurfactant preferentially stimulated the metabolism of the indigenous contaminants, rather than neat hexadecane. Hexadecane degradation was not enhanced to levels above what was achieved by the addition of pre-purified biosurfactant under conditions favoring *in situ* biosurfactant produced by JF2.

In regards to naphthalene degradation in the presence of various levels of pre-purified biosurfactant, there was no clear pattern relating levels of biosurfactant to the amount of naphthalene degraded. The presence of a sediment matrix decreased final naphthalene degradation by almost 45% in comparison to studies conducted in the absence of sediments.

These experiments were the most direct method of testing the effect of biosurfactants on hydrocarbons in the presence of a number of variables. Further research into the relationship between hydrocarbon degradation and biosurfactants is needed. However, these preliminary experiments have indicated how future work should proceed, including the use of low-solubility target hydrocarbons and sediment-free experimental systems. Future investigations will also require

proper microorganisms to be available for use, in either purified culture form or as semi-purified consortiums. These microorganisms must be available in a form absent of any contaminating hydrocarbon. These experiments have identified potential reasons for possible conflicting results in the biosurfactant literature. These experiments have also demonstrated how delicate the relationship is between biosurfactants, hydrocarbons, and environmental conditions.

INTRODUCTION

A review of the biosurfactant literature has underlined areas of research that are deficient in available data regarding the use of these compounds for hydrocarbon degradation. Two of the most prominent areas needing attention are the use of biosurfactants under anaerobic conditions and the use of biosurfactants produced by Gram-positive bacteria. Additionally, this review found conflicting results involving surfactant-enhanced hydrocarbon degradation studies. The following study was designed as a mechanistic approach to these issues. This investigation was a means of examining a broad spectrum of conditions affecting surfactant-enhanced hydrocarbon degradation.

The purpose of this study was to allow for predictions to be made in regards to the effect of *in situ* biosurfactant production on the degradation of hydrocarbons under anaerobic conditions. This investigation would determine if increasing amounts of pre-purified lipopeptide biosurfactant enhances hydrocarbon degradation. Secondly, this study would determine if hydrocarbon degradation increases under conditions favorable to *in situ* biosurfactant production. It was hypothesized that increasing amounts of pre-purified biosurfactant would increase hydrocarbon degradation, and conditions favoring *in situ* production of biosurfactant would increase hydrocarbon degradation more than the addition of pre-purified biosurfactant alone.

MATERIALS AND METHODS

Isolation and Purification of JF2 Biosurfactant

The isolation of purification of the *Bacillus* strain JF2 biosurfactant followed procedures previously described (Javaheri et al., 1985). JF2 was grown for 48 hours in 20 L glass carboys with constant aeration and stirring, at 37 ^{o} C. Aerobic growth was chosen over anaerobic growth to reduce the time necessary for biosurfactant production. Cells were removed by centrifugation of the broth, and the remaining liquid was acidified to a pH of 1.9 to 2.0 with concentrated hydrochloric acid. This was then placed in an ice bath within a 4^{o} C cold room over night. The resulting supernatant was removed by refrigerated centrifugation, and the precipitate was collected and lyophilized over night. The pellet was then suspended in absolute methanol and re-centrifuged. The resulting pellet was dried under a nitrogen gas flow, and lyophilized over night. This crude biosurfactant was suspended in absolute ethanol, and re-centrifuged. The resulting pellet was dried under a nitrogen gas flow and lyophilized over night for a final time. The purified biosurfactant was stored at 0^{o} C in sealed glass vials until use (Javaheri et al., 1985).

Purified biosurfactant collected at various times was composited to minimize required storage space. It was necessary to check the similarity of this composite to the biosurfactant used in previous publications. Thin layer chromatography was performed in a similar manner as previous investigations

with this biosurfactant (McInerney et al., 1990). A 10 µl sample of a 20 mg/ml solution of biosurfactant dissolved in methanol was added in triplicate to two 20 x 20 cm silica gel G plates. The plates were developed with a 65:35:5 (vol/vol) chloroform: methanol: 28% ammonium hydroxide solvent front for one hour. After air drying, one plate was stained with rhodamine B (0.25 g in 100 ml absolute ethanol), dried again, and viewed under UV light for the presence of lipids. The second plate was sprayed with concentrated sulfuric acid, dried at 100 ^{o}C for 10 minutes, and then viewed again for the presence of organic compounds. RF values were calculated. The results showed a high degree of similarity between the newly purified biosurfactant and the biosurfactant originally described (McInerney et al., 1990).

Background Information - Soil

The source of the sediments used in this study was a hydrocarbon - impacted site located near Ft. Lupton, Colorado. The sediments from this site were classified as sand to sandy loam and have been previously characterized in detail (Barker et al., 1996; Borole et al., 1997; Gieg et al., 1999; Sublette et al., 1997). Gas condensate leaked from an underground storage tank into the underlying aquifer during the 1970's. The contaminating gas condensate was composed mostly (96%) of hydrocarbons falling into the C_5 through C_{15} range (Gieg et al., 1999). In the early 1980's, the contamination source was removed, but residual hydrocarbon contamination remained. Monitoring of intrinsic bioremediation of the groundwater and contaminated sediments was initiated in 1993 and continues to date (Gieg et al., 1999). Total petroleum hydrocarbon

levels within the contamination plume averaged 23 mg/L and consisted of a mix of straight and branched - chain alkanes as well as BTEX compounds (Barker et al., 1996; Gieg et al., 1999). A chemical analysis performed at the time of sediment removal determined that the collected sediments for the current study still contained many of the hydrocarbons found in the original contaminating gas condensate (Gieg et al., 1999). Sediments for this study (FL-C) were collected from beneath the shallow water table (~ 1.4 meters) in 1999 and stored under N_2 at 4 ^{o}C. Uncontaminated sediments (FL-U) from a site upgradient of the contamination were also collected and stored under identical conditions.

Background Information – Chemical Analyses

A number of extraction and analytical procedures were performed during this project. Many were developed specifically for this investigation. The analysis of standard stock solutions or gases (i.e. CH_4) accompanied all analytical procedures.

Neat toluene

After incubation, headspace from test serum bottles was injected directly into a Hewlett-Packard (HP) 5890 Gas Chromatograph (GC). This method was run isothermally at 150 ^{o}C using a DB-5 capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The injection temperature was 250 ^{o}C, the detector temperature was 250 ^{o}C.

Neat Hexadecane

When hexadecane was added in neat form to experimental bottles, additional extraction steps were required prior to analysis. These steps would

result in a transfer of hexadecane from the aqueous test environment to a solution in methylene chloride. First, each bottle was given 10 µl of tetradecane as an internal control. The bottle was then shaken vigorously to loosen all soil particles and dislodge attached hydrocarbons. Soil particles were removed by centrifugation. The resulting supernatant was collected in a one liter separatory funnel. Approximately 10 ml of analytical grade methylene chloride was mixed with the aqueous phase. After approximately 20 minutes of resting to allow for phase separation, the heavier organic phase was drained from the separatory funnel through a Whatman ashless filter paper (size 44; 110 mm diameter) cone containing anhydrous sodium sulfate (as a drying agent, to ensure all water had been removed from solution) and into a clean, glass collection vial. The addition of methylene chloride and eventual collection was repeated for a total of three times. The resulting organic phase was then concentrated by drying under a flow of nitrogen gas to approximately two ml. Prior to use, all glassware was thoroughly rinsed with acetone. After each sample was extracted, all glassware was washed, triple rinsed with de-ionized water, rinsed with acetone, and allowed to air dry.

The resulting organic extracts were directly injected into an HP 6890 GC equipped with a flame ionization detector for tetradecane and hexadecane analyses. An HP-5 capillary column was used (30 m x 0.32 mm i.d. x 0.25 µm film thickness). The initial oven temperature was 110 ^{o}C and increased at a rate of 16 ^{o}C per minute until reaching a final oven temperature of 190 ^{o}C. Both the injector and detector temperatures were maintained at 250 ^{o}C.

NAPL toluene

A sample of the toluene-containing, non-aqueous phase liquid (NAPL) was directly injected into a HP 6890 GC equipped with a flame ionization detector for analysis. An HP-5 capillary column was used (30 m x 0.32 mm i.d. x 0.25 µm film thickness). The initial oven temperature was 110 ^{o}C and increased at a rate of 45 ^{o}C per minute until reaching a final oven temperature of 200 ^{o}C. Both the injector and detector temperatures were maintained at 250 ^{o}C.

NAPL hexadecane

A sample of the hexadecane-containing NAPL was directly injected into a HP 6890 GC equipped with a flame ionization detector for analysis using the same parameters established for the analysis of neat hexadecane.

NAPL naphthalene

A sample of the naphthalene-containing NAPL was directly injected into a HP 6890 GC equipped with a flame ionization detector for analysis. An HP-5 capillary column was used (30 m x 0.32 mm i.d. x 0.25 µm film thickness). The initial oven temperature was 160 ^{o}C and increased at a rate of 15 ^{o}C per minute until reaching a final oven temperature of 220 ^{o}C. The injector and detector temperatures were maintained at 200 and 250 ^{o}C, respectively.

Methane

Methane was analyzed via direct headspace injection into a Varian 3300 GC equipped with a flame ionization detector for analysis. This method was run isothermally at 100 ^{o}C using a Porapak Q column (0.18 m x 0.3125 mm i.d.). The

temperature of the injector was 60 ^{o}C and the temperature of the detector was 125 ^{o}C.

Sulfide

Sulfide was measured as a determinant of sulfate reduction. Sulfide was measured by the CHEMets Colormetric Assay for Sulfide (CHEMetrics K-9510D) field kit.

Ammonia

Ammonia was measured as a determinant of nitrate reduction. Ammonia was measured by the CHEMets Colormetric Assay for Ammonia (CHEMetrics K-1510) field kit.

Nitrate

Nitrate was measured as a determinant of nitrate reduction. Nitrate was measured by the CHEMets Colormetric Assay for Nitrate (CHEMetrics K-6902) field kit.

Overview

A series of experiments was designed to test the effect of biosurfactant addition on hydrocarbon degradation in the presence of a number of environmental variables. Variables which were to be tested included: a variety of target hydrocarbons, the delivery method of each hydrocarbon, various anaerobic conditions, different levels of pre-purified biosurfactant within each system, and the mode of biosurfactant delivery to the system. This was achieved through a complex series of experiments originally outlined by Strevett et al. (1998) (Table 2.1). These experiments could be divided into two main categories: those

Table 2.1: Outline of Project Objectives

Objective	HMN	Soil	Added Biosurfactant	Added Biosurfactant Producer
A.	-	-	-	-
B.	+	-	-	-
C.	-	+	-	-
D.	+	+	-	-
E.	-	-	+	-
F.	+	-	+	-
G.	-	+	+	-
H.	+	+	+	-
I.	-	-	-	+
J.	+	-	-	+
K.	-	+	-	+
L.	+	+	-	+

HMN : 2,2,4,4,6,8,8 – heptamethylnonane

+/- denotes the presence or absence of a particular variable

Adapted from : Strevett et al. (1998)

involving pre-purified biosurfactant and those involving *in situ* biosurfactant production.

Overview of experiments using various amounts of pre-purified biosurfactant

The first category of experiments investigated the degradation of target hydrocarbons in the presence of various amounts of pre-purified biosurfactant. Biosurfactant was added in amounts below, at, and above the CMC value of the *Bacillus* strain JF2 biosurfactant (CMC = 10 mg/L). Biosurfactant was added at ¼ the CMC (¼ CMC; 2.5 mg/L) amount and ten times the CMC amount (10 CMC; 100 mg/L) for the below and above CMC values, respectively. These experiments can be further broken down as follows:

1. An analysis of the degradation of neat hydrocarbon in the absence of soil
2. An analysis of the degradation of a hydrocarbon delivered in a NAPL solution in the absence of soil
3. An analysis of the degradation of neat hydrocarbons in the presence of soil
4. An analysis of the degradation of a hydrocarbon in a NAPL solution in the presence of soil

The hydrocarbons of interest included hexadecane, toluene, and naphthalene. These represent the three major classes of hydrocarbons: alkanes, aromatics, and poly-cyclic aromatics. These hydrocarbons also represent a wide solubility range. Each of the target hydrocarbons was delivered directly to the

experimental system (neat form) or was mixed with a 2,2,4,4,6,8,8 - heptamethylnonane (HMN) overlayer, representative of a NAPL solution (Londry and Suflita, 1998). The differences in delivery method of the hydrocarbons were established to mimic various levels of bioavailability of the hydrocarbons (Bedient et al., 1999).

The presence of soil was established in some experimental systems to provide indigenous microbes capable of degrading the target hydrocarbons under anaerobic conditions. The soil also provided a matrix to which the target hydrocarbon could sorb.

Overview of experiments using *in situ* produced biosurfactant

The goal of the second, main category of experiments performed was to test the effect of *in situ* biosurfactant production on hydrocarbon degradation versus the effects of pre-purified, added biosurfactant. This was the main variable of interest for this study, and it relied on the ability for *Bacillus* JF2 to survive and produce the biosurfactant under the chosen experimental conditions.

The reason for pursuing this part of the project was to compare the effect of a small amount of biosurfactant produced over time throughout the experimental system versus a one-time addition. If it was determined that the presence of pre-purified biosurfactant would increase hydrocarbon degradation, the amount of biosurfactant within the system could potentially become a limiting resource due to its biodegradability (Goudar et al., 1999). This would limit the benefit to degradation provided by the surfactant. However, if the biosurfactant

was constantly being replenished (hence, was produced *in situ*), the positive effect on biodegradation could be prolonged. Therefore, in theory, the net amount of hydrocarbon degraded would be greater than if the biosurfactant was simply added in a finite amount at one time. This could have effects far beyond the area of hydrocarbon bioremediation, such as in the area of microbially enhanced oil recovery (MEOR).

Experimental Design - Toluene

Analysis of the degradation of neat toluene, in the absence of soil, with varying amounts of pre-purified biosurfactant added (objectives A and E)

The purpose of these objectives was to study neat hydrocarbon degradation in the absence of soil when various amounts of pre-purified biosurfactant were present (Table 2.2). Twenty five ml of anaerobic Medium E medium (Table 2.3) was added to 60 ml serum bottles with a sterile nitrogen headspace. To each bottle, 8,000 μmol/L of hydrocarbon was added. Biosurfactant was added in the following concentrations: 0 CMC, ¼ CMC, CMC, and 10 CMC. The addition of an appropriate hydrocarbon degrader was necessary because no soil was present to provide an indigenous population. Therefore, *Thauera aromatica* (0.5 ml of an overnight culture, 30^{o} C) was added as indicated (Table 2.2). Each bottle was crimp sealed with a 22 mm butyl stopper (Belco 2048-11800) and incubated upside down at 30^{o} C. Conditions were replicated in triplicate. To ensure that the microbial culture was not

Table 2.2: Experimental Design for the Analysis of the Degradation of Neat Hydrocarbons in the Absence of Soil (Objectives A and E)

Condition	HC	Biosurfactant	*T. aromatica*
A	+	-	+
B	+	¼ CMC	+
C	+	CMC	+
D	+	10 CMC	+
E	-	10 CMC	+
F	+	10 CMC	-
G	+	EtOH	+

Condition: Refers to a group of replicates

HC: Hydrocarbon

+,- : Denotes the presence or absence of a particular variable

EtOH: Because the biosurfactant required addition in an ethanol solution, ethanol was added in the same amount as was added for a 10 CMC biosurfactant addition

CMC: Critical Micelle Concentration

Table 2.3: Anaerobic Medium E (modified from Clark et al, 1981)

Component	Amount per Liter
Nanopure water	990 ml
KH_2PO_4	2.7 g
K_2HPO_4	13.9 g
NaCl	50.0 g
Sucrose	10 g
Yeast Extract *	1.0 g
$NaNO_3$	1.0 g
$(NH_4)_2SO_4$	1.0 g
Proteose Peptone # 3	30 g
Component 2 **	10 ml

* Yeast Extract (Difco 0127-17)

** Component 2 is made by filter sterilizing 5 g $MgSO_4/7\ H_20$ and 25 ml trace metals (Table 2.8) into 75 ml nanopure water. This solution is added after the rest of the medium had been sterilized and has cooled.

becoming nitrate limited, 1 ml of an anaerobic "nutritional supplement" was added to each bottle once per week. This consisted of six grams of yeast extract and six grams of sodium nitrate per liter of nanopure-water, made anaerobic, then sterilized and maintained under a sterile nitrogen headspace. Levels of toluene and ammonia were analyzed over the two-week course of the experiment.

The ability of *T. aromatica* (published as *Pseudomonas* strain K172 in early manuscripts) to degrade toluene is well documented (Altenschmidt and Fuchs, 1991; Heider et al., 1999; Leutwein and Heider, 1999; Tschech and Fuchs, 1987). This organism was capable of growth and toluene degradation in anaerobic Medium E under nitrate-reducing conditions (data not shown).

Analysis of the degradation of toluene, delivered in a NAPL overlayer and in the absence of soil, with varying amounts of pre-purified biosurfactant added or *in situ* biosurfactant production occurring (objectives B, F, and J)

The purpose of Objectives B and F was to study the degradation of a hydrocarbon while in a NAPL solution in the presence of various levels of pre-purified biosurfactant (Table 2.4). Bottles were established similarly to those in Objectives A and E (nitrate reducing conditions). However, instead of the hydrocarbon being added in a neat form, the hydrocarbon was added with HMN to make a hydrocarbon solution (Londry and Suflita, 1998). This was to act as a controlled NAPL layer and was 1/10 the volume of the aqueous portion of the system. Final toluene concentrations in the bottles were 8,000 μmol /L (aqueous).

Table 2.4: Experimental Design for the Analysis of the Degradation of a Hydrocarbon in a NAPL Solution, without Soil (Objectives B, F, J)

Condition	HC	HMN	Biosurfactant	JF2/mutant	*T. aromatica*
A	+	+	-	-	+
B	+	+	¼ CMC	-	+
C	+	+	CMC	-	+
D	+	+	10 CMC	-	+
E	+	+	-	JF2	+
F	+	+	10 CMC	mutant	+
G	+	+	EtOH	mutant	+
H	+	+	-	mutant	+
I	+	+	-	JF2	-
J	+	+	-	mutant	-
K	+	+	-	-	-
L	+	+	10 CMC	-	-

Condition: Refers to a group of replicates

HC: Hydrocarbon

HMN: Heptamethylnonane

CMC: Critical Micelle Concentration

+,- : Denotes the presence or absence of a particular variable

EtOH: Because the biosurfactant required addition in an ethanol solution, ethanol was added in the same amount as was added for a 10 CMC biosurfactant addition

To appropriate bottles, biosurfactant was added in 0, ¼, 1, and 10 times the CMC amount.

Additionally, some bottles were inoculated with 1 ml of an exponential growth phase culture of *Bacillus* strain JF2 or the non-biosurfactant producing JF2 mutant in order to observe the effect of *in situ* biosurfactant production on toluene degradation (Objective J; Table 2.1). The JF2 mutant was added as a control.

To ensure that the microbial cultures were not becoming limited in sources of carbon or nitrate, 1 ml of the nitrate nutritional supplement (described above), amended with 60 g/L sucrose, was added to the bottles once per week for the three week duration of the experiment. The purpose of the sucrose amendment was to provide a carbon source for JF2 or the JF2 mutant. A concern was that *T. aromatica* would utilize the sucrose preferentially over toluene as a carbon source. This could make it difficult to compare the results of the already described neat hydrocarbon experiment and the current experiment. To test this, twelve bottles containing Medium E and *T. aromatica* were established under nitrate-reducing conditions. Six bottles received neat toluene, while six bottles received NAPL toluene. Half of the six bottles containing neat toluene received the unamended nitrate supplement and the remainder received the supplement amended with 60 g/L sucrose. Half of the six bottles containing NAPL toluene received the unamended nitrate supplement and the remainder received the supplement amended with 60 g/L sucrose. After two weeks of incubation, the final amount of toluene degraded was similar for all treatments. The conclusion

was that the sucrose amendment would not significantly alter final toluene degradation results (data not shown).

Levels of toluene and ammonia were analyzed. Conditions were established in triplicate.

Analysis of the degradation of neat toluene, in the presence of soil, with varying amounts of pre-purified biosurfactant (objectives C and G)

The purpose of these objectives was to study neat hydrocarbon degradation in the presence of a soil slurry and various amounts of purified biosurfactant (Table 2.5). Each 125 ml serum bottle received the following: 24 g sediment, 56 ml sterile medium (either medium E, sulfate-reducing or methanogenic) (Tables 2.6, 2.7, 2.8) and a headspace of either nitrogen gas for nitrate-reduction and sulfate-reduction experiments or 80:20 H_2/CO_2 for methanogenic experiments. Additionally, 8,000 μmol/L neat toluene was added. Pre-purified JF2 biosurfactant was added in various amounts to appropriate bottles. Conditions were established in triplicate.

Methanogenic and sulfate-reducing conditions were chosen because the Ft. Lupton sediments already had an established population of microbes capable of degrading a wide variety of hydrocarbons in a number of anoxic conditions (Borole et al., 1996; Elshahed et al., 2001; Rios-Hernandez et al., 2003). All bottles were incubated upside down at room temperature for six months. Levels of toluene, sulfide, and methane were analyzed.

Table 2.5: Experimental Design for the Analysis of the Degradation of Neat Toluene in Soil (Objectives C and G).

Condition	HC	Soil	Biosurfactant
A	+	live	-
B	+	live	¼ CMC
C	+	live	-
D	+	live	CMC
E	+	live	-
F	+	live	10 CMC
G	-	sterile	-
H	-	live	-
I	+	sterile	-
J	+	sterile	CMC
K	+	sterile	EtOH
L	+	live	EtOH
M	+	live	10 CMC *
N	+	sterile	10 CMC *

Condition: Refers to a group of replicates

HC: Hydrocarbon

CMC: Critical Micelle Concentration

+,- : Denotes the presence or absence of a particular variable

EtOH: Because the biosurfactant required addition in an ethanol solution, ethanol was added in the same amount as was added for a 10 CMC biosurfactant addition

* With the exception of these conditions (M and N), all bottles were inoculated with a culture of *T. aromatica* to decrease the time needed for completion of the experiment. This was for nitrate-reducing conditions only.

Table 2.6: Medium for the Culture of Sulfate Reducers

Component	Amount per Liter
SRB Mineral Salts Solution (20X) [a]	0.9375 ml
Trace Metals [a]	5.0 ml
Vitamins [a]	10.0 ml
5% Resazurin	1.0 ml
Nanopure Water	965 ml
TES Buffer	1.0 g
$Fe(NH_4)_2(SO_4)_2$ / $6H_20$	0.2 g

Adjust the pH to 7.3 – 7.5 with KOH.

Sterilize.

Exchange headspace to nitrogen gas while medium is still hot.

Add 50 mg of anaerobic Cysteine Sulfide.

Cool in the anaerobic chamber overnight, then dispense into sterile, anaerobic serum bottles as needed.

a.: See Table 2.8 for recipes.

Table 2.7: Medium for the Culture of Methanogens

Component	Amount per Liter
Mineral Salts [a]	10 ml
Trace Metals [a]	5 ml
Vitamin Stock [a]	10 ml
5% Resazurin	1.0 ml
Nanopure Water	974 ml

Adjust the pH to 7.3 – 7.5 with NaOH.

Buffer with 10 g $NaHCO_3$.

Sterilize.

While the medium is hot, change headspace to N_2 / CO_2 (80:20) .

Anaerobically add 2 ml of filter sterilized reducing agent (per L nanopure water):

4.5 g NaOH
20 g Cysteine HCL
20 g Na_2S / 9 H_2O

Cool over night in chamber, then dispense as needed.

a.: See Table 2.8 for recipes.

Table 2.8: Components of Sulfate-Reducing and Methanogenic Media

Vitamin Solution

Component	mg per L
Pyridoxine – HCl	10
Thiamine – HCl	5
Riboflavin	5
Calcium pantothenate	5
Thioctic acid	5
p- Aminobenzoic acid	5
Nicotinic acid	5
Vitamin B_{12}	5
Biotin	2
Folic acid	2
MESA [a]	10

a.: Mercaptoethanesulfonic acid

Trace Metal Solution

Component	g per L
Nitrilotriacetic acid	2.0
$MnSO_4$ / 6 H_2O	1.0
$Fe(NH_4)_2(SO_4)_2$ / $6H_20$	0.8
$CoCl_2$ / 6 H_20	0.2
$ZnSO_4$ / $7H_20$	0.2
$CuCl_2$ / 2 H_20	0.02
$NiCl_2$ / $2H_20$	0.02
Na_2MoO_4 / $2H_20$	0.02
Na_2SeO_4	0.02
Na_2WO_4	0.02

Mineral Solution

Component	g per L
NaCl	40
NH_4Cl	50
KCl	5
KH_2PO_4	5
$MgSO_4$ / 7 H_20	10
$CaCl_2$ / 2 H_20	2

SRB Mineral Solution (20X)

Component	g per L
NaCl	100
$(NH_4)_2SO_4$	10
$MgSO_4$ / 7 H_20	4
KH_2PO_4	6
$CaCl_2$ / H_20	0.8

Nitrate-reducing conditions were included in order to compare toluene degradation in the presence of various amounts of pre-purified biosurfactant to degradation in the presence of *in situ* biosurfactant production (objective K; Table 2.1). *T. aromatica* (1 ml, log phase culture) was added to bottles to increase the rate of toluene degradation. To ensure that the microbial culture was not becoming limited for a source of nitrate, 1 ml of the nitrate supplement (described above) was added to each bottle once per week for ten weeks. Levels of toluene and ammonia were analyzed. All bottles were incubated upside down at room temperature.

Analysis of the degradation of toluene, delivered in a NAPL overlayer, in the presence of soil and varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives D, H, and L)

The purpose of Objectives D and H was to study the degradation of a target hydrocarbon in a NAPL solution in the presence of various amounts of pre-purified biosurfactant (Table 2.9). Three grams of soil were added to each 60 ml serum bottle along with 25 ml of Medium E and a sterile nitrogen headspace (nitrate-reducing conditions). Toluene was added with HMN in a similar manner as for Objectives B, F, and J. The starting concentration of toluene was 8,000 μmol/L. To appropriate bottles, biosurfactant was added in various amounts. *T. aromatica* was added to guarantee the presence of a nitrate-reducing, toluene degrader.

Table 2.9: Experimental Design for the Analysis of the Degradation of a Hydrocarbon in a NAPL Solution with Soil (Objectives D, H, and L)

Condition	Soil	HC	HMN	Biosurfactant	*Bacillus* JF2/Mutant	*T. aromatica*
A	live	+	+	¼ CMC	-	+
B	live	+	+	CMC	-	+
C	live	+	+	10 CMC	-	+
D	live	+	+	-	-	+
E	live	+	+	-	JF2	+
F	live	+	+	10 CMC	mutant	+
G	live	+	+	¼ CMC	mutant	+
H	sterile	+	+	-	JF2	+
I	live	+	+	-	mutant	+
J	live	-	+	-	-	-
K	sterile	-	+	-	-	-
L	sterile	+	+	-	-	+
M	live	+	-	EtOH	-	+
N	live	-	-	-	-	+
O	sterile	-	-	-	-	+
P	live	+	+	10 CMC	-	-
Q	live	+	+	-	-	-
R	live	+	+	-	JF2	-
S	live	+	+	-	mutant	-

HC: Hydrocarbon
HMN: 2,2,4,4,6,8,8 - heptamethylnonane
CMC: Critical Micelle Value
+, - : Refers to the presence or absence of a variable, respectively
T. aromatica: Added when toluene was the target hydrocarbon
EtOH: Because the biosurfactant required addition in an ethanol solution, ethanol was added in the same amount as was added for a 10 CMC biosurfactant addition
Note: conditions N through S are controls for the addition of *T. aromatica* and were therefore only established when toluene was the target hydrocarbon

The purpose of Objective L was to observe changes to Objectives D and H when biosurfactant was added to the system via *in situ* production by JF2 (Table 2.9). Appropriate serum bottles were established in a similar manner as above except no additional, pre-purified biosurfactant was added. An anaerobic culture of JF2 (one ml, exponential growth phase) was also added.

To ensure that the microbial cultures were not becoming limited in sources of carbon or nitrate, one ml of the nitrate supplement (described above), amended with 60g/l sucrose, was added to each bottle once per week for ten weeks.

Conditions were established in triplicate. Levels of toluene and ammonia were analyzed.

Analysis of the degradation of neat toluene, in the absence of soil, under conditions favoring *in situ* biosurfactant production (objective I)

The purpose of this objective was to observe changes to Objectives A and E when biosurfactant was added to the system via *in situ* production by JF2 (Table 2.10). Serum bottles were established in a similar manner to those in Objectives A and E (Table 2.2), except no additional pre-purified biosurfactant was added. To appropriate bottles, one ml of an exponential growth phase culture of either *Bacillus* strain JF2, the JF2 mutant, and/or *T. aromatica* was added. The starting concentration of toluene was 8,000 µmol/L. To ensure that the microbial cultures were not becoming limited in sources of carbon or nitrate, 1 ml of the nitrate supplement (described above), amended with 60 g/l sucrose, was added to each bottle once per week for two and a half weeks.

Table 2.10: Experimental Design for the Analysis of the Degradation of Neat Hydrocarbons in the Absence of Soil but in the Presence of Biosurfactant Production (Objective I)

Condition	HC	JF2 / Mutant	*T. aromatica*
A	+	-	-
B	+	JF2	-
C	+	JF2	+
D	+	Mutant	-
E	+	Mutant	+
F	+	-	+

Condition: Refers to a group of replicates

HC: Hydrocarbon

+,- : Denotes the presence or absence of a particular variable

Conditions were established in triplicate. Levels of toluene and ammonia were analyzed.

Analysis of the degradation of neat toluene, in the presence of soil and under conditions favoring *in situ* biosurfactant production (objective K)

The purpose of this objective was to observe changes to Objectives C and G (Table 2.5) under conditions favoring *in situ* biosurfactant production by JF2 (Table 2.11). The starting concentration of toluene was 8,000 μmol/L. Serum bottles were established in a similar manner to those in Objectives C and G (nitrate reducing conditions), except pre-purified biosurfactant was added at 2 CMC amounts instead of 10 CMC, in appropriate control bottles. This change would still examine the effects of above CMC levels of biosurfactant on hydrocarbon degradation while conserving the compound for other experiments. A culture of *T.aromatica* was added to appropriate bottles.

Three ml of an exponential phase, anaerobic culture of JF2 were also added. To prevent the out-competition of either JF2 or the mutant, 0.5 ml of a fresh, over-night culture of each was added weekly to the appropriate bottles. To ensure that the microbial culture was not becoming limited in sources of carbon or nitrate, one ml of the nitrate supplement (described above) amended with 60 g/L sucrose, was added to each bottle once per week for five weeks. Conditions were established in triplicate. Levels of toluene and ammonia were analyzed.

Table 2.11: Experimental Design for the Analysis of Hydrocarbon Degradation in the Presence of a Biosurfactant Producing Microorganism (Objective K)

Condition	HC	Soil	Biosurfactant	JF2/mutant
A	+	sterile	-	mutant
B	+	live	-	mutant
C	+	live	2 CMC	mutant
D	+	live	EtOH	mutant
E	+	live	-	JF2
F	+	sterile	-	JF2
G	+	live	-	-
H	+	live	-	JF2 *
I	+	sterile	-	mutant *
J	+	live	2 CMC	-
K	+	sterile	-	-

Condition: Refers to a group of replicates

HC: Hydrocarbon

+,- : Denotes the presence or absence of a particular variable

EtOH: Because the biosurfactant required addition in an ethanol solution, ethanol was added in the same amount as was added for a 2 CMC biosurfactant addition

CMC: Critical Micelle Concentration

* Denotes bottles not inoculated with a culture of *T.aromatica.*

Experimental Design - Hexadecane

Analysis of the degradation of neat hexadecane, in the absence of soil, with varying amounts of pre-purified biosurfactant added (objectives A and E)

Serum bottles were established in a similar manner as the corresponding study involving toluene (Table 2.2). Because no soil was present to provide an indigenous hydrocarbon degrader population, the addition of an appropriate degrader was necessary. To appropriate bottles, 0.5 ml of an exponential growth phase culture of a hexadecane-degrading microbial consortium was added as a replacement for *T. aromatica*. This consortium is well documented for its ability to degrade hexadecane under sulfate-reducing conditions (Caldwell et al., 1998; Callaghan et al., 2003; culture provided by Dr. Lisa Gieg). Medium for Marine SRB (Widdel and Balk, 1992) (Tables 2.12 and 2.13), specific for the cultivation of marine sulfate-reducers, was used instead of anoxic Medium E. The starting concentration of hexadecane was 884 μmol/L. Each bottle was crimp sealed with a 22 mm butyl stopper (Belco 2048-11800) and incubated upside down at 30^{o} C. Levels of hexadecane and sulfide were analyzed over the two month course of the experiment. Conditions were established in triplicate.

Table 2.12: Medium for Marine Sulfate-Reducing Bacteria

Medium for Marine SRB

Mix together:

	Per liter
NaCl	20 g
$MgCl_2 \cdot 6H_2O$	3 g
$CaCl_2 \cdot 2H_2O$	0.15 g
Na_2SO_4	4 g
NH_4Cl	0.25 g
KH_2PO_4	0.2 g
KCl	0.5 g
Trace element	1 mL
Resazurin	1 mL of a 0.1% solution (redox indicator)

Adjust pH to ~7.1-7.3
Boil the above medium for 5 min in the autoclave in order to drive O_2 from the solution
Remove from autoclave and immediately place on ice under 20% N_2/CO_2 to cool and make anoxic
When medium is tepid to cool, mix in $NaHCO_3$ (0.25 g/100 mL) and leave flushing for about 5 min
Dispense appropriate amount into N_2/CO_2-flushed glass bottles or tubes
Cap with appropriate stoppers and seal with aluminum seals
Add cysteine-sulfide (1 to 2 mL/100 mL)
Autoclave to sterilize for 20 min (@121°C and 15 psi)

Once sterile medium has cooled, *aseptically* (e.g., flame everything) add Vitamins, Thiamine, and B12, such that 1 mL of each is added to 1 L medium. For 10 mL tubes, add 0.01 mL (about 2 drops) (Table 2.13)

Table 2.13: Composition of Widdel Trace Elements and Vitamins

Widdel Trace Elements

	Per 500 mL
$FeSO_4 \cdot 7H2O$	1.05 g
H_3BO_3	0.015 g
$MnCl_2 \cdot 4H_2O$	0.05 g
$CoCl_2 \cdot 6H_2O$	0.08 g
$NiCl_2 \cdot 6H_2O$	0.012 g
$CuCl_2 \cdot 2H_2O$	0.001 g
$ZnSO_4 \cdot 7H_2O$	0.072 g
$Na_2MoO_4 \cdot 2H_2O$	0.018 g
$Na_2SeO_3 \cdot 5H_2O$	0.003 g
$Na_2WO_4 \cdot 2H_2O$	0.004 g
NaEDTA	2.6 g

pH to 6 with NaOH

store at 4°C in foil wrapped bottle

Widdel Vitamins

Per 100 mL Na Phosphate buffer, 10 mM, pH 7.1*

4-aminobenzoic acid	4 mg
D(+) biotin	1 mg
Nicotinic acid	10 mg
Calcium D(+) pantothenate	5 mg
Pyroxidine hydrochloride	15 mg

*to make: make 10 mM solutions each of Na_2HPO_4 (0.142 g/100mL) and $NaH_2PO_4 \cdot H_2O$ (0.138 g/100 ml), mix in 5:2 proportions, respectively; pH will be about 7.1

Filter sterilize anoxically*, store under N_2 at 4°C in foil-wrapped bottle (*need an empty, sterile and anoxic bottle to filter the solution into).

(Widdel and Bak, 1992)

Analysis of the degradation of hexadecane, delivered in a NAPL overlayer in the absence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives B, F, and J)

For Objectives B and F, bottles were established similarly to those for the degradation of toluene (Table 2.4) with the following exceptions: hexadecane was the added hydrocarbon, the hexadecane-degrading sulfate-reducing consortium described above was added in place of *T. aromatica*, and the medium used was Medium for Marine SRB. The starting concentration of hexadecane was approximately 890 μmol/L. Levels of hexadecane and sulfide were analyzed over the two month course of the experiment. Conditions were established in triplicate. Conditions E through J could not be established since JF2 would be unable to survive sulfate-reducing conditions. Therefore, Objective J (of Table 2.1; to analyze the effect of biosurfactant produced *in situ* by JF2) was not performed.

Analysis of the degradation of neat hexadecane, in the presence of soil, and varying amounts of pre-purified biosurfactant (objectives C and G)

Conditions were established in a similar manner as the corresponding toluene study under sulfate-reducing and methanogenic conditions (Table 2.14). Methanogenic and sulfate-reducing conditions were chosen because the Ft. Lupton sediments had an established population of microbes capable of degrading

Table 2.14: Experimental Design for the Analysis of the Degradation of Neat Hexadecane in Soil (Objectives C and G).

Condition [(a)]	**Hexadecane Addition**	**Sediment**	**Biosurfactant**[(b,c)]
A	+	live	---
B	+	live	¼ CMC
C	+	live	1 CMC
D	+	live	10 CMC
E	-	sterile	---
F	-	live	---
G	+	sterile	---
H	+	sterile	1 CMC
I	+	sterile	EtOH
J	+	live	EtOH

(a) Each experimental condition was performed in triplicate.

(b) CMC: Critical Micelle Concentration

(c) EtOH: Denotes the addition of ethanol without any biosurfactant. These are control conditions.

a wide variety of hydrocarbons under a number of anoxic conditions (Borole et al., 1996; Elshahed et al., 2001; Rios-Hernandez et al., 2003). For sulfate-reducing conditions, non-marine sulfate-reducing medium (Table 2.6) was used. Marine sulfate-reducing medium (Tables 2.12 and 2.13) could not be utilized because the Ft. Lupton sediments were not marine in nature. The starting concentration of hexadecane was approximately 51 μmol/L. Levels of hexadecane, sulfide, and methane were analyzed over the one year course of the experiments.

The methanogenic portion of this study was repeated in full. The results of both the original and replicate methanogenic experiments were published in Jennings and Tanner (2004).

Analysis of the degradation of hexadecane, delivered in a NAPL overlayer in the presence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives D, H, and L)

Objectives D and H were established to investigate the effect of various amounts of pre-purified biosurfactant on hexadecane degradation. Bottles were established similarly to those for the degradation of neat hexadecane (Table 2.14). However, the hydrocarbon was delivered in a NAPL made up of HMN. Standard sulfate-reducing and methanogenic media were used (Tables 2.6 – 2.8) for reasons as stated above. The starting concentration of hexadecane was

approximately 890 μmol/L. Levels of hexadecane, sulfide, and methane were analyzed. Conditions were established in triplicate.

In regards to Objective L (to investigate the effect of *in situ* biosurfactant production by JF2), JF2 would be unable produce biosurfactant under sulfate-reducing or methanogenic conditions. Therefore, Objective L was not performed.

Analysis of the degradation of neat hexadecane, in the absence of soil, under conditions favoring *in situ* biosurfactant production is occurring (objective I)

The purpose of this objective was to observe changes to Objectives A and E (Table 2.2) when biosurfactant was added to the system via *in situ* production by JF2 (Table 2.10). However, this would require an organism capable of degrading hexadecane under nitrate-reducing conditions (to satisfy the requirements of JF2). No organism was found which could satisfy these necessary qualifications. Therefore, this specific objective was not pursued in regards to hexadecane.

Analysis of the degradation of neat hexadecane, in the presence of soil and under conditions favoring *in situ* biosurfactant production (objective K)

The purpose of this objective was to observe changes to Objectives C and G (Table 2.5) when biosurfactant was added to the system via *in situ* production by JF2. This investigation was established in a similar manner as the corresponding toluene study (Table 2.11). The starting concentration of hexadecane was approximately 890 μmol/L. Levels of hexadecane and ammonia

were analyzed. To ensure that the microbial culture (JF2) was not becoming limited in sources of carbon or nitrate, one ml of the nitrate supplement (described above) amended with 60 g/L sucrose, was added to each bottle once per week for the 5 month duration of the experiment. Conditions were established in triplicate.

Experimental Design - Naphthalene

Because naphthalene is a solid at room temperature, the compound (in neat form) was incompatible with the established experimental system. Therefore, it was decided not to pursue the following objectives in regards to naphthalene: A, C, E, G, I, and K.

Analysis of the degradation of naphthalene, delivered in a NAPL overlayer in the absence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives B, F, and J)

In regards to Objectives B and F, bottles were established similarly to those for the degradation of toluene (Table 2.4) with the following exceptions: Naphthalene was the added hydrocarbon and *Pseudomonas putida* strain PpG7 (ATCC 17485; provided by Dr. Kathleen Duncan) was added in place of *T. aromatica*. This organism is capable of degrading naphthalene under nitrate-reducing conditions (García-Valdés et al., 1988). The starting concentration of naphthalene was approximately 30 μmol/L.

Preliminary studies showed that *P. putida* strain PpG7, in pure culture, did not degrade naphthalene under these conditions. Growth was not visible by eye after 7 days of incubation. It was decided to inject a known amount of sterile air to freshly established bottles in an attempt to stimulate growth and thus degradation. This appeared to work as visible growth began to appear within 24 hours. Naphthalene levels began to decrease after 14 days of incubation.

Preliminary studies were being done during this time to determine the compatibility of strain PpG7 and JF2. A microscopic analysis determined that strain PpG7 exponentially outnumbered JF2 in Medium E after only 36 hours when grown in Medium E under the above microaerophilic conditions. Therefore, the experiment was modified again in an attempt to discern an experimental design where JF2 would no longer be overgrown by the *Pseudomonas*. Each serum bottle containing either JF2 or the JF2 mutant received a one ml inoculation of log-phase JF2 or JF2 mutant twice a week for three weeks. Despite these attempts, a microscopic analysis determined that the *Pseudomonas* culture comprised almost all of the microbial biomass after incubation. JF2 could not compete with the Gram negative culture. Furthermore, no naphthalene degradation was detected during the first two weeks of the trial, and it was assumed that *P. putida* was preferentially consuming a carbon source from Medium E. This could have been a reason for the failure of JF2 or the JF2 mutant to become established.

As a result of these trials, it was decided that the conditions E through J (Table 2.4; established to test the effect of *in situ* biosurfactant production by JF2)

would be removed. The removal of JF2 as a variable allowed for the growth medium to be changed from Medium E to *Pseudomonas* Minimal Medium (Sayler et al., 1985) (Table 2.15). This medium would allow naphthalene to be the only carbon source available to the microorganism, in contrast to Medium E. Additionally, this medium was developed specifically for the growth of *Pseudomonas,* in contrast to Medium E. A new control experiment was established under strict nitrate-reducing conditions. *P. putida* strain PpG7 is capable of degrading naphthalene under nitrate-reducing conditions (García-Valdés et al., 1988). Naphthalene and ammonia were analyzed over the ten week course of the experiment. To ensure that the microbial culture was not becoming limited in sources of nitrate, one ml of the nitrate supplement (described above) was added to each bottle once per week. Conditions were established in triplicate.

Analysis of the degradation of naphthalene, delivered in a NAPL overlayer in the presence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives D, H, and L)

Bottles were established similarly to those for the degradation of toluene (Table 2.9), however the naphthalene-degrading *P. putida* strain PpG7 was substituted for *T. aromatica*. The added hydrocarbon was naphthalene. The starting concentration of naphthalene was approximately 30 μmol/L.

Table 2.15: *Pseudomonas* Minimal Medium

Component	Amount (per L)
$MgSO_4$ - 7 H_20	0.2 g
NH_4Cl	1.0 g
$(NH_4)_2PO_4$ – 2 H_20	2.14 g
KH_2PO_4	1.1 g
$NaNO_3$	1.0 g
Trace Salts (Below)	10.0 ml
Di H_20	bring volume to 1 L

Trace Salts Component	Amount (per L)
$FeSO_4$ – 7 H_2O	0.3 g
$MnCl_2$ – 4 H_2O	0.18 g
$Co(NO_3)_2$ – 6 H_2O	0.13 g
$ZnSO_4$ – 7 H_2O	0.04 g
Na_2MoO_4	0.22 g
$CuSO_4$ – 5 H_20	0.001 g
$CaCl_2$	1.0 g

Because preliminary experiments had determined that *P. putida* was not compatible with JF2 in Medium E, the experiment was further modified: the conditions established to test Objective L (the effect of *in situ* biosurfactant production by JF2; conditions E-I and R-S of Table 2.9) were removed and the incubation medium was changed to *Pseudomonas* Minimal Medium (Sayler et al., 1985) (Table 2.15). It was understood that this would select for a small fraction of the indigenous microbial community of the sediment. However, it would make the presence of sediments the only experimental variable changed from Objectives B and F (Naphthalene).

To ensure that the cultures were not limited for nitrate, one ml of the nitrate supplement (described above) was added to each bottle once per week for the ten week duration of the experiment. Naphthalene and ammonia were analyzed. Conditions were established in triplicate.

RESULTS

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Results- Toluene

Analysis of the degradation of neat toluene, in the absence of soil, with varying amounts of pre-purified biosurfactant added (objectives A and E)

Toluene levels in all test bottles decreased from 8,000 to between 80 and 130 μmol /L (Figure 2.1). This was a 98 - 99% reduction in hydrocarbon.

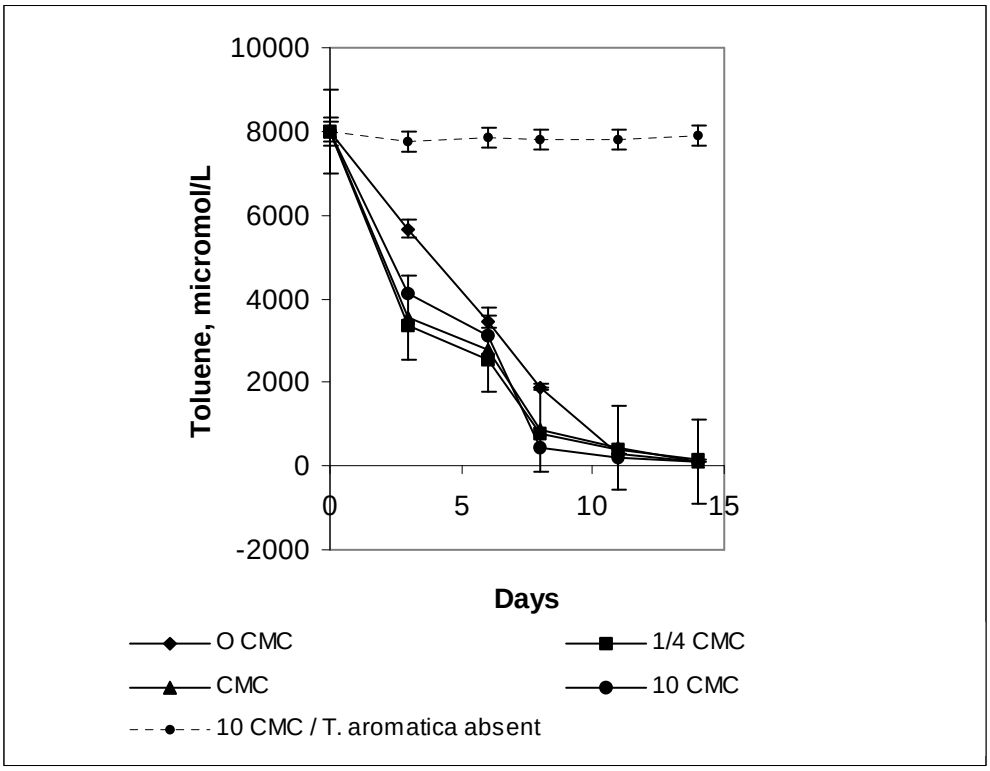

Figure 2.1: Average Neat Toluene in the Presence of Various Levels of Pre-Purified Biosurfactant in the Absence of Soil Under Nitrate-Reducing Conditions (Objectives A and E) after 14 Days.

The control bottles containing 10 CMC pre-purified biosurfactant but not containing *T.aromatica* (condition F) showed a decrease in toluene to a final level of approximately 7,900 μmol/L (1% reduction in hydrocarbon level). The control bottles containing ethanol (as a control for the biosurfactant added while dissolved in the alcohol) demonstrated a similar degree of toluene loss as bottles containing no biosurfactant (and subsequently no ethanol). Ammonia levels rose from 0 to approximately 55 ppm (3 mmol/L) in all bottles containing live cultures.

A concern was that the efficiency with which *T. aromatica* degraded toluene may have overshadowed any effect of the pre-purified biosurfactant. In other words, *T. aromatica* could have been so effective at degrading toluene under these conditions that the absence of biosurfactant was not limiting. A control experiment was devised. Serum bottles were established in an identical manner to those in conditions A through D (Table 2.2). However, only one-fifth of the original amount of *T.aromatica* culture was added to any bottles (0.1 ml) Conditions were monitored for two weeks from the time of inoculation. The amount of toluene degraded in all bottles after one week of incubation was 25-27 % . After two weeks of incubation, the amount of toluene degraded in all bottles ranged from 35-37 %.

Analysis of the degradation of toluene, delivered in a NAPL overlayer in the absence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives B, F, and J)

Bottles which received 0 to 10 CMC biosurfactant or were inoculated with JF2 demonstrated toluene loss from 8,000 to between 185 - 250 μmol /L of target hydrocarbon after 21 days of incubation (Figure 2.2). This is a 97-98% loss of hydrocarbon. Ammonia levels rose from 0 to approximately 85 ppm (4.7 mmol/L) in all bottles containing *T. aromatica*, and to approximately 45 ppm (2.5 mmol/L) in bottles not containing *T. aromatica* but containing JF2 or the JF2 mutant (conditions I and J, Table 2.4). There was no increase in ammonia levels in the absence of live cultures.

Analysis of the degradation of neat toluene, in the presence of soil, with varying amounts of pre-purified biosurfactant added (objectives C and G)

Under nitrate-reducing conditions, toluene levels decreased from 8,000 to between 105-130 μmol /L (98 % depletion) in the presence of 0 or ¼ CMC of added biosurfactant after two and a half months of incubation (Figure 2.3). Levels of hydrocarbon decreased to approximately 250 and 370 μmol /L (97% and 95% loss) in the presence of CMC and 10 CMC levels of biosurfactant, respectively. Live sediments with 10 CMC of biosurfactant but without *T. aromatica* resulted in a 47% decrease in toluene to an average level of 4,194 μmol /L. Ammonia levels increased to approximately 80 ppm (4.4 mmol/L) in bottles containing live soils. No ammonia was detected in any bottles containing sterilized soils.

Under methanogenic conditions, toluene levels decreased by 410 and 490 μmol /L (5% and 6% of the total) in the presence of 0 or ¼ CMC levels of

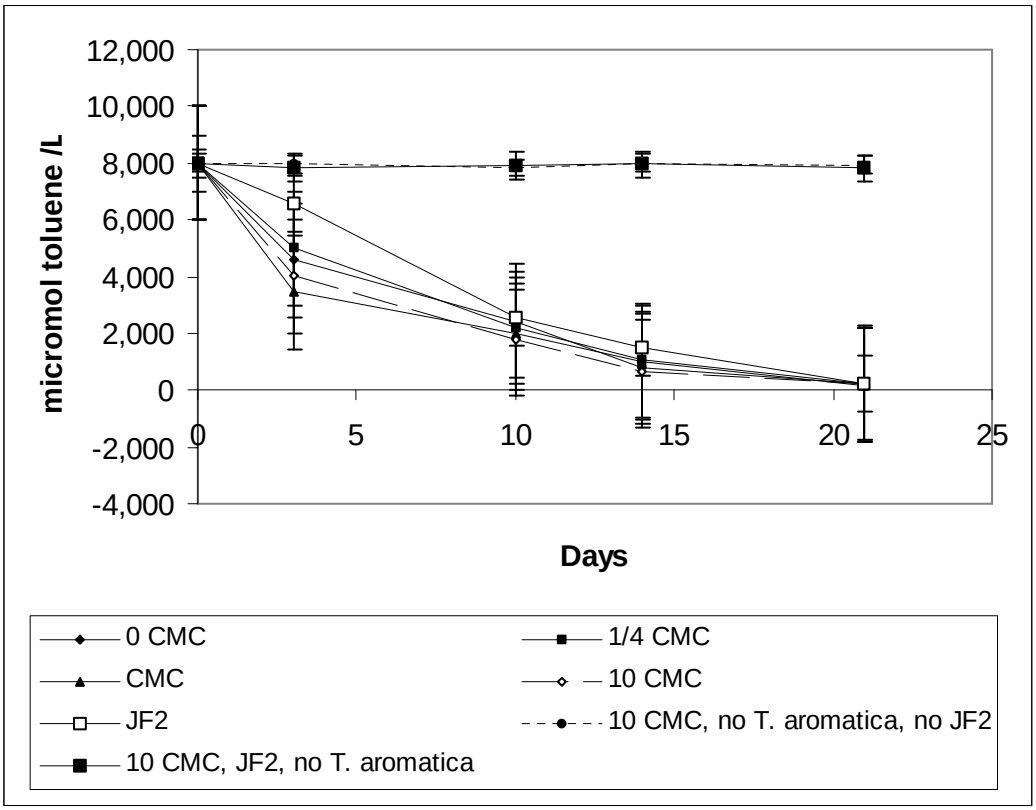

Figure 2.2: Average NAPL Toluene in the Presence of Various Levels of Pre-Purified Biosurfactant or *Bacillus* JF2 in the Absence of Soil Under Nitrate-Reducing Conditions (Objectives B, F, and J) after 21 Days.

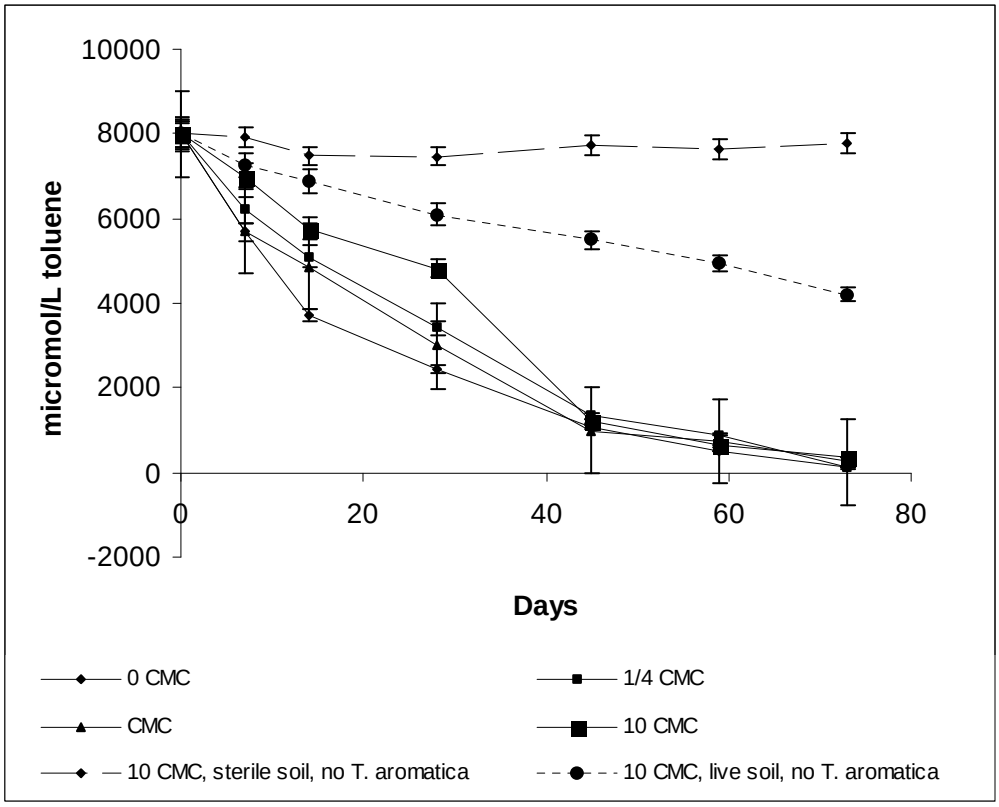

Figure 2.3: Average Neat Toluene in the Presence of Various Levels of Pre-Purified Biosurfactant and Soil Under Nitrate-Reducing Conditions (Objectives C and G) after 73 Days.

biosurfactant after 6 months of incubation (Table 2.16). However, hydrocarbon degradation decreased by between 146-160 μmol /L (1.8 % and 2% of the total) in the presence of CMC or above CMC levels of biosurfactant. This is a similar level to the loss of hydrocarbon in sterilized sediments. Methane increased significantly (2.7 to 4.1 times higher) when CMC or 10 CMC levels of biosurfactant were present, respectively. Methane was not produced in sterilized sediments.

Under sulfate-reducing conditions, the amount of toluene degraded decreased by almost 80% when biosurfactant concentrations were increased from 0 to ¼ CMC (Table 2.17). The amount of toluene degraded in the presence of ¼ CMC (8.66 μmol /L) of biosurfactant was less than that degraded in sterilized sediments (19.70 μmol /L). Degradation capabilities increased in the presence of CMC levels of biosurfactant (84.52 μmol /L) to almost twice the amount of hydrocarbon degraded than in the presence of no biosurfactant. Toluene degradation increased significantly again when 10 CMC levels of biosurfactant (446.19 μmol /L) were added to the system. An average of 447 μmol /L of toluene was degraded (5.6 %) in the presence of these high concentrations of pre-purified biosurfactant. The presence of ethanol (condition L, Table 2.5) did not increase toluene loss over levels found in live soils with no biosurfactant added. Sulfide levels increased to approximately 20 ppm (588 μmol /L) in all bottles with live sediments.

Table 2.16: Average Neat Toluene Loss in the Presence of Various Levels of Pre-Purified Biosurfactant and Soil Under Methanogenic Conditions (Objectives C and G).

Condition, Amount of Biosurfactant	Toluene Loss (μmol /L)	Methane Gain (μmol/L)
A. 0 CMC	410 ± 27	135 ± 8
B. ¼ CMC	490 ± 31	128 ± 9
D. CMC	160 ± 4.6	374 ± 20
F. 10 CMC	146 ± 9.3	561 ± 31
I. 0 (sterile soil)	160 ± 9.1	1 ± 0.1

CMC: Critical Micelle Concentration

Time zero values: 8,100 μmol/L toluene, 0 μmol/L methane

All soils were unsterilized unless noted. The duration of the experiment was approximately 180 days.

Table 2.17: Average Neat Toluene Loss in the Presence of Various Levels of Pre-Purified Biosurfactant and Soil Under Sulfate-Reducing Conditions (Objectives C and G).

Condition, Amount of Biosurfactant	Toluene Loss (µMol /L)	Sulfide Gain (ppm)
A. 0	43.6 ± 2	20 ± 1
B. ¼ CMC	8.66 ± 0.5	20 ± 1
D. CMC	84.5 ± 9	20 ± 1
F. 10 CMC	446 ± 20	20 ± 2
H. 0 (live soil)	0 ± 0	19 ± 0.9±
I. 0 (sterile soil)	19.7 ± 1	2 ± 0.2

CMC: Critical Micelle Concentration

Time zero values: 8,100 µmol/L toluene, 0 ppm sulfide

All soils were unsterilized unless noted. The duration of the experiment was approximately 180 days.

Analysis of the degradation of toluene, delivered in a NAPL overlayer, in the presence of soil with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives D, H, and L)

When 0 to 10 CMC of biosurfactant was present, toluene levels decreased from 8,000 to between 763 and 880 μmol/L (89% to 90 % loss) (Figure 2.4). In the absence of *T. aromatica*, final toluene levels decreased only to approximately 3,000 μmol /L (63% loss) (condition Q). Ammonia levels increased to approximately 75 ppm (4.2 mmol/L) in all bottles containing live cultures.

Analysis of the degradation of neat toluene, in the absence of soil, while *in situ* biosurfactant production is occurring (objective I)

Toluene levels decreased from 8,000 to1,900 μmol /L (76 % loss) in those bottles containing *T.aromatica* and the non-biosurfactant producing JF2 mutant (Figure 2.5). In bottles containing *T.aromatica* and JF2, toluene levels decreased to approximately 1,250 μmol /L (84 % loss).

Ammonia levels increased to approximately 60 ppm (3.3 mmol/L) in all bottles containing live culture.

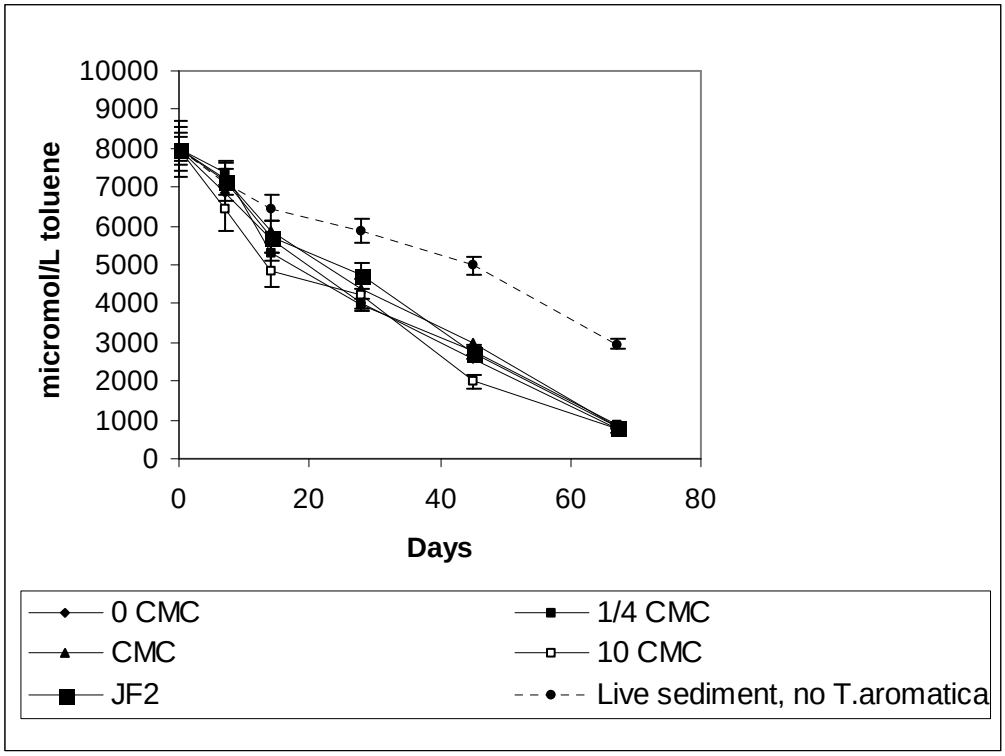

Figure 2.4: Average NAPL Toluene in the Presence of Various Levels of Pre-Purified Biosurfactant and JF2 in Soil Under Nitrate-Reducing Conditions (Objectives D, H, and L) after 67 Days.

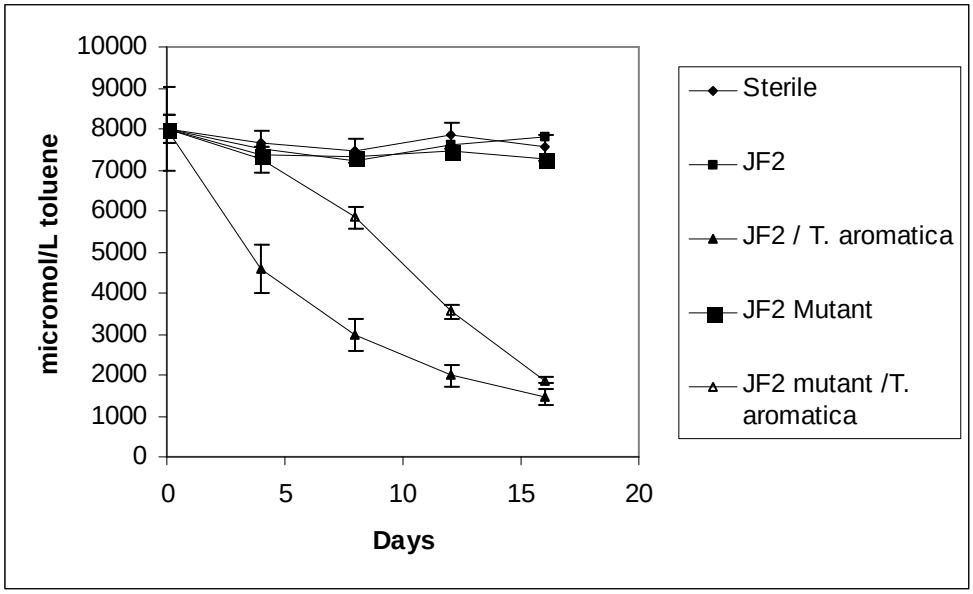

Figure 2.5: Average Neat Toluene Degradation in the Presence of JF2 and in the Absence of Soil (Objective I) after 18 Days Under Nitrate-Reducing Conditions.

Analysis of the degradation of neat toluene, in the presence of soil and under conditions favoring *in situ* biosurfactant production (objective K)

In unamended bottles containing live soil, 0.9 mmol/L (11%)of toluene was degraded (Table 2.18). The amount of toluene degraded in bottles containing live soil amended with JF2 was 0.7 mmol/L (9%). Toluene degradation increased in the presence of the JF2 mutant (2.2 mmol/L; 27% loss). Toluene degradation in the experimental bottles containing live soil amended with 2 CMC of pre-purified biosurfactant (conditions J and C) increased sharply to 5.2 and 5.6 mmol/L (64% and 69% hydrocarbon loss). These test bottles also demonstrated the greatest level of ammonia production (52 and 56 ppm; 3.0 and 3.1 mmol/L) of all test conditions (Table 2.18). All other bottles contained less than 43 ppm (2.4 mmol/L) ammonia after 35 days of incubation.

Results - Hexadecane

Analysis of the degradation of neat hexadecane, in the absence of soil, with varying amounts of pre-purified biosurfactant added (objectives A and E)

In bottles containing 0, ¼, and CMC levels of pre-purified biosurfactant, 226 to 275 μmol /L (26 % to 31%) of neat hexadecane were degraded after two months of incubation (Figure 2.6). An average of 333 μmol /L (38 %) of hexadecane were degraded in bottles amended with 10 CMC of pre-purified biosurfactant.

Table 2.18: Average Neat Toluene Degradation with the Addition of *Bacillus* strain JF2, Under Nitrate-Reducing Conditions (Objective K)

Condition	Average Toluene Loss (mmol/L)	Final Average Ammonium (ppm)
A. Sterile soil, JF2 mutant	1.5 ± 0.1	41.6 ± 3
B. Live soil, JF2 mutant	2.2 ± 0.1	41.6 ± 3
C. Live soil, JF2 mutant, 2 CMC biosurfactant	5.6 ± 0.3	56.3 ± 4
D. Live soil, JF 2 mutant, EtOH	1.9 ± 0.1	42.3 ± 2
E. Live soil, JF2	0.73 ± 0.4	33.1 ± 2
F. Sterile soil, JF2	1.6 ± 0.1	36.3 ± 3
G. Live soil	0.92 ± 0.1	36.1 ± 2
H. Live soil, JF2, No *T. aromatica*	1.1 ± 0.1	31.4 ± 2
I. Live soil, mutant, No *T.aromatica*	1.9 ± 0.1	33.2 ± 3
J. Live soil, 2 CMC biosurfactant	5.2 ± 0.4	51.9 ± 5
K. Sterile soil	0.12 ± 0.01	2.0 ± 0.3

Time Zero: 8.1 mmol/L toluene added
CMC = Critical Micelle Concentration
This experiment lasted for 35 days.

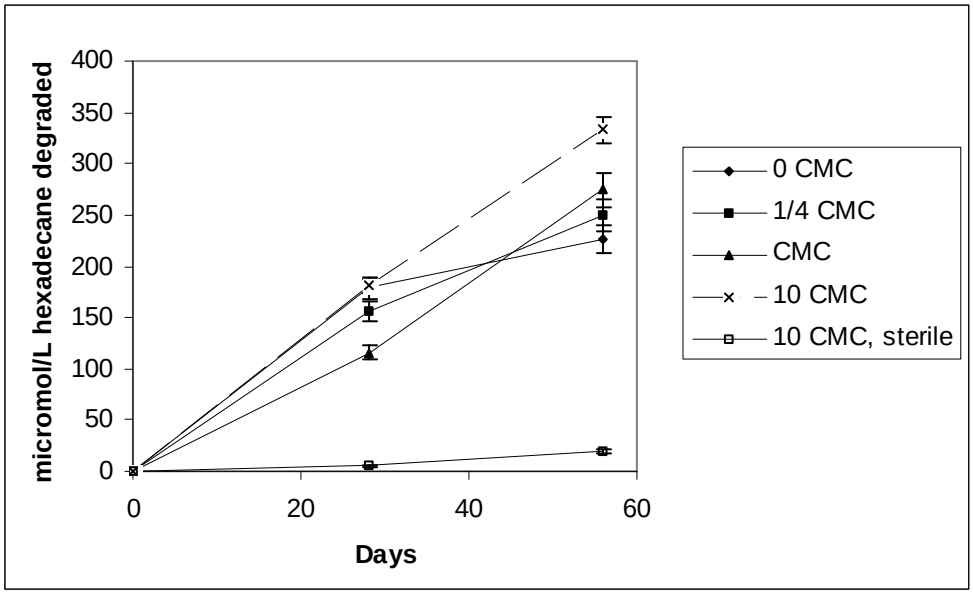

Figure 2.6: Average Neat Hexadecane Loss in the Presence of Various Levels of Pre-Purified Biosurfactant in the Absence of Soil Under Sulfate-Reducing Conditions (Objectives A and E) after 56 Days.

Bottles containing 10 CMC of biosurfactant produced an average of 18 ppm (530 μmol /L) of H_2S while those containing lower levels of pre-purified biosurfactant produced an average of approximately 10 ppm (290 μmol /L) of H_2S.

Analysis of the degradation of hexadecane, delivered in a NAPL overlayer in the absence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives B and F)

Hexadecane levels decreased by 286 – 400 μmol /L (32-46 % total loss) when biosurfactant was added at CMC concentrations or below (Figure 2.6). This difference was not significant. The presence of 10 CMC of pre-purified biosurfactant increased the amount hexadecane degraded by another 35% (550 μmol /L). This was a 62% loss of total hydrocarbon. There was no significant hexadecane loss in bottles not containing the hydrocarbon-degrading microbial consortium.

In bottles containing live culture, levels of produced sulfide increased in a similar pattern as the amount of hexadecane degraded. Average levels of H_2S at the conclusion of this experiment were: 8, 9, 17, and 21 ppm (235, 264, 500, and

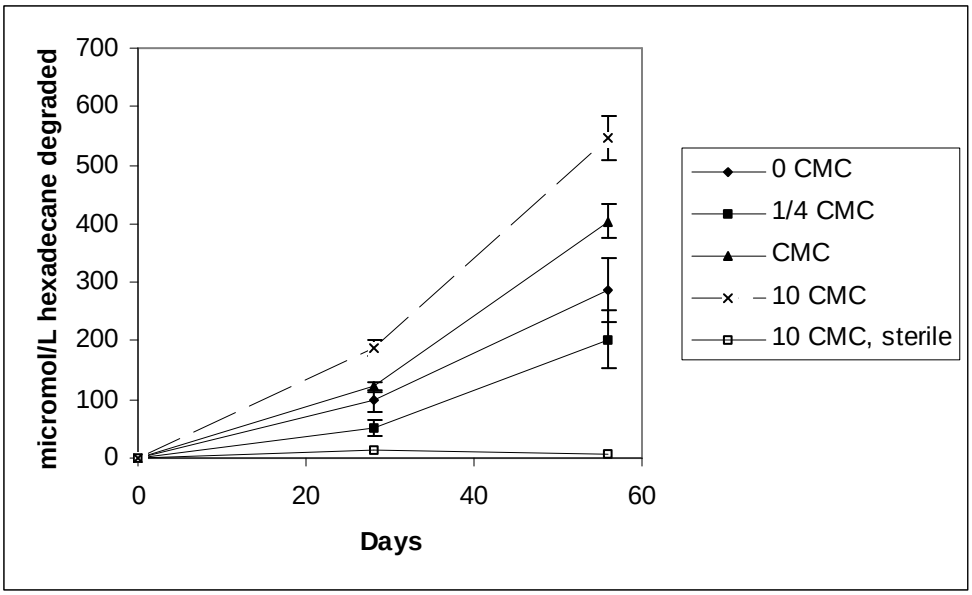

Figure 2.7: Average NAPL Hexadecane Loss in the Presence of Various Levels of Pre-Purified Biosurfactant in the Absence of Soil Under Sulfate-Reducing Conditions (Objectives B and F) after 57 Days.

612 μmol /L; ¼, 0, 1, and 10 CMC levels of pre-purified biosurfactant, respectively).

Analysis of the degradation of neat hexadecane, in the presence of soil, with varying amounts of pre-purified biosurfactant added (objectives C and G)

Under sulfate-reducing conditions, the greatest loss of hexadecane occurred in the presence of CMC levels of biosurfactant. At the conclusion of the experiment, 35 μmol /L (69 %) of the hydrocarbon had been degraded. The least hexadecane loss occurred in the presence of 10 CMC biosurfactant, with 27 μmol /L degraded (53% of the original 51 μmol /L added at the beginning of the experiment). The difference in the amount of toluene degraded was not significant, however. No hexadecane degradation was detected in bottles containing sterilized sediments.

There was a difference in levels of H_2S production in the presence of various amounts of biosurfactant. In bottles containing 0 or ¼ CMC levels of pre-purified biosurfactant, approximately 2 ppm of H_2S (58 μmol /L) was formed. This was also the case for live sediments amended with ethanol (condition L). This level increased as the amount of added biosurfactant reached CMC and 10 CMC levels, resulting in final average H_2S levels of 7 and 9 ppm (206 and 264 μmol /L), respectively.

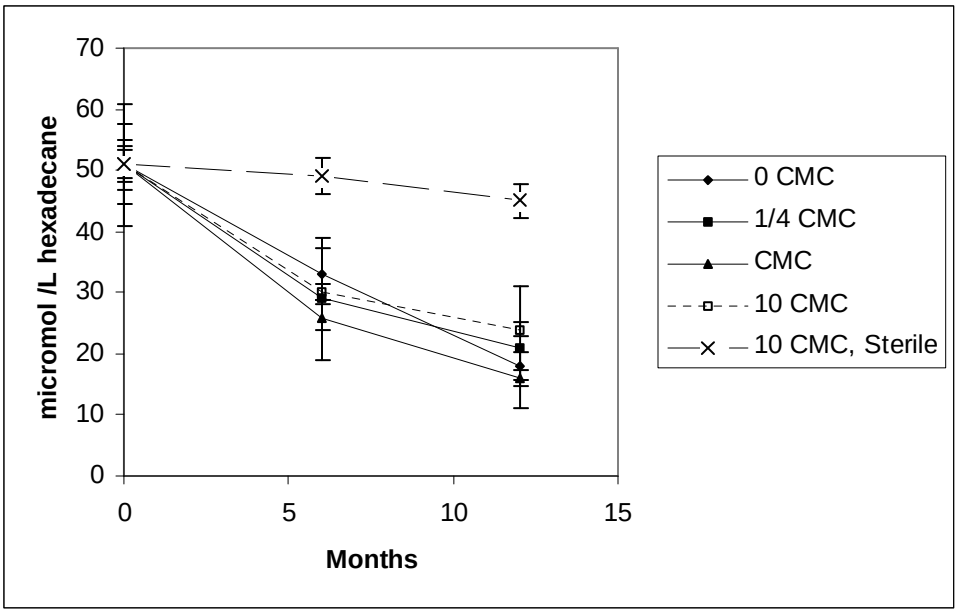

Figure 2.8: Average Neat Hexadecane in the Presence of Various Levels of Pre-Purified Biosurfactant and Soil Under Sulfate-reducing Conditions (Objectives C and G) after One Year.

In regards to the same experiment established under methanogenic conditions, substantial amounts of methane were produced in all bottles that contained live sediments. Methane production increased when any amount of biosurfactant was present, in comparison to those bottles containing no added biosurfactant (Table 2.19). Live sediments containing neither added hexadecane nor biosurfactant (condition F) produced approximately 2,000 µmol of methane (2 %), well over what was expected had hexadecane been added. The data presented in Table 2.19 is normalized against this background value.

In this experiment, the presence of the pre-purified biosurfactant had a significant effect on the amount of hexadecane that was degraded (Table 2.20). In the absence of any biosurfactant (condition A), 18 µmol (36%) of the added hexadecane were removed. However, when ¼ CMC biosurfactant was added (condition B), hexadecane loss increased by approximately 25 µmol. This trend did not continue, however, as increasing amounts of biosurfactant were added. When one or ten times the CMC amounts of biosurfactant were added (conditions C and D), hexadecane degradation decreased to 0 and 6 µmol (0 and 12%) respectively.

Because of the unexpected pattern of hexadecane degradation after one year of incubation, the experiment was repeated. Sediments collected at the same time as for the original experiment, and stored at 4 ^{o}C under N_2, were used and identical experimental conditions were established. After one year of incubation, the analysis of the replicated experiment yielded the same results (Tables 2.19 and

Table 2.19: Average Methane Production in the Presence of Various Levels of Pre-Purified Biosurfactant and Soil (Objectives C and G).

	First Experimental Set		**Second Experimental Set** [b]	
Condition [a]	CH_4 Produced [c] (µmoles)	Expected CH_4 Production [c,d] (%)	CH_4 Produced [c] (µmoles)	Expected CH_4 Production [c,d] (%)
A	6,240 ± 1,118	998	5,160 ± 1,188	826
B	11,600 ± 1,325	1,860	10,900 ± 2,864	1,740
C	16,300 ± 1,172	2,610	18,100 ± 1,962	2,900
D	13,200 ± 1,108	2,110	15,300 ± 2,376	2,450
J	8,300 ± 1,015	1,330	9,900 ± 1,174	1,590

(a) The data presented here are the average of all triplicates, and were normalized to account for the pressure accumulated within each serum bottle. Conditions E, G, H, and I utilized sterile sediments, and produced no methane (Table 9).

(b) The complete experiment was repeated in full, not just those samples shown above. The experiment was repeated approximately one year after the completion of the initial experiment.

(c) Values are corrected for condition F. Condition F utilized live soil without added hexadecane or biosurfactant. These bottles produced an average of 2,000 ± 583µmol CH_4 in the initial study and 2,000 ± 543 µmol CH_4 when the experiment was repeated.

(d) The 51 µmol of hexadecane added was expected to produce 625 µmol of methane.

Table 2.20: Average Neat Hexadecane Loss in the Presence of Various Levels of Pre-Purified Biosurfactant and Soil Under Methanogenic Conditions (Objectives C and G).

	First Experimental Set		Second Experimental Set [b]	
Condition [a]	Hexadecane Consumed [c] (µmol)	Hexadecane Consumed [d] (%)	Hexadecane Consumed [c] (µmol)	Hexadecane Consumed [d] (%)
A	20 ± 4.2	36	23 ± 2.2	46
B	40 ± 6.6	86	37 ± 1.9	72
C	0 ± 1.0	0	0 ± 1.8	0
D	6 ± 1.4	12	7.3 ± 3.1	14
J	20 ± 1.6	45	10 ± 3.3	20

(a) Conditions E and F contained no added hexadecane. Conditions G,H, and I utilized sterile sediments, and no hexadecane was degraded. For a complete description of each condition, see Table 2.14.

(b) The complete experiment was repeated in full, not just those samples shown above. The experiment was repeated approximately one year after the completion of the initial experiment.

(c) Values are mean µmol of consumed hexadecane ± standard deviation

(d) The % of hexadecane consumed is based on 51 µmol of hexadecane added to the incubations.

2.20). Both the original and repeated methanogenic portions of this experiment were published in Jennings and Tanner (2004).

Analysis of the degradation of hexadecane, delivered in a NAPL overlayer in the presence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives D and H)

Under sulfate-reducing conditions, the average amount of hexadecane degraded in the presence of CMC or below levels of pre-purified biosurfactant ranged from 285 to 300 µmol/L (32 % to 34%) after one year of incubation (Figure 2.9). However, the amount increased to 385 µmol/L (44 % hydrocarbon loss) when 10 CMC of pre-purified biosurfactant was added to the system. Hexadecane was not degraded in the absence of live sediments.

Levels of H_2S at the conclusion of the experiment were between 11 and 14 ppm (323 and 412 µmol /L) in the presence of CMC or below levels of pre-purified biosurfactant. This amount decreased by approximately 25% to 10.5 ppm (308 µmol /L) in the presence of 10 CMC biosurfactant. H_2S was not produced in the absence of live sediments.

Under methanogenic conditions, the average amount of hexadecane degraded in the presence of ¼ CMC or below of pre-purified biosurfactant ranged from 346 to 388 µmol/L (39% to 44% loss) after one year of incubation (Figure 2.10). However, the amount increased by approximately 20% to 466 µmol/L (53% hexadecane loss) when CMC concentrations of pre-purified biosurfactant

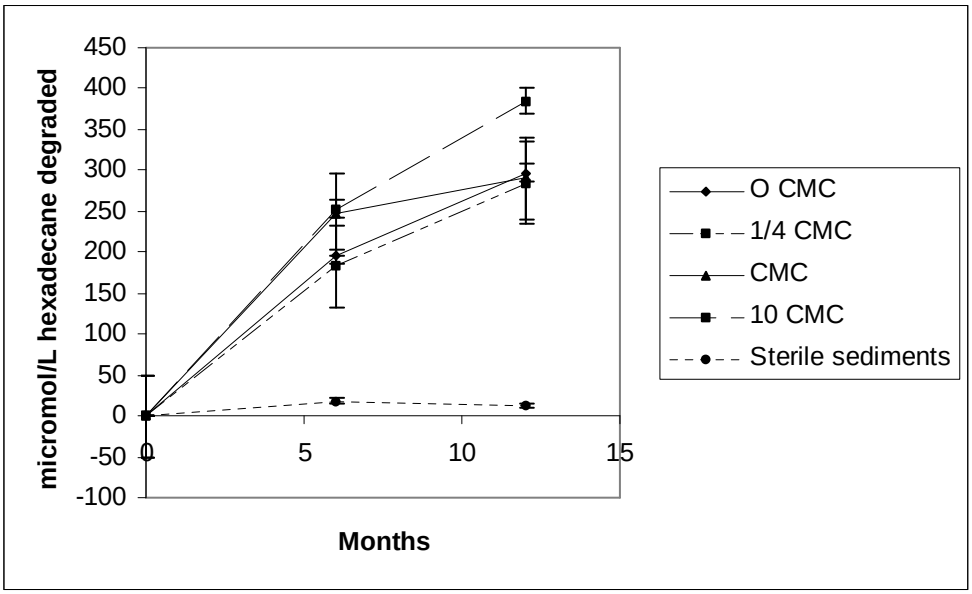

Figure 2.9: Average NAPL Hexadecane Loss in the Presence of Various Levels of Pre-Purified Biosurfactant in Soil Under Sulfate-Reducing Conditions (Objectives D and H) after One Year.

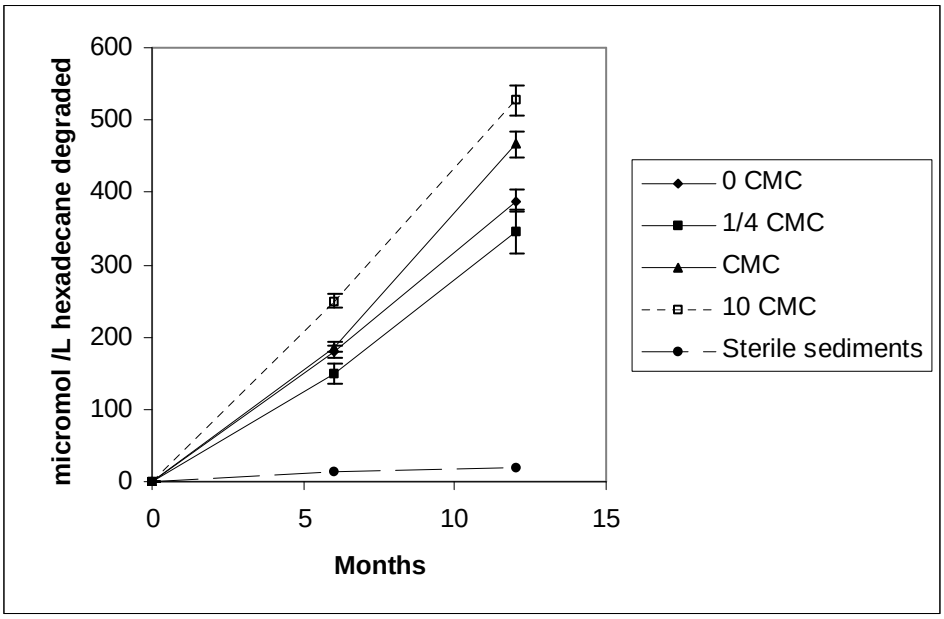

Figure 2.10: Average NAPL Hexadecane Loss in the Presence of Various Levels of Pre-Purified Biosurfactant in Soil Under Methanogenic Conditions (Objectives D and H) after One Year.

were added to the system. This increased further by another 13% to 527 µmol /L (60 % hexadecane loss) when 10 CMC of pre-purified biosurfactant was added. Hexadecane was not degraded in the absence of live sediments.

Methane levels increased from 0 to 4,300 and 4,800 µmol/L in the presence of 0 or ¼ CMC levels of biosurfactant, respectively (Table 2.21). Final methane levels increased by 11% to 5,400 µmol/L in the presence of CMC levels of pre-purified biosurfactant. An additional 18% increase (6,300 µmol/L methane) occurred when biosurfactant levels were added in 10 CMC amounts.

Analysis of the degradation of neat hexadecane, in the presence of soil and under conditions favoring *in situ* biosurfactant production (objective K)

In all bottles containing live sediments, hexadecane levels decreased from approximately 890 µmol/L to between 650 and 639 µmol/L (27 % and 28 % hexadecane loss) after five months of incubation (Figure 2.11). Hexadecane was not degraded in the presence of sterilized sediments. Ammonia levels rose to approximately 70 ppm (3.9 mmol/L) in all bottles containing live sediments with the exception of those amended with pre-purified biosurfactant (conditions C and J, Table 2.11). Ammonia levels rose to an average of 95 ppm (5.3 mmol/L) under these conditions.

Table 2.21: Average Methane Production Resulting from NAPL Hexadecane Degradation in the Presence of Various Levels of Pre-Purified Biosurfactant in Soil Under Methanogenic Conditions (Objectives D and H) after One Year.

Condition	Description	Average Amount of Methane Produced (μmol/L)
A	live soil, ¼ CMC	4,800 ± 97
B	live soil, CMC	5,400 ± 104
C	live soil, 10 CMC	6,300 ± 118
D	live soil, no biosurfactant	4,300 ± 82

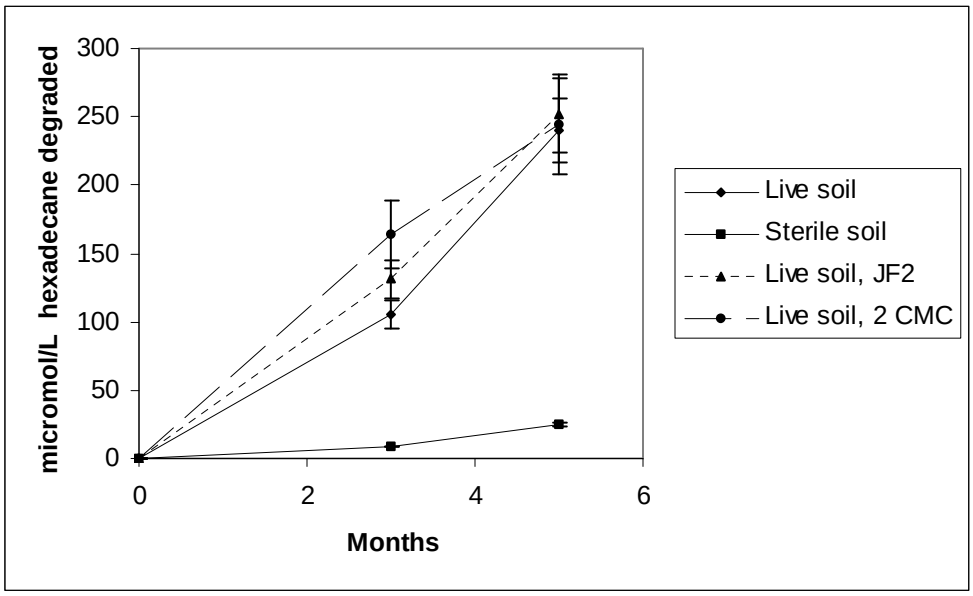

Figure 2.11 Hexadecane Loss in the Presence of Soil and *in situ* Biosurfactant Production under Nitrate-Reducing Conditions (objective K) after Five Months.

Results - Naphthalene

Analysis of the degradation of naphthalene, delivered in a NAPL overlayer, in the absence of soil with varying amounts of pre-purified biosurfactant (objectives B and F)

After 74 days of incubation, 55% of the added naphthalene was degraded without any biosurfactant amendment (16.5 µmol/L) (Figure 2.12). This is similar to the degradation rate in the presence of CMC levels of added biosurfactant (16.2 µmol/L; 54 % loss) and to the average amount degraded in control bottles containing ethanol (16.0 µmol/L; 53 %). This is not significantly different from the average amount of naphthalene degraded in the presence of ¼ CMC of added biosurfactant (13.7 µmol/L, 46 % naphthalene loss) or 10 CMC of added biosurfactant (12.8 µmol/L; 43 % loss). Naphthalene degradation was not detected in sterile bottles lacking a degrading microbial culture.

Between 45 and 84 ppm (2. 4 and 4.7 mmol/L) of ammonia was detected at the conclusion of the experiment in all bottles containing live cultures. Ammonia levels followed the same trend as the amount of hydrocarbon degraded: the two conditions demonstrating the greatest hydrocarbon loss (0 and CMC levels of biosurfactant) had the greatest ammonia production while the condition demonstrating the least hydrocarbon loss (10 CMC biosurfactant) demonstrated the least ammonia production.

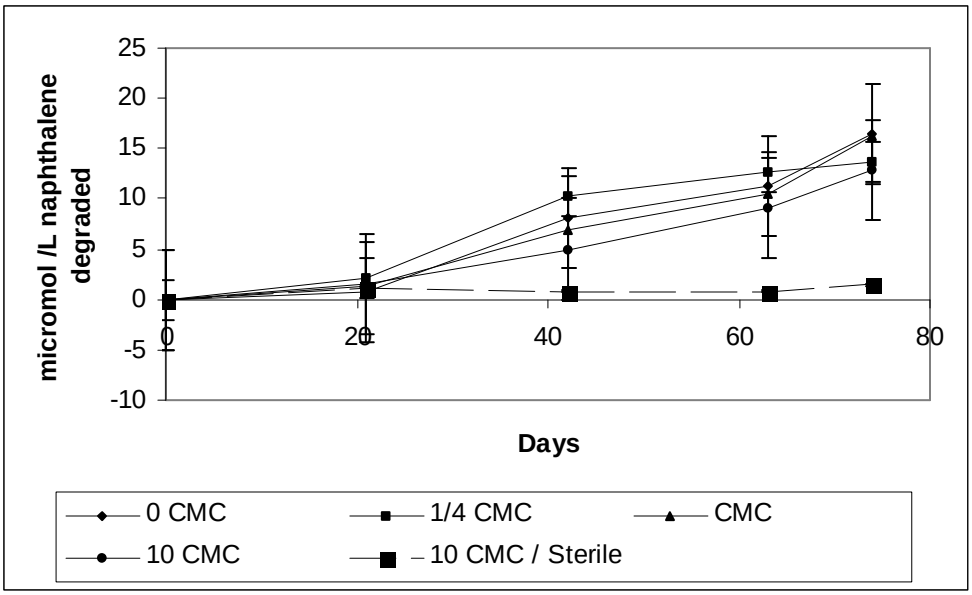

Figure 2.12: Average NAPL Naphthalene Loss in the Presence of Various Levels of Pre-Purified Biosurfactant in the Absence of Soil Under Nitrate-Reducing Conditions (Objectives B and F) after 74 Days.

Analysis of the degradation of naphthalene, delivered in a NAPL overlayer, in the presence of soil with varying amounts of pre-purified biosurfactant (objectives D and H)

Naphthalene degradation ranged from 8.9 to 9.7 µmol/L (30 % to 32% naphthalene loss) in bottles containing 0 to ¼ CMC biosurfactant (Figure 2.13). This degree of hydrocarbon degradation was similar to when CMC levels of biosurfactant were present (10.4 µmol/L ; 35 % loss) or when 10 CMC of biosurfactant was added (9.0 µmol/L; 30% naphthalene loss). Between 75 and 95 ppm (4.2 and 5.3 mmol/L) of ammonia were detected in all bottles containing live cultures. Ammonia levels rose steadily as biosurfactant levels increased.

DISCUSSION

Discussion - Toluene

Analysis of the degradation of neat toluene, in the absence of soil, with varying amounts of pre-purified biosurfactant added (objectives A and E)

The purpose of this objective was to study neat toluene degradation in the presence of various amounts of pre-purified biosurfactant (Table 2.2). The amount of net toluene degraded ranged from 98 - 99% of the toluene initially added to the experiment. The average rate of toluene degradation was 560 $\mu mol \cdot day^{-1} \cdot L^{-1}$. Thus, increasing amounts of pre-purified biosurfactant did not increase toluene degradation (Figure 2.1).

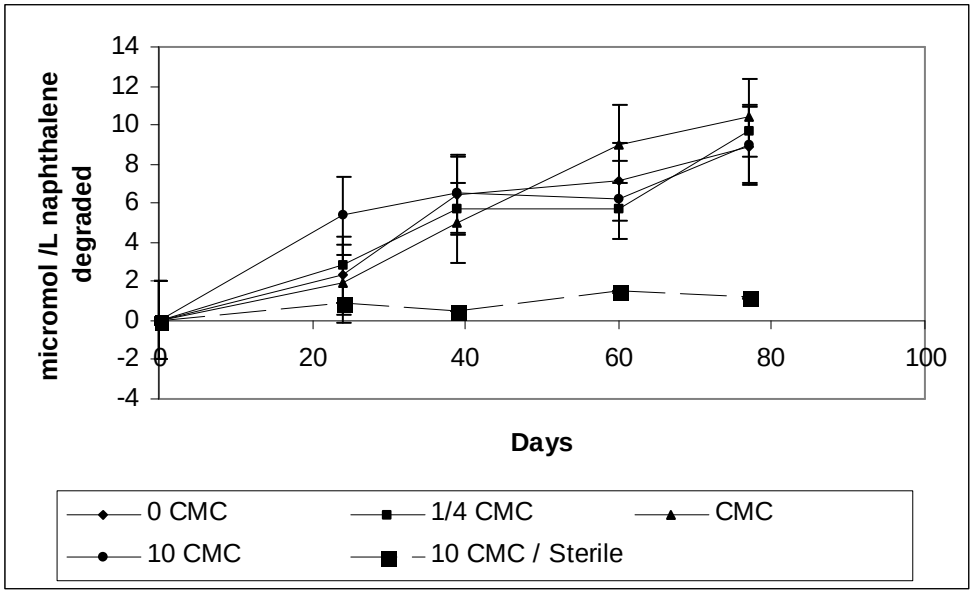

Figure 2.13: Average NAPL Naphthalene Loss in the Presence of Various Levels of Pre-Purified Biosurfactant and Soil Under Nitrate-Reducing Conditions (Objectives D and H) after 77 Days.

Control experiments were performed to determine if the efficiency of *T. aromatica* might have overshadowed any effect of the pre-purified biosurfactant. Much less toluene was degraded in these control experiments than in the initial experiments. The final amount of toluene degraded as well as the rate of hydrocarbon degradation were the same between the four different levels of pre-purified biosurfactant. This trend was identical to what was seen in the full experiment where the degradation rates ranged from 98 – 99% in the presence of the various levels of biosurfactant. The conclusion was that *T. aromatica* was highly effective at degrading toluene under the experimental conditions. However, the various levels of pre-purified biosurfactant were not altering toluene degradation.

Analysis of the degradation of toluene, delivered in a NAPL overlayer in the absence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives B, F, and J)

The purpose of Objectives B, F, and J was to study the effects of various levels of pre-purified biosurfactant or *in situ* biosurfactant production on the degradation of toluene while in a NAPL solution (Table 2.4). As when toluene was added in neat form (condition A, Figure 2.1), there was no difference in overall degradation in the presence of various levels of biosurfactant (Figure 2.2). However, the results clearly demonstrate the ability for *T.aromatica* to degrade toluene when presented to a system within a NAPL overlayer. The average

degradation rate of NAPL toluene was 370 $\mu mol \cdot day^{-1} \cdot L^{-1}$. Additionally, toluene degradation was not increased in conditions favoring *in situ* biosurfactant production (Objective J, Table 2.1).

Analysis of the degradation of neat toluene, in the presence of soil, with varying amounts of pre-purified biosurfactant added (objectives C and G)

The purpose of these objectives was to study neat hydrocarbon degradation in the presence of a soil slurry and various amounts of purified biosurfactant (Table 5). Methanogenic and sulfate-reducing conditions were chosen because the Ft. Lupton sediments already had an established population of microbes capable of degrading a wide variety of hydrocarbons a number of anoxic conditions (Borole et al., 1996; Elshahed et al., 2001; Rios-Hernandez et al., 2003). Nitrate-reducing conditions were included in order to compare toluene degradation in the presence of various amounts of pre-purified biosurfactant to when *in situ* biosurfactant production was occurring (objective K) (Table 1).

Under nitrate-reducing conditions, bottles containing no biosurfactant consistently resulted in more toluene loss than all other bottles. Bottles containing 10 CMC biosurfactant consistently resulted in less toluene loss than all other bottles. After 28 days of incubation, bottles containing no pre-purified biosurfactant had an average toluene degradation rate of 200 $\mu mol \cdot day^{-1} \cdot L^{-1}$, while bottles containing 10 CMC biosurfactant had an average degradation rate of 110 $\mu mol \cdot day^{-1} \cdot L^{-1}$. After approximately 42 days of incubation, however, this difference became insignificant (Figure 2.3). At the conclusion of the experiment

(73 days), the average hydrocarbon degradation rate for all conditions was 110 $\mu mol \cdot day^{-1} \cdot L^{-1}$.

The presence of soil increased the amount of time necessary for a given level of neat toluene degradation to be achieved under nitrate reducing conditions. Approximately 8 mmol/L of neat toluene was degraded in 14 days in the absence of sediments (Figure 2.1), with an average degradation rate of 560 $\mu mol \cdot day^{-1} \cdot L^{-1}$. Similar levels of neat toluene were degraded after 73 days in the presence of sediments (Figure 2.3), resulting in an average hydrocarbon degradation rate of 110 $\mu mol \cdot day^{-1} \cdot L^{-1}$. The presence of sediments was the only variable which was different between these two experimental sets.

Under methanogenic conditions, neat toluene degradation was not stimulated by ¼ CMC levels of biosurfactant (Table 2.16). The average rate of hydrocarbon degradation when zero or ¼ CMC biosurfactant was present was 2.7 $\mu mol \cdot day^{-1} \cdot L^{-1}$. However, toluene degradation declined by 30 – 40 % in the presence of CMC and 10 CMC levels of biosurfactant. In bottles containing 10CMC levels of biosurfactant, toluene loss was similar to the apparent abiotic loss observed in sterilized soils, with an average degradation rate of 0.84 $\mu mol \cdot day^{-1} \cdot L^{-1}$ (Table 2.16). In contrast, methane production increased as the level of added biosurfactant increased. This suggests that the presence of increasing biosurfactant concentrations stimulated the degradation of indigenous contaminants within the experimental sediments. However, target hydrocarbon degradation was not stimulated.

Under sulfate-reducing conditions, neat toluene degradation was significantly inhibited by ¼ CMC levels of biosurfactant (Table 2.17). The average rate of toluene degradation decreased from 0.24 $\mu mol \cdot day^{-1} \cdot L^{-1}$ to 0.048 $\mu mol \cdot day^{-1} \cdot L^{-1}$ (zero and ¼ CMC biosurfactant, respectively). Above this biosurfactant concentration, however, the degradation of toluene increased as biosurfactant concentrations increased. Approximately 85 µmol/L were degraded in the presence of CMC levels of biosurfactant, with an average hydrocarbon degradation rate of 0.47 $\mu mol \cdot day^{-1} \cdot L^{-1}$. The amount of toluene degraded in the presence of 10 CMC biosurfactant was almost ten times that degraded in the presence of no biosurfactant, and the average toluene degradation rate was 2.5 $\mu mol \cdot day^{-1} \cdot L^{-1}$.

Final sulfide levels appeared to stay relatively constant among conditions containing live sediments and various levels of pre-purified biosurfactant. This would seem to suggest that although toluene degradation was enhanced with the addition of CMC and above levels of biosurfactant, degradation of the overall hydrocarbon content of the system (indigenous contaminants as well as added target hydrocarbon) remained fairly constant across the various amounts of added pre-purified biosurfactant. However, methane production was detected in bottles containing live sediments. Methane levels followed the trend observed under true methanogenic conditions (Table 2.16), and increased as biosurfactant concentrations increased. Final methane concentrations ranged from 112 to 463 µmol/L (zero and 10 CMC biosurfactant, respectively). Therefore, it is possible that the addition of increasing concentrations of pre-purified biosurfactant

stimulated the degradation of the total hydrocarbon content of the system, but did so via methanogenic pathways.

Analysis of the degradation of toluene, delivered in a NAPL overlayer in the presence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives D, H, and L)

The purpose of Objectives D and H was to study the degradation of a target hydrocarbon in a NAPL solution in the presence of soil without the addition of biosurfactant or when various amounts of purified biosurfactant were added Between 89 - 90 % of the added toluene was degraded under test conditions (Figure 2.4). As with the corresponding study that did not contain sediment (Figure 2.2), the presence of various levels of pre-purified biosurfactant did not appear to increase the rate or overall amount of toluene degraded. The average rate of hydrocarbon degradation was 110 $\mu mol \cdot day^{-1} \cdot L^{-1}$. Furthermore, *in situ* biosurfactant production by JF2 did not appear to increase toluene degradation. This was similar to the results of the non-sediment study.

The presence of sediments delayed NAPL toluene degradation in comparison to the corresponding study that did not contain sediment (Figures 2.2, 2.4, and 2.14). This observation was similar to that made in regards to experiments involving neat toluene degradation (Figures 2.1, 2.3, and 2.14). As with the neat toluene studies, this delay of NAPL toluene degradation appeared to be indifferent to the source of the biosurfactant (i.e. the addition of pre-purified

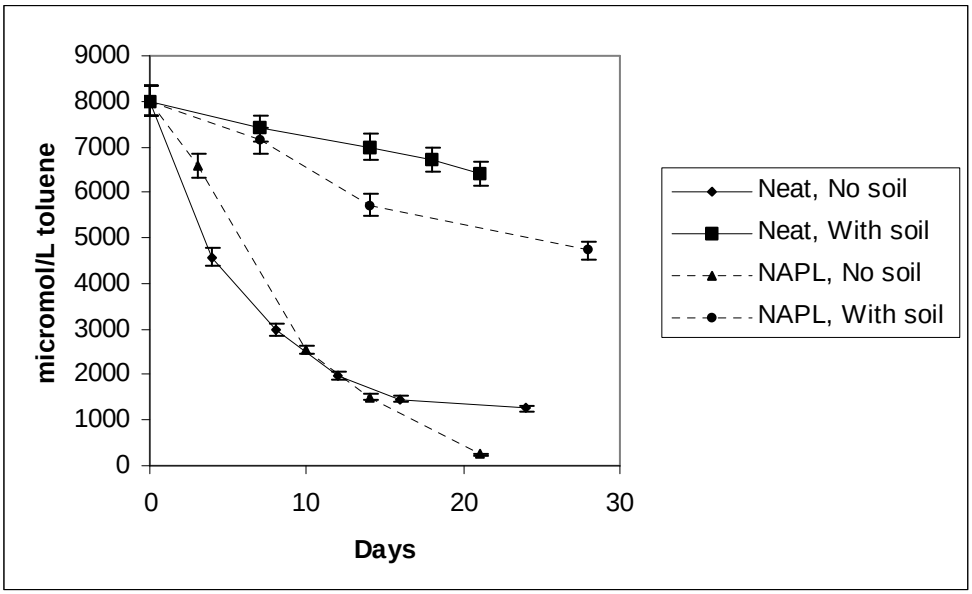

Figure 2.14. A Comparison of Neat and NAPL Toluene Degradation, With and Without Sediments, under Conditions Favoring the *in situ* Biosurfactant Production by JF2.

biosurfactant versus *in situ* production by JF2. Neat toluene was degraded at an average rate of 280 $\mu mol \cdot day^{-1} \cdot L^{-1}$ in the absence of sediments. In the presence of sediments, however, the hydrocarbon degradation rate averaged 76 $\mu mol \cdot day^{-1} \cdot L^{-1}$. NAPL toluene was degraded at an average rate of 370 $\mu mol \cdot day^{-1} \cdot L^{-1}$ in the absence of sediments. In the presence of sediments, however, NAPL hydrocarbon degradation rate averaged 120 $\mu mol \cdot day^{-1} \cdot L^{-1}$.

Analysis of the degradation of neat toluene, in the absence of soil, under conditions favoring *in situ* biosurfactant production (objective I)

The purpose of this objective was to observe changes to Objectives A and E when biosurfactant was added to the system via *in situ* production by JF2. Final toluene levels were similar between the condition containing JF2 and the condition containing the non-biosurfactant producing JF2 mutant (both with *T. aromatica*) (Figure 2.5). However, a noticeable difference was observed between the amount of toluene degraded in the presence of JF2 and the JF2 mutant during the first days of the experiment. In the presence of JF2, toluene was degraded at an average rate of 850 $\mu mol \cdot day^{-1} \cdot L^{-1}$ during the first four days of incubation. In this same time period, the average rate of hydrocarbon degradation in the presence of the non-biosurfactant producing JF2 mutant was 190 $\mu mol \cdot day^{-1} \cdot L^{-1}$. Between days four and eight of the experiment, the average rates of toluene degradation were 401 and 352 $\mu mol \cdot day^{-1} \cdot L^{-1}$ (JF2 and JF2 mutant, respectively). Between days eight and 16 of the experiment, average hydrocarbon degradation rates were 190 and 500 $\mu mol \cdot day^{-1} \cdot L^{-1}$ (JF2 and JF2 mutant, respectively). Thus,

the degradation rate of neat toluene in the presence of JF2 slowed over the duration of the experiment while the degradation rate in the presence of the JF2 mutant increased (Figure 2.5). This allowed for the net loss of toluene to be similar between the two conditions at the conclusion of the experiment.

Although biosurfactant production was not measured during this investigation, experimental conditions were designed to favor production by JF2 (for example, the use of Medium E and nitrate-reducing conditions). Assuming that biosurfactant production was occurring, this would be the only variable different between the JF2 and JF2 mutant conditions. Therefore, biosurfactant production by JF2 could be associated with the increase in toluene degradation over that which occurred in the presence of the JF2 mutant. However, this difference decreased over time until final degradation levels were similar. This suggests that JF2 may have had a difficult time maintaining biosurfactant production under these experimental conditions. These difficulties could possibly be due to competition with *T. aromatica* for nutrients within Medium E or due to biosurfactant degradation.

The final amount of toluene degraded after two weeks in the presence of JF2 was much less than that degraded in the same time but in the presence of pre-purified biosurfactant (Figures 2.1 and 2.5). At the conclusion of the pre-purified biosurfactant experiments, between 83-137 μmol/L of toluene remained. However, approximately 1,500 μmol/L toluene remained in the presence of JF2. This is over ten times the amount of toluene remaining when pre-purified biosurfactant was added to the system.

This experiment was repeated with similar results. Microscopic observations made during this repeated attempt found that the number of *T.aromatica* cells increased throughout the duration of the experiment in all bottles inoculated with the organism. The number of both JF2 and the JF2 mutant vegetative cells declined after approximately the first week. By the conclusion of the experiment, *T. aromatica* was out-competing JF2 and the JF2 mutant by numbers. This observation gives merit to the suggestion that JF2 had a difficult time maintaining biosurfactant production under these experimental conditions due to competition with *T. aromatica*. The end result was that final toluene levels were equal across conditions containing *T.aromatica*, regardless of whether JF2 or the JF2 mutant were present after 16 days (Figure 2.5).

Analysis of the degradation of neat toluene, in the presence of soil and under conditions favoring *in situ* biosurfactant production (objective K)

The purpose of this objective was to observe changes to Objectives C and G when biosurfactant was added to the system via *in situ* production by JF2. Inoculation with a culture of JF2 to stimulate *in situ* biosurfactant production did not enhance toluene degradation (Table 2.18). The presence of JF2 (conditions E and H) appeared to result in a decline in toluene degradation in comparison to bottles amended with the JF2 mutant (conditions B and I). The rate of toluene degradation in the presence of JF2 averaged 26 $\mu mol \cdot day^{-1} \cdot L^{-1}$. In the presence of the JF2 mutant, the average hydrocarbon degradation rate was 58 $\mu mol \cdot day^{-1} \cdot L^{-1}$. This was regardless of the presence of *T. aromatica* (conditions H and I).

Therefore, the only variable different was the presence of JF2 versus the JF2 mutant, and toluene degradation was inhibited by the presence of the biosurfactant producer. This portion of the experiment was repeated three times and this result was constantly obtained.

The presence of 2 CMC biosurfactant stimulated both toluene degradation as well as total ammonia production (Table 2.18). This is different from what was observed when 10 CMC of pre-purified biosurfactant was added under similar conditions (Figure 2.3). The only variable changed was the amount of biosurfactant over the CMC value that was added (2 CMC versus 10 CMC). Therefore, this portion of the experiment was repeated three times, adding 0, 2 and 10 CMC of biosurfactant to bottles containing media, soil, and *T. aromatica*. Bottles containing O CMC and 10 CMC of biosurfactant demonstrated similar toluene degradation levels. However, almost twice this amount was degraded in bottles containing only 2 CMC of biosurfactant. This suggests that the phrase "above CMC levels of biosurfactant" may not be specific enough, under some circumstances, when examining the effect of various levels of biosurfactant on the degradation of a particular hydrocarbon. The degree of how much the biosurfactant supersedes the CMC concentration may also be important.

Conclusions - Toluene

The degradation of toluene was slowed when the hydrocarbon was delivered as a component of a NAPL overlayer (Figures 2.1 through 2.5; Table 2.18). Significant quantities of toluene were degraded in both delivery conditions,

neat and NAPL, regardless of other experimental variables (sediment/no sediment, biosurfactant addition, etc.). This is likely due to the fact that toluene is one of the most easily degradable hydrocarbons, as a result of its relatively high solubility, and can be degraded under a wide variety of environmental conditions (Churchill et al., 1995; Lovely and Londergan, 1990; Meckenstock, 1999; Rouse et al., 1994; Strong-Gunderson and Palumbo, 1995; Thauer et al., 1977, Wilson et al., 1995). However, as a component of a NAPL, toluene is less biodegradable than if present in neat form. This is because NAPL toluene is less soluble than neat. This coincides with reports presented in Bedient et al. (1999).

Overall, the presence of various levels of pre-purified JF2 biosurfactant did not significantly impact the amount of toluene degraded. This was regardless of the presence or absence of sediments or if the hydrocarbon was delivered to the experimental system in neat or NAPL form. These results agreed with Churchill et al. (1995) but contradicts the results of Jain et al. (1992). There were some exceptions. For example, under methanogenic conditions, CMC and above levels of biosurfactant inhibited neat degradation. Under sulfate-reducing conditions, ¼ CMC levels of biosurfactant inhibited toluene degradation. These results disagree with many of the published studies on this topic (Churchill et al., 1995; Strong-Gunderson and Palumbo, 1995). However, at the same time, these results support the findings of others (Rouse et al., 1994).

The presence of JF2, for the purpose of *in situ* production of biosurfactant, did not affect NAPL toluene degradation (Figure 2.2 and 2.4). The degradation of neat toluene, however, was significantly less over a given amount of time in the

presence of JF2 than in the presence of pre-purified biosurfactant (Figure 2.1, 2.3, 2.5; Table 2.18).

The presence of a soil (sediment) consistently slowed down the degradation of toluene. This was regardless of if the hydrocarbon was presented to the system in neat or NAPL form (Figures 2.1- 2.4), or if JF2 was added to the system as a means of *in situ* biosurfactant production (Figure 2.5, Table 2.18). In the absence of sediment, toluene experiments were concluded in two to three weeks. In the presence of sediments, equivalent toluene degradation took as long as ten weeks. It is suspected that the sediments provided binding sites to which toluene can sorb, making the hydrocarbon less bioavailable (Bedient et al., 1999).

Therefore, it can be concluded that in this experimental system, toluene degradation was not enhanced by the presence of pre-purified *Bacillus* species JF2 biosurfactant. Additionally, *in situ* production of the JF2 biosurfactant did not necessarily enhance toluene degradation. Under some circumstances, such as if toluene was delivered in neat form, *in situ* biosurfactant production actually decreased the degradation of the hydrocarbon. Thus, the two main hypotheses of this large, multi-component experiment are determined to be false in regards to toluene.

Discussion - Hexadecane

Analysis of the degradation of neat hexadecane, in the absence of soil, with varying amounts of pre-purified biosurfactant added (objectives A and E)

The purpose of this study was to observe changes in neat hexadecane degradation in the presence of varying levels of pre-purified biosurfactant. There appeared to be a positive relationship between the amount of hexadecane degraded and the amount of pre-purified biosurfactant in a system (Figure 2.6). The presence of ¼ CMC biosurfactant increased hydrocarbon degradation by approximately 10% over no biosurfactant addition. Approximately 225 μmol/L of hexadecane were degraded in the absence of biosurfactant at an average rate of 4.1 $\mu mol \cdot day^{-1} \cdot L^{-1}$. In the presence of ¼ CMC, 250 μmol/L of hydrocarbon was degraded at an average rate of 4.4 $\mu mol \cdot day^{-1} \cdot L^{-1}$ (Figure 2.6). The presence of CMC levels of biosurfactant increased hexadecane degradation by another 10% over that occurring in the presence of ¼ CMC biosurfactant to a final loss of 275 μmol/L at an average rate of 4.9 $\mu mol \cdot day^{-1} \cdot L^{-1}$. The presence of 10 CMC of biosurfactant increased the amount of hexadecane degradation by at least 21% over all other the other biosurfactant concentrations to a net loss of 333 μmol/L at an average rate of 5.9 $\mu mol \cdot day^{-1} \cdot L^{-1}$ (Figure 2.6).

The results of this study also clearly demonstrated the ability for the microbial consortium to successfully degrade hexadecane under sulfate-reducing conditions. Neat hexadecane analysis required the destruction of the sample. Therefore, the results of the investigation were not known until all microcosms were destroyed, and no biology was recovered. However, a stock culture of the consortium added to the experimental bottles was maintained at all times.

Analysis of the degradation of hexadecane, delivered in a NAPL overlayer in the absence of soil, with varying amounts of pre-purified biosurfactant added or under conditions favoring *in situ* biosurfactant production (objectives B and F)

The purpose of this experiment was to observe how various levels of pre-purified JF2 biosurfactant would change the amount of hexadecane degraded when the hydrocarbon was delivered in a NAPL overlayer. Unlike the results for neat hexadecane (Figure 2.6), NAPL hexadecane degradation was not stimulated until CMC or above levels of biosurfactant was present (Figure 2.7). When no biosurfactant was added and when ¼ CMC biosurfactant were present, 200 -286 µmol/L of hexadecane were degraded at an average rate of 4.35 $\mu mol \cdot day^{-1} \cdot L^{-1}$. In contrast, CMC levels of biosurfactant stimulated hexadecane degradation by 41% over levels without biosurfactant addition, increasing the amount of hydrocarbon degraded from 286 to 404 µmol/L at an average rate of 7.2 $\mu mol \cdot day^{-1} \cdot L^{-1}$. The presence of 10 CMC of biosurfactant further stimulated hexadecane degradation another 35% up to 547 µmol/L, at an average rate of 9.7 $\mu mol \cdot day^{-1} \cdot L^{-1}$.

Furthermore, more NAPL hexadecane was degraded than neat when in the presence of almost every tested level of biosurfactant after the same amount of incubation time. The only exception was when ¼ CMC biosurfactant was added. Hexadecane degradation increased by 47 and 64% (CMC and 10 CMC biosurfactant), when added in NAPL form over neat. This is in comparison to the 26% increase in NAPL hexadecane degradation over neat hydrocarbon in the absence of any added biosurfactant. In the presence of ¼ CMC of pre purified

biosurfactant, however, 19% less hexadecane was degraded when presented in NAPL form rather than neat form.

Analysis of the degradation of neat hexadecane, in the presence of soil, with varying amounts of pre-purified biosurfactant added (objectives C and G)

In regards to the sulfate-reducing portion of this experiment, the average amount of hexadecane degraded in the presence of ¼ CMC levels of pre-purified biosurfactant was higher than that in the presence of CMC biosurfactant but lower than 10 CMC biosurfactant. This difference, however, was not significant. The conclusion was that there is no clear relationship between the level of added biosurfactant and the amount of neat hexadecane degraded in the presence of sediments under these conditions. The average rate of hexadecane degradation was 2.6 $\mu mol \cdot months^{-1} \cdot L^{-}1$. The amount of H_2S produced was positively related to the amount of pre-purified biosurfactant present. Methane was detected in many of the bottles, but values were not related to the concentration of biosurfactant present. These results are dissimilar to the corresponding study involving neat toluene degradation in the presence of soil under sulfate-reducing conditions.

In regards to the methanogenic portion of this experiment, substantial amounts of methane were produced in all bottles that contained live sediments. Methane production increased when any amount of biosurfactant was present, in comparison to those bottles containing no added biosurfactant (Table 2.19).

According to the equation below, 12.25 moles of methane should be produced for every one mole of hexadecane oxidized, if complete degradation to carbon dioxide (in the form of bicarbonate) occurred.

$$C_{16}H_{34} + 11.25\ H_2O \rightarrow 12.25\ CH_4 + 3.75\ HCO_3^- + 3.75\ H^+$$

Therefore, it would be expected that 625 µmol of methane could be produced from the hexadecane added in each incubation containing both hexadecane (51 µmol) and live sediments. However, all produced much greater quantities than this (Table 2.19). This excess methane production is likely attributable to the degradation of residual hydrocarbon contamination within the sediments. Live sediments containing neither added hexadecane nor biosurfactant (condition F) produced approximately 2,000 µmol of methane (2 %), well over what was expected had hexadecane been added. The data presented in Table 2.19 are normalized against this background value.

The addition of ethanol alone to bottles containing live sediment stimulated methanogenesis (condition J, Table 2.19). However, a comparison of those results withmethane production in bottles treated with the biosurfactant (conditions B, C and D, Table 2.19) suggests that the majority of methane production was a result of the added biosurfactant and not the accompanying ethanol.

The results in Table 2.20 show that over 35% of the added hexadecane added to live sediment was microbially degraded, and that this was increased to more than 70% when some biosurfactant was added (conditions A and B, Table 2.20). The average rate of hexadecane degradation in the absence of added

biosurfactant was 1.7 μmol·month^{-1}·L^{-}1. In the presence of ¼ CMC biosurfactant, the average rate of hydrocarbon degradation increased to 3.3 μmol·month^{-1}·L^{-1}. However, the results clearly show that most of the methane produced from the live sediments originated from the biodegradation of hydrocarbons already present in this sediment (Table 2.19). Bottles containing sterilized sediment performed as expected, showing neither hexadecane degradation nor methane production.

The biodegradation of the added hexadecane, however, decreased when the amount of lipopeptide biosurfactant added to bottles was increased from one fourth of the CMC to one or ten times the CMC (Table 2.20). The rate of hexadecane degradation remained below 0.5 μmol·month^{-1}·L^{-1} when CMC or 10 CMC biosurfactant were present. A similar effect was observed in a study using a *Pseudomonas* rhamnolipid biosurfactant (Grimberg et al., 1996). The addition of rhamnolipid below CMC increased the biodegradation of hexadecane in a sand core, but biodegradation essentially ceased when the rhamnolipid was added above the CMC.

Analysis of the degradation of hexadecane, delivered in a NAPL overlayer in the presence of soil, with varying amounts of pre-purified biosurfactant added (objectives D and H)

Objectives D and H investigated the effect of various amounts of pre-purified biosurfactant on NAPL hexadecane degradation in the presence of sediments. Under sulfate-reducing conditions, degradation was not enhanced

until 10 CMC levels of biosurfactant were added to the system (Figure 2.9). Between 285 and 297 µmol/L of hexadecane were degraded when below this was added, at an average hydrocarbon degradation rate of 24 $\mu mol \cdot month^{-1} \cdot L^{-1}$. However, 385 µmol/L was degraded at an average degradation rate of 32 $\mu mol \cdot month^{-1} \cdot L^{-1}$ when 10 CMC of biosurfactant was added. This is at least a 30% increase in hydrocarbon loss.

The delivery of hexadecane in NAPL form appeared to stimulate the degradation of hexadecane to levels higher than if the hydrocarbon was delivered in neat form (Figures 2.8 and 2.9). This trend was also observed in the corresponding hexadecane experiments lacking sediments (Objectives A, B, E, and F). The total amount of hexadecane degraded in the presence of sediments after 12 months when added in NAPL form (sulfate-reducing conditions) is almost ten-fold the amount degraded in the same time period when delivered in neat form (Figures 2.8 and 2.9). This increase is significantly higher than in those studies lacking sediments, and is similar to the results found when this experiment was performed under methanogenic conditions.

In regards to experiments conducted under methanogenic conditions, between 346 and 388 µmol/L of hexadecane was degraded in the presence of zero or ¼ CMC biosurfactant at an average rate of 30 $\mu mol \cdot month^{-1} \cdot L^{-1}$. Degradation increased by 20% in the presence of CMC of biosurfactant (466 µmol/L), and another 13% in the presence of 10 CMC biosurfactant (527 µmol/L). The rates of hydrocarbon degradation in the presence of CMC and 10 CMC biosurfactant were 38 and 44 $\mu mol \cdot month^{-1} \cdot L^{-1}$, respectively. These levels were at least nine-fold the

amount of hexadecane degraded when the hydrocarbon was delivered in neat form (Table 2.20). Methane production in the presence of NAPL hexadecane increased as the level of biosurfactant increased (Table 2.21). This is similar to methane production patterns found when neat hexadecane was degraded in the presence of sediments (Table 2.19). Both conditions demonstrated methane production more indicative of total hydrocarbon loss (including those hydrocarbons found in the sediments) rather than just from hexadecane degradation alone.

Analysis of the degradation of neat hexadecane, in the presence of soil and under conditions favoring *in situ* biosurfactant production (objective K)

The purpose of this objective was to observe changes in hexadecane degradation under conditions favorable for *in situ* biosurfactant production by JF2. The fact that JF2 was involved dictated a need for nitrate-reducing conditions. Levels of hexadecane declined from 890 to approximately 650 μmol/L (27 % loss) in all bottles containing live soils after five months of incubation at an average degradation rate of 0.035 $\mu mol \cdot month^{-1} \cdot L^{-1}$ (Figure 2.11). The conclusion was that the degradation of hexadecane was not affected by the presence of JF2. Levels of ammonia increased to approximately 70 ppm in all bottles containing active cultures except those containing added pre-purified biosurfactant as a control. Ammonia levels increase to approximately 95 ppm (36% increase) when above CMC levels of pre-purified biosurfactant was added.

This is likely due to the enhanced degradation of indigenous hydrocarbon contaminants within the sediments.

Conclusions – Hexadecane

The degradation of hexadecane appears to be clearly influenced by the way in which the hydrocarbon is delivered to an experimental system. More hexadecane was degraded when the hydrocarbon was delivered as a component of a NAPL than when the hydrocarbon was delivered in neat form. This was regardless of if the experimental system contained a sediment matrix or not (Figures 2.6 - 2.9). This is likely due to the strong, hydrophobic nature of hexadecane. Neat hexadecane tended to form a condensed bead floating at the top of the aqueous solution. This resulted in a limited surface area exposed to the degrading microorganisms. In a NAPL form, however, the hydrocarbon was dispersed throughout the thin NAPL layer that completely covered the aqueous surface. This provided more surface area for the degrading microbes to contact the hydrocarbon and thus more opportunity for the hydrocarbon to be metabolized (Bedient et al., 1999). These results were supported by the findings of others (Bai et al., 1998; Sekelsky and Shreve, 1999). These results contradict those found in Efroyman and Alexander (1991) that reported that NAPL hexadecane degradation occurred at a slower rate than neat hydrocarbon in the presence of surfactants.

In regards to the degradation of hexadecane in the presence of various amounts of pre-purified biosurfactant, NAPL hexadecane degradation was positively influenced when biosurfactant was provided at CMC concentrations or

above. This follows the results of Jain et al. (1992) and Moran et al. (2000), a study which used surfactin as its test biosurfactant. In contrast, the degradation of neat hexadecane was not affected. Because *Bacillus* strain JF2 can only grow anaerobically under nitrate-reducing conditions, there was a limitation to the type of experiments that could help determine the effect of *in situ* biosurfactant production on the degradation of hexadecane. The presence of JF2 did not increase hexadecane degradation above levels achieved in corresponding studies involving various levels of pre-purified biosurfactant. Furthermore, the presence of JF2 did not increase hexadecane degradation above levels found in unamended systems. These results were in contrast with the findings of Ramsay et al., (1998), MacDonald et al. (1981), and Whyte et al. (1999) but are in agreement with the findings of Jain et al. (1992). The overall conclusion, however, is that the degradation of hexadecane under these conditions was not altered by a change in the delivery of biosurfactant (pre-purified versus conditions favoring *in situ* production).

The presence of sediments significantly impacted the degradation of hexadecane when the hydrocarbon was delivered in neat versus NAPL form (Figures 2.8 and 2.9; Tables 2.19 and 2.20). These results agree with the findings of Herman et al. (1997). In the presence of sediments, the degradation of NAPL hexadecane was exponentially more than that of neat hexadecane under both sulfate-reducing and methanogenic conditions (Figure 2.14). This was not unexpected. Neat hexadecane will tightly sorb to the sediment matrix. This can limit the bioavailability of the hydrocarbon to degrading microorganisms, even

with the addition of surfactants. Evidence for this is seen in various studies where neat hexadecane degradation was stimulated only in the presence of CMC or above CMC levels of biosurfactant (Bedient et al., 1999, Herman et al., 1997; Hisatsuka et al., 1971; Nakahara et al., 1981).

If an organic NAPL is available, however, the hydrocarbon may preferentially traverse from the sediment particles to the NAPL overlayer (Bedient et al., 1999). This can result in an increase in available surface area for contact between the target hydrocarbon and degrading microorganisms (Sekelsky and Shreve, 1999). Additionally, it may be easier for added surfactants to solubilize the target hydrocarbon out of a NAPL layer than from a sediment particle (Bedient et al., 1999; Sekelsky and Shreve, 1999). Thus, there may be an increased potential for the stimulation of target hydrocarbon degradation from a NAPL layer than in neat form in the presence of sediments. Evidence for this was seen in studies where NAPL hexadecane degradation was significantly stimulated by similar biosurfactants as those in the above neat studies, but at much lower biosurfactant concentrations (Bai et al., 1998; Colores et al., 2000; Ito et al., 1982; Bruheim et al., 1997; Bruheim et al., 1999).

In the absence of sediments, the amount of neat and NAPL hexadecane degraded was quite similar (Figures 2.6 and 2.7). Because hexadecane was insoluble in water, the hydrocarbon would essentially form its own, one-component “NAPL” within test bottles when the hydrocarbon aggregated together at the surface. This would be the only location of the hexadecane in the absence of any sediment matrix to attract the hydrocarbon,. Therefore, it was not

surprising that neat hexadecane and NAPL hexadecane behaved in a remarkably similar manner to one another in the absence of a soil or sediment matrix.

In conclusion, the degradation of hexadecane was stimulated by the presence of pre-purified biosurfactants when the hydrocarbon was in a NAPL form and the biosurfactant was at or above CMC concentrations. In the presence of hydrocarbon-contaminated sediments, the presence of various levels of the pre-purified biosurfactant stimulated the metabolism of the indigenous contaminating compounds. This degradation was more reflective of the amount of pre-purified biosurfactant added than was the degradation of the target hydrocarbon, hexadecane. Thus, the first main hypothesis of this large, multi-component experiment was upheld under certain conditions in regards to hexadecane. The presence of biosurfactant produced *in situ* by JF2 did not enhance hexadecane degradation to levels above what was achieved by the addition of pre-purified biosurfactant.

Discussion - Naphthalene

Analysis of the degradation of naphthalene, delivered in a NAPL overlayer in the absence of soil, with varying amounts of pre-purified biosurfactant added (objectives B and F)

There was no relationship between the amount of pre-purified biosurfactant added to the system and the level of naphthalene degraded. In bottles containing no biosurfactant, approximately 16.5 µmol/L of naphthalene

was degraded in the absence of biosurfactant (Figure 2.12). In the presence of 10 CMC biosurfactant, 12.8 µmol/L of naphthalene degraded. This difference is not significant. The average rate of naphthalene degradation was 0.20µmol·days^{-1}·L^{-1}. Thus, the presence of pre-purified biosurfactant did not stimulate naphthalene degradation. Final ammonia levels reflected the level of hydrocarbon degradation.

Analysis of the degradation of naphthalene, delivered in a NAPL overlayer, in the presence of soil with varying amounts of pre-purified biosurfactant added (objectives D and H)

In comparison to degradation occurring in the absence of any pre-purified biosurfactant, naphthalene degradation increased by 1- 9% in bottles containing ¼ CMC to 10 CMC biosurfactant. The difference between conditions is not significant. The average degradation rate of NAPL naphthalene in the presence of sediments was 0.12 µmol·month^{-1}·L^{-1} , almost half of the degradation rate of naphthalene in the absence of sediments.

The overall amount of naphthalene degraded appeared to be much less in bottles containing sediments (Figure 2.13) than in those not containing sediments (Figure 2.12). In the presence of no biosurfactant, 46% less naphthalene was degraded in the presence of sediments than without (8.9 and 16.5 µmol/L, respectively). In the presence of ¼ CMC biosurfactant, 29% less was degraded (13.7 and 9.7 µmol/L, without and with sediments respectively). In the presence of CMC levels of biosurfactant, the difference is 35 % (16.2 and 10.4 µmol/L,

without and with sediments, respectively). When 10 CMC of biosurfactant was added, 30% less was degraded (12.8 and 9 µmol/L, without and with sediments, respectively).

Between 75 and 95 ppm (4.2 and 5.3 mmol/L) of ammonia were detected in all bottles containing live cultures. Ammonia levels rose steadily as biosurfactant levels increased, including 10 CMC of biosurfactant. Therefore, it is presumed that the trend in ammonia formation is the product of the degradation of both the target hydrocarbon as well as those indigenous to the sediments.

Conclusions – Naphthalene

There was no clear pattern relating levels of pre-purified biosurfactant within a system and the amount of naphthalene degraded. This was true both in the presence of sediments and not (Figures 2.12 and 2.13). This disagreed with other reports of naphthalene-enhanced remediation (Edwards et al., 1991). The similarity of the patterns (sediment and non-sediment) may be a result of the already high aqueous solubility of the hydrocarbon (relative to other hydrocarbons) which can result in an increased bioavailability to degrading microorganisms (in comparison to hexadecane, for example) (Churchill et al., 1995; Deziel et al., 1996; Garcia – Junco et al., 2003; Liu et al., 1995; Riser-Roberts, 1998; Strong-Gunderson and Palumbo, 1995).

The presence of a sediment matrix, however, clearly decreased the amount of naphthalene that was degraded. The amount of naphthalene degraded decreased by as much as 45% when sediments were present (Figures 2.12 and 2.13). This contradicts one of the findings of Strong-Gunderson and Palumbo

(1995). These results are similar, however, to the results found in this investigation when toluene (another a highly soluble hydrocarbon) was the target for degradation.

In conclusion, it was determined that the addition of various amounts of pre-purified JF2 biosurfactant did not significantly increase naphthalene degradation. Additionally, there was no consistent effect (positive or negative) of increasing pre-purified biosurfactant levels on naphthalene degradation. This was true both in the presence and absence of a sediment matrix. Therefore, the first main hypothesis of this large, multi-component experiment was not upheld in regards to naphthalene.

FINAL CONCLUSIONS:

This series of experiments clearly demonstrated the need for further research into the relationship between hydrocarbon degradation and biosurfactants. The relationship between a hydrocarbon and a biosurfactant is affected by more than just these two variables alone. Hydrocarbons interact with soil particles and any other hydrocarbons that may be present. Hydrocarbons also can be affected by the type, amount, and mode of delivery of the tested biosurfactant. These interactions, taken together, will determine if the desired result of hydrocarbon degradation is achieved in an experimental system.

This series of experiments was the most direct method to begin to answer questions regarding the environmental variables that affect hydrocarbon degradation. The first main conclusion that can be drawn from these experiments is that future experiments will need to be performed on hydrocarbons with a low aqueous solubility. Toluene and naphthalene were too water-soluble to show clear, conclusive results for many of the test variables. The most clear results were seen when hexadecane was the target hydrocarbon, following the conclusions of Diallo et al. (1994) and Pennell et al. (1993). The second main conclusion that can be drawn from these studies is that new experimental designs need to be developed. Specifically, hydrocarbon degradation needs to be first tested in the absence of sediments. This will require either purified degrading cultures or "semi-purified" degrading consortiums absent of any contaminating hydrocarbon and will eliminate the numerous variables brought by sediments. Sediments involve potential hydrocarbon binding sites, alternative nutrient

sources for the hydrocarbon degrader, and microbial populations that may act antagonistically against an added hydrocarbon degrading microorganism or biosurfactant producer (Biedent et al., 1999).

These experiments have identified potential reasons for possible conflicting results in the biosurfactant literature. The results of these experiments also raise questions regarding the act of adding either pre-purified biosurfactant or biosurfactant-producing microorganisms to a remediation system. For many environments, it may simply be more practical to enhance indigenous biosurfactant-producing microorganisms in an attempt to stimulate hydrocarbon degradation. In order to enhance the indigenous biosurfactant-producing community, however, researchers must first determine if such a community exists at a location and what conditions will stimulate the desired effect of either enhanced hydrocarbon recovery or degradation.

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CHAPTER THREE

Biosurfactant – Producing Bacteria in Hydrocarbon – Contaminated Soils

ABSTRACT

Proposed benefits to biosurfactant-producing bacteria include enhancing the bioavailability of hydrophobic nutrients and/or acting as biocides against competing bacteria or fungi. Prior research indicated that the number of bacterial biosurfactant producers in soil may be influenced by the presence of hydrocarbon contamination as well as levels of organic matter and fungi. The purpose of this study was to determine the relationship between soil organic matter levels, hydrocarbon contamination, numbers of fungi, and numbers of indigenous biosurfactant producers. Six soils were used: two hydrocarbon impacted soils, each paired with uncontaminated soil, and two pristine soils with different levels of organic matter. Gross numbers of fungi were higher in soils with higher levels of organic matter. Soils containing higher numbers of fungi contained a greater percentage of biosurfactant-producing aerobic heterotrophs, but only in the absence of a hydrocarbon. The percentage of biosurfactant producers was greater when hydrocarbon contamination was present. Additionally, the presence of the *Bacillus subtilis srf*A gene, which encodes a highly conserved region of the surfactant synthetase complex, was monitored directly in soils for the first time by PCR amplification and Southern hybridization.

INRODUCTION

Biosurfactants are microbially produced surface-active compounds. These are amphiphilic molecules with both hydrophilic and hydrophobic regions, and are capable of decreasing interfacial surface tension (Banat, 1995a; Georgiou et al., 1992; Lin, 1996; Volkering et al., 1998). Although the primary role of biosurfactants in the ecology of microorganisms is not fully understood, it is known that these secondary metabolites can enhance nutrient transport across membranes, providing an advantage over other microbes competing for these nutrients (Banat, 1995a; Banat, 1995b; Lin, 1996). Many biosurfactants also have the potential to act as biocides or fungicides. Such an activity could provide protection to the producing organism by suppressing or eliminating competitors for soil nutrients such as fungi or other bacteria (Cooper and Zajic, 1980; Haferburg et al., 1986; Katz and Demain, 1977; Zuber et al., 1993).

The genetic basis of biosurfactant production has been most thoroughly examined in two microorganisms. *RhlA* and *rhlB* are genes in the *Pseudomonas aeruginosa* rhamnolipid biosurfactant production pathway (Lang and Wullbrandt, 1999) and have been used to identify gram-negative isolates as *P. aeruginosa* (Bodour et al., 2003). *Bacillus subtilis* is the best studied system in Gram-positive bacteria (Nakano et al., 1988; Nakano and Zuber, 1993; Peypoux et al., 1999; Sullivan, 1998). *B. subtilis* produces surfactin, a lipopeptide biosurfactant. A three-subunit enzyme complex regulating the formation of the peptide region of the biosurfactant molecule is called surfactin synthetase (Peypoux et al., 1999). The surfactin synthetase complex is coded for by three of the four open reading

frames within the *srfA* operon (Cosmina et al., 1993; Vollenbroich et al., 1994). Therefore, a highly conserved region of the *srfA* gene could function as a potential marker for biosurfactant production by *Bacillu*s species related to *B. subtilis*.

Despite the interest in both the genetics controlling and functions of biosurfactants, there has been relatively little research performed investigating the distribution of biosurfactant-producing bacteria indigenous to soils. The majority of studies have centered on using biosurfactants in tertiary oil recovery or enhanced biodegradation of various hydrocarbons (Karanth et al., 1999; McInerney et al., 1990; Rouse et al., 1994; Volkering et al., 1998). In one study investigating the presence of indigenous biosurfactant-producing bacteria, various strains of bacteria were isolated from a single site contaminated with poly-aromatic hydrocarbons (PAH). These were tested for the ability to degrade a variety of hydrocarbons (Willumsen and Karlson, 1997). In 1998, Bodour and Miller-Maier used bacteria isolated from different soils to refine the drop-collapse method for biosurfactant screening initially described by Jain et al. (1991). The bacterial isolates were taken from four locations: one uncontaminated soil with low organic matter, one uncontaminated soil with high organic matter, one soil contaminated with waste oil, and one soil contaminated with cadmium and lead (Bodour and Miller-Maier, 1998). Bacterial isolates were not identified or correlated to the soil sources used for the initial isolation in these papers.

In a more recent study, Bodour et al. (2003) screened 20 soil types for biosurfactant-producing bacteria and examined the phylogenetic diversity of the resulting isolates. A wide range of soil sources was used, including undisturbed

soils, hydrocarbon impacted soils, and metal contaminated soils, ranging from sand to silty clays. Soil chemistry was not correlated with the biosurfactant-producing bacteria isolated from each soil. PCR amplification of the *rhlB* gene of *Pseudomonas aeruginosa* was performed to distinguish Gram-negative isolates from *P. aeruginosa*. A comparable molecular analysis of the Gram-positive isolates was not available. Relative levels of *rhlB* in the soil samples were not determined (Bodour et al., 2003). One study examining *Pseudomonas* rhamnolipid producers isolated from two non-contaminated, agricultural soils found that soils containing higher amounts of organic matter contained lower numbers of biosurfactant producing bacteria than low organic matter soils (Nielsen et al, 2002). *Pseudomonas* was the only genus analyzed in the investigation.

In order to directly investigate the microbial ecology of biosurfactant-producing organisms and in particular its relationship to certain features of the chemical environment (e.g. organic matter, presence / absence of hydrocarbon contamination), biosurfactant-producing bacteria were obtained from six soils. These six soils had a wide range of soil organic matter levels as well as two different types of hydrocarbon contamination. Soil fungi were enumerated. Potential biosurfactant-producing bacteria were enumerated, isolated, and identified. Biosurfactant production was confirmed through a number of methods including a drop-collapse test, an oil spreading test, and the observation of surface tension reduction. Total soil DNA as well as the DNA of each isolate was

screened for *srfA*-like genes to estimate the presence of biosurfactant producers related to *B. subtilis*.

It was hypothesized that a greater fraction of bacteria would be biosurfactant-producers in the presence of hydrocarbon contamination than would be predicted from soil organic matter levels alone. It was also hypothesized that higher soil organic matter levels would result in higher numbers of soil fungi. The number of bacterial biosurfactant producers was predicted to increase with elevated numbers of fungi.

MATERIALS AND METHODS

Source of Soil Samples

Six soils taken from three different locations were used in this study and chosen to provide a wide range of organic matter levels and soil types.

The source of two samples (Fort Lupton, contaminated and uncontaminated, or FL-C and FL-U, respectively) is a hydrocarbon - impacted site located near Ft. Lupton, Colorado. The sediments from this site are classified as sand to sandy loam and have been previously characterized in detail (Gieg et al., 1999; Sublette et al., 1997). Gas condensate leaked from an underground storage tank into the underlying aquifer during the 1970's. In the early 1980's, the contamination source was removed, but residual hydrocarbon contamination remained. Total petroleum hydrocarbon levels averaged 23 mg/L and consisted of a mix of straight and branched - chain alkanes as well as BTEX compounds

(Barker et al., 1996; Gieg et al., 1999). A chemical analysis performed at the time of sediment removal determined that the collected sediments for the current study still contained many of the hydrocarbons found in the original contaminating gas condensate (Gieg et al., 1999). Sediments for this study (FL-C) were collected from beneath the shallow water table (~ 1.4 meters) in 1999 and stored under N_2 at 4 ^{o}C. Uncontaminated sediments (FL-U) were collected from a site upgradient and stored under identical conditions.

A second set of soils (Tallgrass Prairie, contaminated and uncontaminated, or TGP-C and TGP-U, respectively) collected for this study were from within the Tallgrass Prairie (TGP) Preserve in Osage County, Oklahoma, and are classified as silty loam. The Preserve is owned by The Nature Conservancy and has been an area of active oil production since the 1920's. Approximately 70 barrels of dewatered crude oil were spilled in January 1999. Samples of both contaminated and neighboring uncontaminated soils were collected in September of 2003 and stored at 4^{o}C until use (within 24 hours).

The final two soil samples (RST low, RST high) were taken from a piece of unimproved property located in Garvin County, Oklahoma. Both soils are classified as loamy clay. One sample was taken from an earthen dam and was low in organic matter. The other sample was taken from a seasonal creek bed and was rich in organic matter. Samples were taken from just below the soil surface and stored at 4^{o} C until use (within 24 hours). These soils have no known hydrocarbon contamination.

Approximately 500 grams of each of the soil samples was analyzed by the Oklahoma State University Cleveland County Oklahoma Cooperative Extension Service. The following were determined: pH, buffer index, percent organic matter, nitrate (ppm), phosphorus (ppm), potassium (ppm), sulfate (ppm), calcium (ppm), and magnesium (ppm). The percent water for each soil sample was determined gravimetrically by standard methods (Brady and Weil, 1999). The textural classification of each soil was determined according to U.S. Department of Agriculture protocols (Brady and Weil, 1999; USDA-NRSC, 1993).

Enumeration of total heterotrophic bacteria and fungi

One gram (wet weight) of sediment/soil from each of the six sites was serially diluted in triplicate into 9 mL of 0.85% sterile saline. Gross numbers of heterotrophic aerobic bacteria were estimated from aliquots plated on R2A Agar (Difco). Gross numbers of fungi were calculated from samples prepared as above and plated onto Potato Dextrose Agar (Difco). Plates were incubated at 30^{o} C and counted every 24 hours for four days.

Enumeration and isolation of potential biosurfactant producing bacteria

Blood agar (BA) plates made from Blood Agar Base (Difco) and 5% sheep blood were used for the screening and isolation of potential biosurfactant producing bacteria (Bernheimer and Avigad,1970; Lin, 1996). Samples were prepared as above and plated onto BA. Beta-hemolytic colonies were counted after 24 and 48 hours incubation at 30^{o}C. Colonies from the most dilute plates

demonstrating beta hemolysis were restreaked to obtain pure culture isolates. Colony morphology, Gram stain, and cell morphology characteristics were recorded for the isolates. Isolates were placed in groups on the basis of microscopic analysis and colony morphology (Gerhardt et al., 1994). A representative from each group was isolated and selected for further analysis and identification

Identification of potential biosurfactant producing bacteria

For each Gram-positive isolate demonstrating beta-hemolysis on BA, Tryptic Soy Broth Agar (TSBA) slants were inoculated for identification via Fatty Acid Methyl Ester (FAME) analysis by Microcheck, Inc. (Northfield, VT) (Sassar, 1990). Simmon's citrate agar (EM Industries, Inc., Gibbstown, NJ), lactose fermentation, and glucose fermentation tests were used to further identify isolates.

DNA from each Gram-positive isolate was extracted using a standard phenol - chloroform extraction followed by precipitation with ethanol and sodium acetate (Ausubel et al., 1992; Sambrook et al., 1989). PCR amplification of 16S rDNA (Sambrook et al., 1989) was performed on a Robocycler Gradient 40 Temperature Cycler (Stratagene, La Jolla, CA) using the universal eubacterial primers GM5F and D907R (Muyzer et al., 1993), which amplify approximately 500 bp of the 16S rRNA gene. The PCR products from each isolate were purified using Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA) and sent to the Oklahoma Medical Research Foundation (Oklahoma City, OK) for sequence

analysis. Sequencing was performed on an ABI Model 3777 automated sequencer using Ampli-TaqFA DNA polymerase and fluorescent-labeled dNTP's in a cycle-sequencing kit (ABI Prism Dye Terminator Kit, PE Applied Biosystems, Inc., Foster City, CA). A sequence comparison was made by a nucleotide BLAST search to determine the identity of each isolate (Altschul et al., 1997; Warren and States, 1993).

For those Gram-positive isolates identified as being the same species by 16S rDNA sequence analysis and other tests, a repetitive sequence-based polymerase chain reaction (REP-PCR) was performed using the BOXA1R primer, using the amplification conditions specified by Versalovic et al., (1994) to determine if the isolates were likely to be members of the same clone. REP-PCR was performed using the Universal Mutation Detection System electrophoresis unit (Bio-Rad Laboratories, Hercules, CA). PCR products were applied to a 5% polyacrylamide gel in 1X TAE buffer (Sambrook et al., 1989). Electrophoresis was performed at 85 V for 135 minutes at 58^{o}C, followed by 45 V for an additional 45 minutes at 28^{o}C, and a final 45 V for 45 minutes at 28^{o}C. After electrophoresis, the gel was stained with ethidium bromide (0.5 mg/L) and viewed under UV illumination.

Gram-negative isolates demonstrating beta-hemolysis on BA were transferred to Biolog Universal Growth (BUG) medium (Biolog, Inc., Hayward, CA) and prepared for identification according to Biolog protocols (Solit, 1999).

Determination of the biosurfactant capabilities of each isolate

Beta-hemolytic isolates were grown in both Medium E broth and Tryptic Soy Broth (TSB) medium (Difco) at both 30^{o}C and 37^{o}C for 24 hours. Medium E broth was developed as a growth medium specifically for the cultivation of surfactant-producing *Bacillus* strain JF2 (McInerney et al., 1990). Isolates were subjected to a drop-collapse test (Bodour and Miller-Maier, 1998; Jain et al., 1991; Youssef et al., 2004) and an oil spreading test (Morikawa et al., 2000, Youssef et al., 2004) to determine if an isolate produced a biosurfactant under any of these four conditions. Assays were performed in triplicate. *Bacillus* strain JF2 (ATCC 39307) and a non-biosurfactant producing mutant of JF2 (Javaheri et al., 1985) were grown under each of the conditions as controls.

The ability for each isolate to produce biosurfactant was confirmed by analyzing surface tension via the Du Nouy ring tensiometer method (McInerney et al., 1990) on a Surface Tensiomat 21 ring tensiometer (Fisher Scientific). Isolates were grown for 24 hours in TSB at 30^{o}C. Cultures were centrifuged, and the resulting supernatant was tested for surface tension reduction (Youssef et al., 2004). Isopropanol and pure water were used to calibrate the tensiometer. Uninoculated medium was tested as another negative control. All measurements were made in triplicate.

Correlations between soil microorganisms and chemistry were made using an analysis of variance (ANOVA) test, a Tukey-Kramer Multiple Comparisons test, and linear regressions.

Determination of the presence of *srfA* gene in the six soil samples and biosurfactant-producing isolates

0.25 g of each soil sample was aseptically removed from a homogenized bulk sample and placed into a sterile 1.7 ml microfuge tube for storage at -40^{o}C. This sub-sampling was performed within 24 hours of the initial sampling and was performed in triplicate. Two of the three sub-samples were used for DNA extraction for each soil. The third tube was kept at -40^{o}C as a reserve, if necessary. Two soils, FL-U and FL-C, were obtained prior to the inception of this project, and thus the soils had been maintained differently, e.g. stored under nitrogen at 4^{o}C.

For each of the soils, both duplicate samples were subjected to DNA extraction using the Powersoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA) according to the manufacturer's directions. Extraction products of replicates were pooled and stored at -20^{o}C.

The presence of the *srfA* gene in each of the six soil samples and the Gram-positive isolates was tested by performing a PCR using primers designed for the conserved region between nucleotide 17807 and 17983 in the chromosomal *srfA* operon (McInerney et al., 2001) as determined from the sequence of *Bacillus subtilis* 168 (ATCC 23857) (Cosmina et al., 1993). The sequence of the forward primer (5' to 3'), srfA1F, was: GCGGTAGAAAAACTGCTTGC. The sequence of the reverse primer (5' to 3'), srfA1R, was: TGTGAATCAAACGCACCAAT (McInerney et al., 2001). PCR

reactions were performed according to protocols established by McInerney et al. (2001) on a Techne Genius Thermocycler (Techne Incorporated, Princeton, New Jersey) with the following parameters: initial denaturation at 94^{o}C for 4 minutes followed by 35 cycles of 94 ^{o}C for 1 minute, 59 ^{o}C for 1 minute, 72 ^{o}C for 1 minute. The final extension was at 72 ^{o}C for 7 minutes. This procedure was repeated with a range of DNA amounts added to each PCR reaction. In one set of tests intended to detect *srfA*-like sequences that differed slightly in the primer region, PCR amplification was repeated for all six soils but with less stringent annealing temperatures of 54^{o}C and then 50^{o}C.

Southern hybridization was performed to determine if a labeled *srfA* probe would bind to DNA from any of the soil samples or potential-biosurfactant producing isolates (Amann et al., 1997; Sambrook et al., 1989). The probe DNA was a PCR product amplified from JF2 DNA and labeled with digoxigenin-dUTP using the DIG High Prime DNA Labeling and Detection Starter Kit 1 (Roche Diagnostics, Germany). One hundred nanograms of DNA extracted from each soil sample were cross-linked to the membrane using UV irradiation (UV cross-linker, Ultra-Lum, Inc., Carson, CA). Hybridization was at a medium stringency of 50^{o}C. Detection followed the manufacturer's instructions (Roche Diagnostics Manual, Roche Diagnostics, Germany).

DNA was isolated from *B. cereus* (ATCC strain 14579) and *Bacillus megaterium* (ATCC strain 14581) via a phenol-chloroform extraction for use as *srfA* -negative controls (Sambrook et al., 1989; Ausubel et al., 1992). DNA was

extracted from *Bacillus* strain JF2 was the positive control. A PCR reaction using the *srf*A primers as described above was performed on control organisms.

RESULTS

Soil Chemistry and Gross Numbers of Organisms

The soil chemistry for the six test soils is summarized in Table 3.1. The sandy Fort Lupton contaminated (FL-C) sediments contained minimal organic matter and have been contaminated for over 25 years. In contrast, contamination at the Tallgrass Prairie site (TGP-C) has been present for a much shorter period of time at the time of collection (approximately four and a half years) and contained approximately 6% organic matter. The pristine, high organic matter soil (RST-H) contained much more organic matter (>43%) than any of the other five test soils.

The pH values of the six soils ranged widely, from 5.7 (RST-H) to 6.8 (FL-C) as did the levels of sulfate (4.0 to 289.5 ppm, RST-L and FL-U, respectively (Table 3.1). The two Tallgrass Prairie soils contained the greatest concentration of calcium, and the RST-high organic matter soil (RST-H) contained almost three times the concentration of calcium as did the RST-low organic matter soil (RST-L).

Of the six soils, the lowest number of aerobic heterotrophs was found at the FL-C site and the highest at the RST-high organic content soil (4.3×10^4 and 1.62×10^6, respectively, #CFU/gram soil, dry wt.) (Table 3.2). An analysis of variance (ANOVA) test showed significant differences for the mean number of

TABLE 3.1: Chemistry of Soils Sampled

Soil Sample[a]	pH	Sulfate (ppm)	Calcium (ppm)	Magnesium (ppm)	Nitrate (ppm)	Phosphorous (ppm)	Potassium (ppm)	Water Content (%)	Organic Matter (%)	Soil Classification
FL – C	6.8	72	280	48	2.0	4.5	23	12	0.12	Sand to Sandy loam
FL – U	6.7	290	600	45	0.5	6.5	17	1	0.07	Sand to Sandy loam
TGP – C	6.2	27	2,100	460	5.0	3.5	120	16	5.77	Silty loam
TGP – U	5.8	12	2,300	480	2.0	8.0	110	17	5.13	Silty loam
RST – High	5.7	57	1,000	160	1.0	2.5	120	51	43.0	Silty loam
RST – Low	6.5	4	380	39	3.0	6.5	63	7	0.55	Sandy loam

[a] Soil Samples are abbreviated as follows: Fort Lupton, Contaminated (FL-C); Fort Lupton, Uncontaminated (FL-U); Tall Grass Prairie, Contaminated (TGP-C); Tall Grass Prairie, Uncontaminated (TGP-U); Uncontaminated private property, high organic level (RST-High); Uncontaminated private property, low organic level (RST-Low).

TABLE 3.2: Gross numbers of aerobic heterotrophic bacteria, beta-hemolytic bacteria, and fungi from soil samples used in this study.

Soil Sample [a]	Number of Heterotrophs [b]	Number of Beta Hemolytic Bacteria [b]	Number of Fungi [b]	Percentage of β-Hemolytic Heterotrophs [b,c]
FL – C	$4.3 \times 10^4 \pm 2.0 \times 10^{2\,d}$	$3.4 \times 10^4 \pm 5.7 \times 10^{3\,e}$	$6.3 \times 10^4 \pm 4.3 \times 10^{3\,g}$	79.1
FL – U	$9.3 \times 10^4 \pm 1.9 \times 10^{4\,d}$	$1.0 \times 10^3 \pm 8.2 \times 10^{2\,f}$	$6.6 \times 10^4 \pm 4.9 \times 10^{3\,g}$	1.1
TGP – C	$2.1 \times 10^5 \pm 6.1 \times 10^{3\,d}$	$7.1 \times 10^4 \pm 7.3 \times 10^2$	$1.8 \times 10^5 \pm 3.9 \times 10^{3\,h}$	35.1
TGP – U	$2.1 \times 10^5 \pm 1.2 \times 10^{4\,d}$	$6.7 \times 10^3 \pm 7.8 \times 10^{2\,f}$	$1.7 \times 10^5 \pm 1.1 \times 10^{4\,h}$	3.2
RST – High	$1.6 \times 10^6 \pm 9.8 \times 10^4$	$1.6 \times 10^5 \pm 6.6 \times 10^4$	$3.4 \times 10^5 \pm 1.6 \times 10^4$	10.1
RST – Low	$9.5 \times 10^5 \pm 3.7 \times 10^4$	$3.4 \times 10^4 \pm 1.4 \times 10^{3\,e}$	$8.6 \times 10^4 \pm 1.0 \times 10^{3\,g}$	3.6

[a] Soil Samples are abbreviated as follows: Fort Lupton, Contaminated (FL-C); Fort Lupton, Uncontaminated (FL-U); Tall Grass Prairie, Contaminated (TGP-C); Tall Grass Prairie, Uncontaminated (TGP-U); Uncontaminated private property, high organic level (RST-High); Uncontaminated private property, low organic level (RST-Low).

[b] Values given are the average of three replicates plus or minus one standard deviation. The number of organisms given are based on per gram of dry weight of soil.

[c] Equals the number of beta hemolytic heterotrophs divided by the gross number of heterotrophs.

[d-h] Superscripts indicate groups of means that are significantly different ($p<0.05$, Tukey-Kramer Multiple Comparisons Test).

aerobic heterotrophs among the six samples (log_{10} transformed $F_{5, 12}$ = 1,272.6, P<0.0001). Both the RST-H and RST L soils had significantly higher numbers of aerobic heterotrophs than the other four soils. The RST-H soil contained more aerobic heterotrophs than the low organic matter soil.

The lowest number of beta-hemolytic bacteria was found in the FL-U sediment (1.0×10^3) while the highest number was found in the RST-high organic matter soil (1.6×10^5). An ANOVA test showed significant differences for mean number of beta hemolytic bacteria among the six samples (log_{10} transformed $F_{5, 12}$=23.7 , P<0.0001). The RST-H soil had significantly higher numbers of beta hemolytic bacteria than the other five soils.

The number of fungi isolated from the six sites ranged from 6.3×10^4 to 3.4×10^5 per gram of dry soil (FL-C and RST-H soils, respectively) (Table 3.2). An ANOVA test showed significant differences for mean number of fungi among the six samples (log_{10} transformed $F_{5,12}$=150.5, P<0.0001). A Tukey-Kramer Multiple Comparisons Test showed no significant difference between the mean number of fungi in the FL-C, FL-U, and RST-L sites. There was also no significance difference between the TGP-C and TGP-U soils. The RST-H soil contained significantly higher numbers of fungi than the other soils studied.

Correlation of number of microorganisms with soil organic matter

The number of aerobic heterotrophic bacteria and percent organic matter in a soil were positively correlated (r^2 = 0.4827, log_{10} transformed number of aerobic heterotrophic bacteria versus log percent organic matter). The greatest

number of bacteria was isolated from the RST-H soil (Table 3.2). The lowest number of aerobic heterotrophs was found at the FL-C site. The linear regression of numbers of fungi showed an even higher positive correlation with soil organic matter ($r^2 = 0.9648$, $\log_{10}$ transformed number of fungi versus log percent organic matter). The numbers of beta-hemolytic bacteria had the weakest positive linear correlation with soil organic matter, ($r^2 = 0.4102$, $\log_{10}$ transformed number of beta-hemolytic bacteria versus log percent organic matter).

In order to normalize differences in the fraction of bacteria that produce beta hemolysis, the percentage of aerobic heterotrophs that were also beta-hemolytic was calculated. The percent beta hemolytic bacteria and soil organic matter did not show a significant correlation when all six soil samples were considered (r^2=0.03480, slope = -5.175, percent beta-hemolytic bacteria versus log percent organic matter). However, the two hydrocarbon-contaminated sites (FL-C and TGP-C) appeared to be responsible for the negative slope. When a second linear regression was performed using the four uncontaminated soils, the correlation between the levels of soil organic matter and the percent beta hemolytic bacteria was higher ($r^2 = 0.7751$) but the slope was not significantly different from 0 (slope = 2.842, p = 0.2430), indicating that a higher percent organic matter in uncontaminated soil did not select for a significantly higher percentage of beta hemolytic bacteria. When all six sites were included, the percent of beta hemolytic bacteria was negatively correlated with the (log) number of fungi ($r^2 = 0.04482$, slope = -21.785), again due to the two hydrocarbon-contaminated sites. A highly significant positive linear regression of

the percent beta hemolytic bacteria with the ($\log_{10}$) number of fungi was seen using the four uncontaminated sites ($r^2 = 0.8044$, slope = 10.925).

Identification of Isolates

Bacteria demonstrating beta hemolysis on red blood agar were isolated and purification was confirmed by observation of colony morphology after repeated streak plating and by microscopic analysis. Isolates were grouped together based on characteristics outlined in the Materials and Methods section. A total of eight isolate-groups were found. Five of these groups were Gram-positive rods, one was a Gram-positive coccus, and two were Gram-negative rods.

A preliminary identification of the Gram-positive isolates was performed by FAME analysis (Sassar, 1990) (Table 3.3). Most were identified as *Bacillus* species: isolate A as *B. sphaericus*, isolate B as *B. megaterium* GC subgroup A, isolate E as *B. cereus* GC subgroup A, and isolate H as *B. cereus* GC subgroup A. Isolate J was identified as *Paenibacillus lentimorbus*. Isolate P was identified as *Enterococcus faecium* GC subgroup A.

The 16S partial analysis showed similar results to the FAME analysis for isolates with SI values above 0.800, with the exception of Isolate J. The 16S rDNA sequence from Isolate A had an identical match to 587 base pairs of *B. sphaericus* strain 205y. Isolate B had an identical match to 587 base pairs of *B. megaterium* strain KL-197. Isolates E and H had an identical match to 588 base pairs of *B. cereus* ATCC strain 14579 as well as many other *B. cereus* group species including *B. mycoides* and *B. anthracis*. Isolate J was identified as either

TABLE 3.3: Potential Gram-Positive Biosurfactant-Producing Microorganisms – Identification, Source, and Ability to Demonstrate Surfactant Production

Isolate	ID (16 S)	ID (FAME)	Soil Source [a]	Dilution Source	Location of Similar Isolates [a]	Oil Spreading (TSB), cm [b]	Drop Collapse (TSB) [b]	Surface Tension (mN/m) [b,c]
A	*Bacillus sphaericus*	*Bacillus sphaericus*	RST-H	10^{-5} TGP-C	FL-C	1.00 ±0.12	+++	41.7 ±1.11
B	*Bacillus megaterium*	*Bacillus megaterium*	RST-H	10^{-5}	FL-U TGP-U TGP-C	0.57 ±0.29	+	57.3 ±0.44
E	*Bacillus cereus* group	*Bacillus cereus*	RST-H	10^{-4}	FL-C TGP-C	2.92 ±0.03	+++	40.3 ±0.89
H	*Bacillus cereus* group	*Bacillus cereus*	RST-L	10^{-4}	------	0.43 ±0.10	+	51.3 ±0.44
J	*Bacillus subtilis Bacillus amyloliquefaciens*	*Paenibacillus lentimorbus*	FL-C	10^{-3}	RST-L	0.77 ±0.06	+++	44.3 ±0.44
P	*Leuconostoc mesenteroides*	*Enterococcus faecium*	TGP-U	10^{-4}	TGP-C	0.29 ±0.06	-	58.7 ±0.44

[a] Soil source for isolate tested. Soil Samples are abbreviated as follows: Fort Lupton, Contaminated (FL-C); Fort Lupton, Uncontaminated (FL-U); Tall Grass Prairie, Contaminated (TGP-C); Tall Grass Prairie, Uncontaminated (TGP-U); Uncontaminated private property, high organic level (RST-High); Uncontaminated private property, low organic level (RST-Low).

[b] The oil spreading, drop collapse, and surface tension data reported are for cultures grown at 30°C. No isolates showed any surfactant activity when cultured at 37°C. Drop collapse test on ME: JF2 = ++++; JF2 mutant = +; medium blank = ---. Medium abbreviations are as follows: Tryptic Soy Broth (TSB); Medium E (ME).

[c] This test was performed on cultures grown in TSB.

a *B. subtilis* or *B. amyloliquefaciens* by 16S rDNA (100% match to 588 base pairs of *B. subtilis* strain BGSC 3A23 as well as to *B. amyloliquefaciens* strain Ba-S13). The culture most closely matched *Paenibacillus lentimorbus* by FAME analysis. Isolate J was tested for the ability to grow on Simmon's Citrate Agar, as members of the *Bacillus* utilize citrate, unlike members of the genus *Paenibacillus* (Ash et al., 1993; Holt et al., 1994). Isolate J grew on citrate and also produced acid from lactose fermentation, consistent with identification as *B. amyloliquefaciens* (Holt et al., 1994).

The partial 16S rDNA sequence of Isolate P had an excellent match (>99% identity, 586/587 base pairs) to *Leuconostoc mesenteroides* but a low level of similarity to *Enterococcus faecium* by FAME analysis. The production of CO_2 from glucose fermentation supports the identification of Isolate P as a member of the genus *Leuconostoc* (Holt et al., 1994).

Gram-negative rods were identified by a Biolog Microlog Analysis (Solit, 1999) (Table 3.4). One of the isolates was identified as *Enterobacter nimipressuralis* (100% probability). The second was identified as *Pseudomonas syringae* (96% probability). Colony morphology, microscopic examination, and FAME analysis results of the Gram-negative isolates were consistent with the Biolog identifications.

TABLE 3.4: Potential Gram-Negative Biosurfactant-Producing Microorganisms – Identification, Source, and Ability to Demonstrate Surfactant Production

Isolate	ID	Soil Source [a]	Dilution Source	Location of Similar Isolates [a]	Oil Spreading (TSB), cm [a,b]	Drop Collapse (TSB) [a,b]	Drop Collapse (ME) [a,b]	Surface Tension (mN/m) [b]
T	*Enterobacter nimipressuralis*	RST-H	10^{-4}	TGP-C	1.07 ±0.04	+	-	56.3 ±0.44
W	*Pseudomonas syringae*	RST-H	10^{-3}	--------	1.47 ±0.18	+	-	55.3 ±0.44

[a] Soil Samples are abbreviated as follows: Fort Lupton, Contaminated (FL-C); Fort Lupton, Uncontaminated (FL-U); Tall Grass Prairie, Contaminated (TGP-C); Tall Grass Prairie, Uncontaminated (TGP-U); Uncontaminated private property, high organic level (RST-High); Uncontaminated private property, low organic level (RST-Low).
[b] The oil spreading, drop collapse, and surface tension data reported are for cultures grown at 30°C. No isolates showed any surfactant activity when cultured at 37°C.
Medium abbreviations are as follows: Tryptic Soy Broth (TSB); Medium E (ME).

Confirmation of Biosurfactant Producing Ability

Each isolate was subjected to three different tests to determine if they produced a biosurfactant. Reported results were the average of three replicates, for both isolates and controls (Table 3.3).

Each isolate was subjected to a drop collapse test. The test was performed on cultures grown at both 30^{o}C and 37^{o}C, both in Medium E and in TSB (Tables 3.3 and 3.4). *Bacillus* strain JF2 was successful in producing drop collapse and acted as a positive control. Drops of uninoculated media (both types, incubated at both temperatures) did not collapse when placed on the oil film. Drops of media (both types) inoculated with the non-biosurfactant producing mutant of JF2 and incubated at both test temperatures remained standing for at least 10 minutes and were therefore negative for drop collapse (Youssef et al., 2004).

Both Gram-negative isolates demonstrated partial drop collapse in TSB when incubated at 30^{o}C (Table 4). Drops of TSB medium containing Isolates A and E, when grown at 30^{o}C, completely collapsed (Table 3.3). Growth temperature and medium each had an effect on the ability of strains to produce drop collapse. No soil isolate demonstrated any drop collapse at 37^{o}C in either growth medium.

An oil-spreading test was also performed as a test for biosurfactant production. JF2 grown at 37^{o}C in Medium E had an average diameter of clearing measuring 1.5 cm and acted as a positive control. The JF2 mutant had a diameter of clearing less than 0.45 cm. This measurement or less was considered a

negative result for biosurfactant production (Youssef et al., 2004). Isolates A, B, E, J, T, and W all had average diameters of clearing greater than 0.57 cm when grown at 30^{o}C in TSB (Table 3.3). The greatest diameter of clearing (2.92 cm) was produced by Isolate E, almost twice the diameter produced by JF2. Isolates had an average diameter of clearing less than 0.45 cm when grown at 37^{o}C, in agreement with the results from the drop collapse method.

A third test for biosurfactant production was measuring surface tension. Pure water was used as a negative control (surface tension = 71.6 mN/m) and isopropanol was a positive control (surface tension = 31.6 mN/m). Uninoculated Medium E and TSB had average surface tensions of 66.5 and 64.8 mN/m, respectively. *Bacillus* strain JF2 and the non-biosurfactant producing JF2 mutant lowered surface tension to approximately 30 and 53 mN/m, respectively. Three isolates demonstrated an average surface tension of under 45 mN/m when grown in TSB at 30^{o}C: Isolates A, E, and J (Tables 3.3). All other isolates had average surface tension measurements range from between 50 to 59 mN/m when grown at 30^{o}C in TSB (Tables 3.3 and 3.4).

Determining the presence of *srfA* in isolates and DNA extracted from soil samples

DNA was isolated from each soil sample as well as from each isolate. Each DNA sample was tested for the absence of PCR inhibitors by performing a PCR reaction using universal eubacterial 16S rRNA primers. All DNA samples resulted in strongly positive reactions. A PCR reaction shown to amplify a region

of the *srfA* gene from *B. subtilis* was performed on DNA extracted from the isolates and soil samples using annealing temperatures of 59 ^{o}C, 54 ^{o}C, and then 50 ^{o}C. Amplification was observed for *Bacillus* strain JF2 under stringent annealing conditions. Amplification was not observed for any of the isolates or soils. Additional PCR reactions at both the normal and lower stringency temperatures indicated no amplification in the two negative control organisms, *B. cereus* and *B. megaterium*.

Southern hybridization was performed as an additional test to detect the *srfA* gene in DNA from both the soils and isolates. DNA from the isolates did not show a positive reaction to the *srfA* probe. The only soil DNA sample to clearly indicate the presence of the *srfA* gene was that of the uncontaminated Tallgrass Prairie (TGP-U) sample.

DISCUSSION

Correlation of numbers of microorganisms with percent organic matter

Prior work with both the Fort Lupton and Tallgrass Prairie Soils provided the basis for this investigation (Jennings and Tanner, 1999; Appendix 1) (Jennings and Tanner, 2000; Appendix 2). Soil organic matter (SOM) has long been known to be a major controlling factor in the productivity of soil (Paul and Clark, 1989). The results strongly support a central role for SOM in determining the numbers of microorganisms found in these soils. The correlation was highest between percent SOM and fungal numbers, reflecting a role of fungi as primary decomposers of

organic matter. This was consistent with observations made in studies comparing fungal species diversity and activity levels to decomposition rates of various organic substrates (Setala and McLean, 2004; Subuke et al., 2004). However, both of these studies used biomass as a measure of fungal numbers and the primary interests of the studies were species diversity and rhizosphere activity. These studies did not correlate the numbers of fungi to other aspects of soil chemistry or to the activity of soil bacteria.

Other soil parameters that affect fungal growth also co-varied with percent SOM. Calcium stimulated fungal growth (Brady and Weil, 1999), and the three soils with the three highest gross numbers of fungi (TGP-C, TGP-U, and RST-H) had the three highest soil calcium levels (1,083-2,316 ppm). Of the six soils tested, the FL-C site contained both the lowest gross number of fungi and amount of calcium.

There was a weaker but still significant positive relationship between percent SOM and numbers of aerobic heterotrophic bacteria. The greatest number of bacteria were isolated from the RST-H soil (Table 3.2), as expected due to the high nutrient levels of the soil as well as its low sand content (Brady and Weil, 1999). The lowest number of aerobic heterotrophs was found at the FL-C site, and is likely a reflection of soil composition (Brady and Weil, 1999). The FL-U and FL-C values are similar to those previously reported (Gieg et al., 1999). Other parameters varied among the soils, such as the sand/silt/clay soil composition, moisture content, and soil chemicals (Table 3.1) and likely affected the numbers of heterotrophic bacteria. The FL-C sediments, however, had both

the lowest percent SOM of the tested soils and typically had low levels of dissolved oxygen (Barker et al., 1996; Gieg et al, 1999). This was reflected by the low number of aerobic heterotrophic bacteria found at that site.

A significant but modest positive linear relationship was seen between percent SOM and the gross number of beta-hemolytic aerobic heterotrophs (r^2 = 0.4102). Lower numbers of these bacteria were found in soils with low percent SOM. However, hydrocarbon contamination over-rides the trend produced by SOM; both Tallgrass Prairie soils contained very similar levels of organic matter but the hydrocarbon-contaminated site had approximately ten-fold higher average levels of beta-hemolytic bacteria than did the uncontaminated site. Likewise, the FL-C site had more than 10-fold higher average levels of beta-hemolytic bacteria than did the FL-U sediments, with only a slightly higher percent SOM (Table 3.2).

Influence of hydrocarbon contamination and fungi on bacterial biosurfactant production

Results indicated different patterns of association between the percentage of beta hemolytic, heterotrophic bacteria and other factors depending upon whether or not the soil was contaminated with hydrocarbon. The two hydrocarbon-contaminated soils (TGP-C and FL-C) contained significantly higher percentages of beta-hemolytic heterotrophs than the rest. Over 35% of the heterotrophs found in the TGP-C soil were beta-hemolytic (Table 3.2). This is approximately ten times the levels found in the TGP-U soil. Even more

remarkable was that 79% of heterotrophs found in the FL-C sediment were capable of beta-hemolysis versus only one percent in the uncontaminated, background sediment. The percent of beta-hemolytic heterotrophs found in the remaining four uncontaminated soils ranged from 1.1-10.1%. Therefore, the percent of heterotrophs capable of beta hemolysis is much higher in the presence of a hydrocarbon contaminant than in its absence.

It has been demonstrated that the presence of biosurfactants can increase hydrocarbon degradation (Haferburg et al., 1986; Jennings and Tanner, 2004; Rouse et al., 1994). Such degradation can provide nutrients to the bacteria, and thus the ability to produce a biosurfactant would be advantageous. Therefore, the selection of a biosurfactant-producing population in a contaminated environment such as the TGP-C or FL-C samples may be due to competition for nutrients. When comparing the two contaminated soils, the FL-C site has been contaminated for almost six times as long as the TGP-C site and has over twice the percentage of beta-hemolytic heterotrophs as does the TGP-C soil. Furthermore, on a percentage basis, the FL-C sediment has more than twice the percent SOM than the FL-U sediments (0.12% versus 0.07%), suggesting that a large proportion of the organic matter at the FL-C site may be in the form of hydrocarbons. In contrast, a relatively small fraction of the organic matter at the TGP-C site is in the form of hydrocarbons (TGP-C:5.77%, TGP-U: 5.13%), and the exposure to hydrocarbons was a one-time event, in contrast to the continuous exposure at the FL-C location (Mehta, 2004).

In uncontaminated soils, the percentage of potential biosurfactant producers appears to be positively correlated with both percent SOM and to the gross number of fungi. In organic rich soils, microorganisms tend to be metabolically stimulated (Brady and Weil, 1999). The production of compounds that aid in transporting nutrients across cell membranes, such as biosurfactants, should increase as bacterial metabolism is increased (Banat, 1995a; Banat, 1995b; Lin, 1996). This may account for the positive but non-significant relationship seen between soil organic matter and the percent of potential biosurfactant producers. However, a highly significant positive relationship was found between the numbers of fungi and the percent of beta hemolytic bacteria. We hypothesize that higher levels of organic matter stimulate the growth of fungi, which results in an increase in the competition for the soil nutrients between fungi and bacteria. Bacteria that can produce anti-fungal compounds, such as biosurfactants, are expected to be more successful in competing with fungi for nutrients (Katz and Demain, 1977; Koumoutsi et al, 2004).

In summary, the results suggest the following: higher levels of soil organic matter result in higher numbers of soil fungi. The increased presence of fungi select for bacteria capable of competing against the fungi through the use of anti-fungal biosurfactants. However, a high percentage of aerobic heterotrophic bacteria were found to be beta-hemolytic when isolated from hydrocarbon contaminated soils. The advantage of producing biosurfactants in these circumstances may be due to increasing the aqueous solubility of hydrocarbons that can be used as carbon and energy sources. Hydrocarbon contamination

appears to override the effects of other environmental and ecological factors such as soil organic matter and fungi.

Biosurfactant-producing isolates

All of the isolates identified were species commonly found in soils (Brenner et al., 1986; Carter, 1945; Dye, 1969; Holt et al., 1994; Pettersson et al., 1999; Wilson et al., 1999). Isolates E and H were identified as members of the *B. cereus* group of species by FAME analysis and 16S sequence analysis. The difference in biosurfactant-production capability between the two isolates may be a result of the loss of the secondary phenotype.

Confirmation of biosurfactant producing ability

The ability to lyse red blood cells is an established method for the initial screening of bacteria for their ability to produce biosurfactants (Bernheimer and Avigad, 1970; Carillo et al., 1996; Yonebayashi et al., 2000). Biosurfactant production by both Gram-positive and Gram-negative bacteria has been detected by beta-hemolysis (Bernheimer and Avigad, 1970; Johnson and Boese-Marrazzo, 1980; Moran et al., 2002). However, the ability to lyse red blood cells is not adequate proof of biosurfactant capabilities (Mulligan et al., 1984; Youssef et al., 2004). Three other ways of detecting biosurfactant production include a drop collapse test (Bodour and Miller-Maier, 1998; Jain et al., 1991), an oil-spreading analysis (Morikawa et al., 2000), and measurement of surface and/or interfacial tension (Cooper and Zajic, 1980; Haba et al., 2000).

Isolates representing three colony morphology groups demonstrated complete drop collapse, significant oil spreading and a significant decrease in surface tension (below 45 mN/m): Isolates A, E, and J. Isolate A, identified as *B. sphaericus*, demonstrated complete drop collapse and the second highest oil-spreading measurement of all isolates tested (Table 3.3). There is no literature documenting biosurfactant activity from *B. sphaericus*, and it should also be noted that these measurements were taken from cultures freshly revived from the frozen stock culture.

Isolate E produced the most robust biosurfactant of all isolates. Identified as *B. cereus*, this isolate demonstrated the largest oil spreading capability and the greatest decrease in surface tension of all isolates tested, lowering surface tension to approximately 40 mN/m (Table 3.3). These results support previous research showing that the genus *Bacillus* contains many species capable of producing biosurfactants, including a number of *B. cereus* strains (Cooper and Goldenberg, 1987; Cooper and Zajic, 1980; Peypoux et al., 1999).

Isolate J, identified as *B. amyloliquefaciens*, produced the third best biosurfactant in regards to oil spreading and surface tension reduction ability (Table 3.3). Although a surfactant was produced, the nucleotide sequence coding for the surfactant was apparently different from that of *B. subtilis srfA* as there was no amplification using Isolate J's DNA as a template and no hybridization to the *srfA* probe. This result is similar to that found in another study with *B. amyloliquefaciens* strain FZB42 (Koumoutsi et al., 2004).

Thus, not all of the beta hemolytic isolates in this study were found to produce a biosurfactant when tested by other means. These results are consistent with Youssef et al. (2004) which concluded that lysis of blood agar is not a good method for the initial screening of biosurfactant producers. There is an important difference, however, between the test conditions of the Youssef et al. (2004) study and the current investigation. The former study was conducted on pre-isolated bacterial strains. In contrast, the current investigation used soil containing a mixed community of bacteria as its starting material. In order for the current study to be fully comparable to the Youssef et al. (2004) paper, organisms from the test soils would first have to be diluted onto agar, grown, isolated and purified before any biosurfactant-detection tests could begin. These steps would take additional time, defeating the attempt to quickly screen soils for potential biosurfactant producing bacteria.

Screening for the *Bacillus subtilis srfA* gene in isolates and total soil DNA

A secondary phenotype such as biosurfactant production could be lost as a result of changes in environmental factors such as temperature and salinity (McInerney et al., 1990). Therefore, a molecular probe was utilized to determine if a *B. subtilis*-like*srfA* gene was present but not active in the isolates or in total soil DNA extracted from each research site (McInerney et al., 2001; Youssef et al., 2004).

In this investigation, Isolate J was the bacterium most closely related to *B. subtilis* by 16S sequence but a *B. subtilis*-like*srfA* gene was not detected (Table

3.3). The *srfA* gene was only detected in the TGP-U soil. This was the first time *srfA* had been used to test environmental samples, as opposed to purified environmental isolates (Youssef et al., 2004). The conclusion is that monitoring the *B. subtilis*-like*srfA* gene in these soils did not indicate the presence of biosurfactant producers related to *B. subtilis*. This is in accord with the results that suggest that *B. subtilis* and closely related species were not the dominant biosurfactant producing microorganisms in these soils (Bodour et al., 2003).

In conclusion, this study has demonstrated that higher levels of soil organic matter are correlated with higher numbers of soil fungi. There was a direct relationship between the numbers of fungi and the numbers of potential biosurfactant producers in the absence of a hydrocarbon contaminant. This study has also demonstrated that screening soils for a *B. subtilis*-like*srfA* gene was not an adequate method of determining the biosurfactant-producing capabilities of these soils.

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APPENDIX ONE

Biosurfactant Producing Bacteria in Hydrocarbon Impacted Soils

ABSTRACT

Microbially produced surfactants have been often studied for their potential use in Microbially Enhanced Oil Recovery (MEOR) and hydrocarbon bioremediation. However, most of these studies have involved biosurfactants produced by a small number of pure-culture microbes that were isolated in a laboratory and then applied to environmental samples. Here, we determine whether biosurfactant-producing microorganisms were naturally present at two hydrocarbon-impacted sites – one in northwestern Oklahoma and the other near Ft. Lupton, Colorado. We also examined neighboring uncontaminated materials from these sites for biosurfactant producers. This study sought to enumerate and identify those microbes that produce biosurfactants and whether these organisms were ubiquitous to a variety of sediments.

INTRODUCTION

Biosurfactants are microbially produced surface acting compounds. They are amphiphilic molecules with both hydrophilic and hydrophobic regions causing them to aggregate at interfaces between fluids with different polarities (such as water and hydrocarbons), forming micelles. This molecular film allows for a decrease in interfacial surface tension (Lin, 1996; Shafi and Khanna, 1995; Rouse et al., 1994; Volkering et al.,1998; Fiechter, 1992; Georgiou et al., 1992). Although the entire role of biosurfactants is not fully understood, it is known that these secondary metabolites can enhance nutrient transport across membranes, act in various host – microbe interactions, and provide biocidal and fungicidal protection to the producing organism (Lin, 1996; Banat, 1995a; Banat,1995b).

In observing the effectiveness of a biosurfactant, the concentration required for micelle formation is measured. At or above this Critical Micelle Concentration (CMC), the surfactant is effective at drawing hydrophobic compounds into the micelle interior and thus increasing the overall solubility of the compound in a hydrophilic environment (Rouse et al., 1994; Banat, 1995a; Banat, 1995b; Lin, 1996; Shafi and Khanna, 1995). At concentrations below the CMC, the surfactant monomers remain individual and in solution.

However, it is the formation of micelles, with hydrophilic exteriors and hydrophobic interiors, which is important to tertiary oil recovery and bioremediation efforts. This micelle formation increases hydrocarbon solubilization and emulsification, therefore allowing for the capture of these compounds in an aqueous environment for either recovery or degradation (Lin,

1996; Rouse et al., 1994; Volkering et al., 1998). Studies have shown that many of the identified biosurfactant producers are also hydrocarbon degrading organisms, although observations of a second microbe using the surfactant compound as a co-substrate for hydrocarbon metabolism have also been reported (Rouse et al., 1994; Willumsen and Karlson, 1997; Volkering et al., 1998).

In the past decade, many studies have reported the effects of microbial produced surfactants on bioremediation and enhanced oil recovery (Jack, 1988; Jenneman et al., 1984; Volkering et al., 1998). However, these studies typically involved a single microbe or group of microbes isolated and identified in a laboratory and then applied to either *ex-situ* soil core experiments or injected into existing oil reservoirs for observation. In addition, the majority of these studies involve a small number of well studied microbial species such as *Bacillus licheniformis* strain JF-2, *Bacillus subtilis*, or *Pseudomonas fluorescens*, utilizing the large amounts of information available on these relatively few microbes to design experiments whose main objective is typically to enhance the desirable effects of the organisms (Banat, 1995a; Banat, 1995b; Lin, 1998, McInerney et al., 1990). Few studies, though, have analyzed the natural, indigenous biosurfactant producing microbes present in oil recovery or bioremediation sites. The purpose of this study is to observe the biosurfactant producing microbes naturally occurring in two different terrestrial hydrocarbon contaminated sites and to not only compare the numbers of microbes found, but also the various genera.

MATERIALS AND METHODS

Sources of Environmental Samples

Sediments for this study were collected from two different locations. The first location is at an active natural gas production site near Ft. Lupton, Colorado (northeast of Denver) (Gieg et al., 1999). The soil in this area is classified as a sandy to sandy loam, used primarily for agriculture. During the 1970's multiple gas condensate contamination incidents occurred, including leaks from an underground produced water storage sump. In 1980, the contamination source was removed, but hydrocarbons continue to flow towards the South Platte River located only 90 meters downgradient. Monitoring of intrinsic bioremediation of the groundwater and contaminated soils was initiated in 1995 and continues to date (Gieg et al., 1999). Sediments for this study were collected from beneath the shallow water table (~ 1.4 meters) in March, 1999 and stored at 4^{o} C. In addition to contaminated sediments, upgradient, uncontaminated sediments were also collected and stored under identical conditions.

The second set of sediments collected for this study were from within the Tall Grass Prairie Preserve in Osage County, Oklahoma. This portion of NE Oklahoma is owned by The Nature Conservancy and was a large oil field until 1989 when the organization purchased the land. Although oil production does continue to occur on a small scale, the primary function of the land is to recreate a native Oklahoma tall grass prairie ecosystem. In January 1999, 70 barrels of

dewatered crude oil were spilled. The primary spill site overflowed from a natural silty loam clay basin, traveled across a road, and settled in a second natural basin. This contamination pattern has allowed for two different treatments of the spill site to be easily examined. Current plans call for one half of the spill site to be actively remediated (tilling plus fertilizer) while the other half will be subjected to tilling alone. Five days after the spill, the contaminated soil was tilled to a depth of approximately one meter. However, fertilizer and subsequent tilling did not occur until two months after samples were collected.

Sediments from this site, like those from Ft. Lupton, were also collected in March, 1999. Soils were stored at 4^{o} C. Both contaminated and neighboring uncontaminated soils were collected for analysis.

Media Used

Blood Agar plates were used for the isolation of potential biosurfactant producing bacteria (Bernheimer and Avigad,1970; ; Banat, 1995a; Banat, 1995b; Lin, 1996). Preparation included the addition of 40 g/L Blood Agar Base (Difco Laboratories; beef heart infusion 500 g/L, bacto tryptose 10 g/L, NaCl 5 g/L, bacto agar 15 g/L) to nanopure water that was then autoclaved. When the medium cooled to between 45-50^{o} C, 60 ml/L sterile sheep blood (Brown Laboratories; Topeka, KS) were added and the plates were then poured.

Plate count agar (PCA) was used for the maintenance of isolated biosurfactant-producing bacterial colonies and for counting the total number of aerobic heterotrophic bacteria (Duncan et al., 1997). Preparation included the

addition of 17 g/L Plate Count Broth Base (Difco Laboratories; bacto yeast extract 5 g/L, bacto tryptone 10 g/L, bacto dextrose 2 g/L) to 16 g/L of Purified Agar (Becton Dickinson) and nanopure water. This was then autoclaved and the plates poured.

Tryptic Soy Broth Agar (TSBA) slants were made to grown biosurfactant producing colonies for Fatty Acid Methyl Ester (FAME) analysis. TSB Base (15g/L Difco; pancreatic digest of casein 17 g/L, papaic digest of soybean meal 3.0 g/L, dextrose 2.5 g/L, sodium chloride 5.0 g/L, dipotassium phosphate 2.5 g/L) was added to 16 g/L purified agar (Becton Dickinson) and nanopure water, autoclaved, then poured into sterile slant tubes.

Biolog Universal Growth medium (BUG) plus 5% sheep blood (Biolog, Inc; Hayward, CA, USA) was used for the preparation of both the Gram positive and Gram negative isolates for the Biolog analysis. One Gram negative isolate was also cultured on Tryptic Soy Agar (TSA) to compare analysis results with the medium supplied by Biolog, Inc.. This was prepared by adding 20g/L of TSA (Difco; Bacto tryptone 15 g/L, Bacto Soytone 5 g/L, sodium chloride 5g/L, Bacto agar 15 g/L) to nanopure water and then sterilizing.

Isolation of Biosurfactant Producers

One gram (wet weight) of contaminated sediment from the Ft. Lupton site was added to 10 ml of sterile 0.85% saline solution (NaCl) in sterile 50 ml centrifuge tubes which were then vortexed on high for two minutes. One hundred microliters were removed from this slurry and added to a second centrifuge tube

containing 0.9 ml of 0.85% NaCl saline solution. This second tube was then vortexed for two minutes on high, and 0.1 ml was removed for further 1:10 dilutions. All dilutions were performed in triplicate.

Dilutions were spread-plated on Blood Agar plates (prepared one day prior) with final dilutions ranging from 10^{-1} through 10^{-4}. These were incubated at 30^{o} C and counted after 24 and 48 hours.

After 48 hours, colonies exhibiting beta hemolysis were transferred again onto fresh Blood Agar plates to separate any colonies that needed further isolation. This was continued until single colonies of beta hemolytic bacteria were isolated. Due to extreme overgrowth on many of the lower dilution plates, isolates were mainly chosen from higher dilutions. After isolation, colonies were maintained at 30^{o}C on PCA plates.

The above isolation of beta hemolytic bacteria was repeated for uncontaminated sediments from Ft. Lupton, and uncontaminated and contaminated soils from the Tall Grass Prairie site.

Observations in reference to colony morphology, growth, microscopic characteristics were made. Similar colonies were grouped together on the basis of microscopic analysis and colony morphology. For example, isolate A included flat, slightly moist, cream colored colonies with light feathering at the edges. Microscopic analysis of the bacteria in each of the colonies revealed non-motile, Gram positive rods sometimes linked in short chains. A representative colony of each group was then selected for further analysis.

In addition to the dilution plating of sediments onto Blood Agar plates, positive and negative controls were also plated. As a positive control, *Bacillus licheniformis* JF-2 (ATCC 39307) was used. This bacterium is a well studied biosurfactant producer (McInerney et al., 1990). As a negative control, a mutant form of *Bacillus licheniformis* JF-2 was plated. This mutant has lost the ability to produce any biosurfactant (McInerney et al., 1990).

Enumeration of Total Aerobic Heterotrophs

Dilution plating was performed on all four soil samples (Tall Grass Prairie and Ft. Lupton, both contaminated and uncontaminated) in a similar manner as with the isolation of biosurfactant-producing bacteria with the exception of substituting PCA for Blood Agar (Duncan et al., 1997). Final plate dilutions ranged from 10^{-2} to 10^{-5}. Total colony counts were taken after 24 and 48 hours of incubation at 30^{o}C.

Identification of Selected Cultures – FAME Analysis

Those colonies selected to represent the isolated biosurfactant producing bacteria were first subjected to a Fatty Acid Methyl Ester (FAME) analysis for identification. This involved first growing the individual colonies on TSBA slant tubes for 24 hours at 30^{o} C such that growth was visible (Sassar, 1990). Cultures were then shipped on ice to Microcheck, Inc. (Northfield, VT; USA) for analysis.

Biolog Analysis

Representative colonies for each group were analyzed by the Biolog Microlog System for comparative identification. All isolated colonies were first plated on BUG + Blood Agar. The non-biosurfactant producing *Bacillus licheniformis* JF-2 mutant was also plated as a system control. In addition, one Gram-negative isolate was also plated onto TSA agar as a medium control. After overnight incubation at 30^{o} C, all Gram positive cultures were then used to inoculate Biolog Gram Positive MicroPlates and Gram negative isolates were used to inoculate Biolog Gram Negative MicroPlates according to Biolog, Inc. protocols (Solit, 1999). After all MicroPlates had incubated at 30^{o} C for 24 hours, they were analyzed by the Biolog Microlog Software and Microstation System.

RESULTS AND DISCUSSION

Bacterial Numbers

The numbers of total aerobic heterotrophs were 10 to 100-fold higher for the Tall Grass Prairie samples than in the Ft. Lupton samples (Table A1.1). Initially, from the values obtained from both uncontaminated sites, it was thought that the higher numbers at the Tall Grass Prairie site may reflect the somewhat higher soil temperatures in Oklahoma (not recorded) than in Colorado (12^{o} C) during the month of March. However, prior research at the Ft. Lupton site during the summer of 1997 showed almost identical aerobic heterotroph values as found in this study (Gieg et al., 1999). Thus, the lower values from the Ft. Lupton site

Table A1.1. Numbers of aerobic heterotrophs and biosurfactant producing bacteria from each of the soil samples.

	Average # Aerobic Heterotrophs	Average # Biosurfactant Producers
Tall Grass Prairie, OK		
Uncontaminated	2.3×10^6	1.6×10^4
Contaminated	1.0×10^6	1.4×10^4
Ft. Lupton, CO		
Uncontaminated	1.3×10^5	5.5×10^2
Contaminated	6.0×10^4	1.4×10^3

may be a reflection of the effect of the soil quality, structure, and contamination history on microbial activity rather than the effect of temperature.

The importance of the soil structure at the Ft. Lupton site may be considered when the numbers of aerobic heterotrophs in both the contaminated and background soils are observed. Sandy soils will typically have lower microbial activity than soils with higher clay or silt contents (Tate, 1995). However, it would be expected that the addition of a contaminant, if present in toxic quantities, would further lower the number of microbes in a soil in comparison to a similar, uncontaminated soil. In this situation, though, the contamination has been present for over two decades and a population of hydrocarbon degrading heterotrophs may have become so well adapted to this environmental condition that their numbers now mimic their background soil counterparts. In addition, the contaminant may be below toxic levels and may actually be providing an additional carbon source for the microbes.

In contrast, the Tall Grass Prairie soil has a high clay content (28-40%). This makes contaminant infiltration much more difficult due to the increased number of micropores. When this is coupled with the fact that the site had been contaminated for less than three months at the time of sampling, the effect of the contamination on microbes may not yet be severe. This is reflected in the fact that the contaminated Tall Grass Prairie soil has almost an identical number of aerobic heterotrophs as the background soil (Table A1.1). However, just as infiltration of the oil into the clay-rich soil is slower and more difficult than in a sandy soil, so will be the infiltration of any fertilizer intended to stimulate

degradation. Thus, future monitoring of aerobic heterotrophs may indicate rising microbial numbers in the uncontaminated soil as warmer soil temperatures prevail, and relatively low numbers may result in the contaminated zone as the presence of crude oil takes effect until a strong hydrocarbon degrading population becomes established.

The numbers of biosurfactant-producing bacteria found in the two uncontaminated soils tested were different by two orders of magnitude (Table A1.1). It was expected that the population trend would mimic that of the aerobic heterotrophs because, having never been contaminated, neither soil has experienced conditions which would select for microbes with an enhanced ability to utilize hydrocarbons as a nutrient source. However, because sampling at both sites occurred within two weeks apart in the early spring time, soil temperatures in Colorado may have been low enough to prevent strong encouragement of microbial growth. Thus, the numbers of biosurfactant producing bacteria were different between the two uncontaminated sites, and that difference was quite larger than the difference in aerobic heterotrophs.

The low and similar levels of biosurfactant producers between the contaminated sites at Ft. Lupton and the Tall Grass Prairie were a surprise when compared to levels of aerobic heterotrophs (Table A1.1). Both contaminated sites had similar conditions in that both are hydrocarbon impacted. The presence of hydrocarbons was expected to select for microorganisms able to survive and thrive in such an environment, such as surfactant producers, many of which are also capable of metabolizing hydrocarbons (Rouse et al., 1994; Willumsen and

Karlson, 1997; Volkering et al., 1998). This is particularly true for the contaminated Ft. Lupton site, which has been contaminated for at least 20 years. This continued, long term contamination at Ft. Lupton was expected to select for a significantly higher number of surfactant producing bacteria than the newly contaminated Tall Grass Prairie soil.

In regards to the Tall Grass Prairie site, the similar numbers of biosurfactant producers in both the contaminated and uncontaminated soils is not as questionable. The contamination occurred only three months prior to sampling. However, the timing of the contamination was in January, when cold air and soil temperatures provided little thermal push for microbial activity. Thus, although there may have been a new chemical selection for hydrocarbon degraders (and biosurfactant producers) to grow, the relatively short time span and low temperatures may have been enough to suppress this push and thus no significant difference between the contaminated and uncontaminated soil was seen at the time of sampling.

Identification of Biosurfactant Producing Bacteria

The initial isolation of suspected biosurfactant producers was done on Blood Agar plates, utilizing the ability of many biosurfactants to lyse erythrocytes, resulting in a band of beta hemolysis surrounding biosurfactant producing bacterial colonies (Bernheimer and Avigad, 1970; Banat, 1995a; Banat, 1995b; Lin, 1996). Such colonies were isolated and then maintained on PCA plates. Colony morphologies, growth patterns on various media, and microscopic

analyses indicated that although there were many colonies isolated, there were similarities among many of them. When the isolated colonies were grouped according to these similarities, nine well isolated colonies were chosen to represent each of these groups (Table A1.2).

Although the source of each of the colonies chosen for FAME analysis happened to be soils (contaminated or uncontaminated) from Ft. Lupton, all beta hemolytic colonies isolated on Blood Agar using soil from the Tall Grass Prairie were similar to one of the eleven different groups of colonies and thus are represented.

Of the nine different colony types analyzed, two genera predominated: *Bacillus* and *Pseudomonas* (Table A1.2). This is not surprising for two main reasons: first, the ability for members of both genera to produce biosurfactants is well documented (Banat, 1995a; Banat, 1995b; Georgiou, 1992; Rouse et al., 1994; Shafi and Khanna, 1995). Secondly, both genera are well known for members able to degrade or at least withstand exposure to a wide variety of hydrocarbons. Both the*Bacillus* and *Pseudomonas* species identified were common to both the contaminated and uncontaminated soils of the Ft. Lupton and Tall Grass Prairie sites.

The identification of colonies using Biolog gave similar results to those using FAME analysis (Table A1.2). Again, the vast majority of the identified cultures were either *Bacillus* or *Pseudomonous* species. It is worth noting that isolate A, identified by the FAME analysis as *Bacillus cereus*, was not tested by the Biolog system. This was because the colony was adhered too tightly to the

Table A1.2. Species of biosurfactant producing bacteria according to FAME and Biolog analyses.

Species Identification Via FAME Analysis	Species Identification Via Biolog Analysis
A. *Bacillus cereus*	(unable to analyze)
B. *Pseudomonas aureofaciens*	*Pseudomonas fluorescens*
C. *Pseudomonas fluorescens*	*Pseudomonas fluorescens*
D. *Pseudomonas fluorescens*	*Burkholderia vietmaniensis*
E. *Bacillus cereus*	*Bacillus cereus/thuringiensis*
F. *Bacillus thuringiensis*	*Bacillus mycoides*
G. *Bacillus cereus*	*Bacillus cereus/thuringiensis*
H. *Enterobacter*	*Enterobacter nimipressuralis*
I. *Pseudomonas putida*	*Burkholderia vietmaniensis*

BUG agar surface and thus removing the colony without attached media proved impossible. Since the first step in plating a culture onto the Biolog MicroPlate is to make a bacterial suspension with a precise turbidity, isolation of the colony from the media was essential.

According to Biolog protocols, the BUG medium should be the growth medium used immediately prior to inoculation of the MicroPlates. Isolate I was prepared in this manner as well as being plated onto a TSA plate prior to being analyzed by the Biolog system. Although the final species identity was the same, the statistical probabilities associated with the different media were different. The reported similarity to the system's data base for the bacteria cultured on the BUG medium was 0.53 while for the culture grown on TSA it was only 0.41.

A comparison of FAME identifications versus the Biolog identifications, although similar, reflects current debates in microbial taxonomy (Table A1.2). For example, the genus *Burkholderia* (found by Biolog) contains species that many consider to actually belong to the *Pseudomonas* genus (found for the same isolates, but by the FAME analysis). To add to the confusion of isolate identification, analysis of the 16S rRNA has led some to describe *Bacillus cereus* and *Bacillus thuringiensis* as "virtually identical" with "no differential features" (Priest, 1993). Similarly, some believe *Bacillus mycoides* to be a species separate from *Bacillus cereus* while others feel it is only a subspecies (Priest, 1999). Thus, with this in mind, it can be said that the differences between the FAME and Biolog identifications of the isolates are minimal.

In regards to the ability of the isolates to produce a biosurfactant, it is felt that enough evidence exists to believe that they do. In addition to the beta hemolytic characteristics of the colonies, there is an abundance of literature to support each of the identified genera (Kosaric, 1993; Banat, 1995a; Banat, 1995b; Georgiou, 1992; Rouse et al., 1994). The exception to this is isolate H, identified by both FAME and Biolog analyses as a member of the *Enterobacter* genus. It is believed that this isolate, common on the spread plates from the Ft. Lupton site, may have had its source in the agricultural nature of the area. Since *Enterobacter* is associated with animals, plants and soils, it can be hypothesized that the source of the bacteria is an area of farming whose groundwater flows into the monitored site. In June, 1998, *Enterobacter cloacae* was actually the most numerous cultured bacterium from impacted samples analyzed on both aerobic and anaerobic heterotrophic medium (Tanner, 1998). The mistaken beta hemolysis on the initial spread plate may have been an extension of a zone of clearing from a neighboring colony (which was also sampled off of the original plate) that extended into the agar around the area of the *Enterobacter*, causing both colonies to appear beta hemolytic.

Conclusions

In conclusion, biosurfactant-producing microorganisms are indigenous to soils and this presence is not exclusive to soils contaminated by hydrocarbons, as seen by the results in both the Ft. Lupton and Tall Grass Prairie background soils. The fact that these microbes are found in uncontaminated soils may reflect the

activities of biosurfactants other than that of hydrocarbon emulsification. This presence, though, is one that may be useful for such things as landfarm exercises where uncontaminated soil is added to a contaminated soil in order to lower contaminant levels to below toxicity so that remediation can begin. In addition, their presence may be helpful when searching for a potential microbe for use in tertiary oil recovery or remediation by increasing the sources from where researchers can look.

However used, though, this study shows that uncontaminated soils may be an untapped source of biosurfactant producing microbes which have not yet under gone selection pressures towards degradation of one specific contaminant class. This “flexibility” may allow for the selection of microbes that can, someday, act on entirely new compounds previously thought impossible to bioremediate.

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APPENDIX TWO

Biosurfactant-Producing Bacteria Found In Contaminated and Non-Contaminated Soils

ABSTRACT

Microbially produced surfactants have been studied for microbially enhanced oil recovery (MEOR) and the bioremediation of hydrocarbons . However, most of these studies have used biosurfactants produced by one of a small number of pure-culture microbes isolated in a laboratory. In previous work we determined that biosurfactant-producing microorganisms were naturally present at two hydrocarbon-impacted sites. In this study, we examine the prevalence of biosurfactant producers in uncontaminated soils. Biosurfactant producing bacteria were found to constitute a significant proportion (up to 35%) of aerobic heterotrophs. Biosurfactant producers were isolated. Isolates were identified primarily as strains of *Bacillus* and *Pseudomonas*.

INTRODUCTION

Biosurfactants are microbially produced surface active compounds. They are amphiphilic molecules with both hydrophilic and hydrophobic regions causing them to aggregate at interfaces between fluids with different polarities such as water and hydrocarbons (Banat, 1995a; Fiechter, 1992; Georgiou, 1992; Kosaric, 1993; Karanth et al., 1999). These biomolecules may also decrease interfacial surface tension (Lin, 1996; Shafi and Khanna, 1995; Rouse et al., 1994; Volkering et al.,1998; Fiechter, 1992; Georgiou et al., 1992; Karanth et al., 1999). Although the function of biosurfactants in microorganisms is not fully understood, it is known that these secondary metabolites can enhance nutrient transport across membranes, act in various host – microbe interactions, and provide biocidal and fungicidal protection to the producing organism (Lin, 1996; Banat, 1995a; Banat,1995b).

However, it is the ability of the biosurfactant producers to reduce interfacial surface tension that has important tertiary oil recovery and bioremediation consequences (Lin, 1996; Rouse et al., 1994; Volkering et al., 1998). Many of the known biosurfactant producers are also hydrocarbon-degrading organisms (Rouse et al., 1994; Willumsen and Karlson, 1997; Volkering et al., 1998).

In the past decade, many studies have reported the effects of microbially produced surfactants on bioremediation and enhanced oil recovery (Jack, 1988; Jenneman et al., 1984; Volkering et al., 1998). However, these studies typically

involved a single microbe or group of microbes isolated and identified in a laboratory and then applied to either *ex-situ* soil core experiments or injected into existing oil reservoirs for observation. In addition, the majority of these studies tested for enhanced biosurfactant production or hydrocarbon recovery and were conducted with only a few species such as *Bacillus licheniformis* strain JF-2, *Bacillus subtilis*, or *Pseudomonas fluorescens* (Adkins et al., 1992; Banat, 1995a; Banat, 1995b; Lin, 1998, McInerney et al., 1990). Few studies, though, have evaluated the presence of natural, indigenous biosurfactant producing microbes in oil recovery or bioremediation sites.

In a prior study, we reported the surprising presence of a large variety and number of biosurfactant producers isolated from two hydrocarbon-impacted sites (Jennings and Tanner, 1999). In this study, we isolated and identified biosurfactant producers from two additional soils that are unconnected to any hydrocarbon contamination and determined the proportion of aerobic heterotrophs that are biosurfactant producers in these soils.

MATERIALS AND METHODS

Sources of Environmental Samples

Sediments for this study were collected from two different locations. The first sample (RST soil) was taken from a piece of unimproved property located in Garvin County in central Oklahoma. This site is in a pristine, uncontaminated condition, and is comprised of a loamy clay soil rich in organic matter. Samples

were taken from just below the soil surface and stored at 4^{o} C until use (within 48 hours).

The second sampling location was the Tulsa Rose Garden in Tulsa, OK. Because the area is a professional, formal botanical garden, the soil system is highly manipulated. This manipulation includes the regular addition of heavy mulch layers to the flower beds as well as fungicides and pesticides. Samples of the Tulsa Rose Garden soil were taken from where the mulch litter meets the soil to a depth of approximately 10 cm deep. Samples were kept at 4^{o} C until use (within 48 hours).

Because comparisons will be made between these two samples and samples collected for the aforementioned study (Jennings and Tanner, 1999), brief descriptions of the prior samples follow: The first sample is from an active natural gas production site near Ft. Lupton, Colorado (Gieg et al., 1999). The soil in this area is classified as a sandy to sandy loam with a low organic content. During the 1970s, gas condensate contamination occurred and, although the source has been removed, residual contamination exists both in the soil and groundwater. Sediments for this study were collected in 1999 from beneath the shallow water table (~ 1.4 meters). Upgradient, uncontaminated sediments were also collected in addition to contaminated sediments.

The second set of soil samples collected for the 1999 study were from within the Tall Grass Prairie Preserve in Osage County, Oklahoma. In January 1999, 70 barrels of dewatered crude oil were spilled into a silty loam clay basin. Sediments from this site, like those from Ft. Lupton, were also collected in

March of 1999. Again, both contaminated and neighboring uncontaminated soils were collected for analysis.

Media Used

Blood Agar plates were used for the screening and isolation of potential biosurfactant producing bacteria (Bernheimer and Avigad,1970; ; Banat, 1995a; Banat, 1995b; Lin, 1996).

Full strength Plate Count Agar (PCA; Difco Laboratories, number 0751-17-2) was used for the maintenance of isolated biosurfactant-producing bacterial colonies and for counting the total number of aerobic heterotrophic bacteria (Atlas and Parks, 1993).

Tryptic Soy Broth Agar (TSBA; Difco product number 0369-17-6) slants were inoculated with colonies for a Fatty Acid Methyl Ester (FAME) analysis by Microcheck, Inc. (Sassar, 1990).

Biolog Universal Growth (BUG) medium (Biolog, Inc., Hayward, CA) was used for the preparation of isolates for the Biolog analysis according to Biolog protocols (Solit, 1999).

METHODS

Isolation of Biosurfactant Producers

One gram (wet weight) of sediment from the Tulsa Rose Garden site was serially diluted in 0.85% sterile saline. All dilutions were performed in triplicate.

Dilutions were spread-plated on Blood Agar plates (prepared one day prior) with final dilutions ranging from 10^{-1} through 10^{-4}. These were incubated at 30^{o} C and counted after 24 and 48 hours. After isolation, colonies were maintained at 30^{o}C on PCA plates.

The above screen for beta hemolytic bacteria were repeated for the RST soil sample.

Colony morphology, growth, and microscopic characteristics were recorded for isolates. Similar colonies were grouped together on the basis of microscopic analysis and colony morphology (Tate, 1995; Gerhardt et al., 1994)). For example, isolate A included colonies showing a flat, slightly moist, cream colored morphology with light feathering at the edges. Microscopic analysis of the bacteria in each of the colonies grouped together and designated "isolate A" revealed non-motile, Gram-positive rods sometimes linked in short chains. A representative colony of each group was then selected for further analysis.

In addition to the dilution plating of sediments onto Blood Agar plates, positive and negative controls were also plated. As a positive control, *Bacillus licheniformis* JF-2 (ATCC 39307) was used (McInerney et al., 1990). A mutant form of *Bacillus licheniformis* JF-2 that does not produce biosurfactant was plated as a negative control.

Enumeration of Total Aerobic Heterotrophs

Dilution plating was performed on both the Tulsa Rose Garden and RST soil samples in a similar manner as with the isolation of biosurfactant-producing

bacteria with the exception of substituting PCA for Blood Agar (Atlas and Parks, 1993). Final plate dilutions ranged from 10^{-2} to 10^{-5}.

Biolog Analysis

Representative colonies for each of the above groups were analyzed by the Biolog Microlog System for comparative identification according to Biolog, Inc. protocols (Solit, 1999).

Identification of Selected Cultures – FAME Analysis

Certain colonies that were difficult to identify by the Biolog Analysis were identified by using FAME analysis by Microcheck, Inc. (Northfield, VT) (Sassar, 1990).

RESULTS AND DISCUSSION

Bacterial Numbers

The number of total aerobic heterotrophs were very similar between the Tulsa Rose Garden and RST soil samples, ranging from 9.2×10^5 to 1.1×10^6, respectively (Table A2.1). This similarity was not surprising, given that both soil samples contained high amounts of organic matter. Biosurfactant producers ranged from 1.0×10^5 to 3.8×10^5 (Table A2.1).

Until recently, biosurfactant producers were thought to be restricted to contaminated soils where conditions would select for microbes with an enhanced

Table A2.1. Numbers of aerobic heterotrophs and biosurfactant producing bacteria from each of the soil samples.

	Average # Aerobic Heterotrophs	Average # Biosurfactant Producers	Percent Biosurfactant Producers
RST Soil			
Uncontaminated	1.1×10^6	3.8×10^5	35.0 %
Tulsa Rose Garden			
Uncontaminated	9.2×10^5	1.0×10^5	10.1 %
Tall Grass Prairie, OK [a]			
Uncontaminated	2.3×10^6	1.6×10^4	0.7 %
Contaminated	1.0×10^6	1.4×10^4	1.4 %
Ft. Lupton, CO [a]			
Uncontaminated	1.3×10^5	5.5×10^2	1.1 %
Contaminated	6.0×10^4	1.4×10^3	9.1 %

a: These two soils were initially studied as part of a prior project (Jennings and Tanner, 1999).

ability to utilize hydrocarbons (Rouse et al., 1994; Willumsen and Karlson, 1997; Volkering et al., 1998). However, small levels of biosurfactant production has been demonstrated in unimpacted soils and may be a reflection of the other roles biosurfactants play in a soil ecosystem, functioning as biocides, fungicides, and nutrient transport molecules (Lin, 1996; Banat, 1995a; Banat, 1995b; Jennings and Tanner, 1999). Therefore, detecting a biosurfactant producer population from within the RST and Tulsa Rose Garden soils was not necessarily surprising.

Based on prior observations, we expected to recover a significant population of biosurfactant producing bacteria (Table A2.1). However, the extent of the biosurfactant producing population surpassed expectations in these two hydrocarbon-unimpacted soils. Biosurfactant producers constituted between 10 – 35 % of the aerobic heterotrophic bacterial population (Tulsa Rose Garden and RST soils, respectively) (Table A2.1). The reason that these numbers are so impressive is that the majority of the Ft. Lupton and Tall Grass Prairie samples had biosurfactant-producing populations at approximately 1 % (Jennings and Tanner, 1999) (Table A2.1).

The high values for the Tulsa Rose Garden and RST soils may be explained by the relationship between the amount of soil organic matter and the size of the biosurfactant population. For example, Ft. Lupton and Tall Grass Prairie sediments that are characterized by low organic matter, had low percentages of biosurfactant producers. In contrast, the two soils with the higher organic matter content, the Tulsa Rose Garden and RST soils, had high fractions of biosurfactant producers (Table A2.1).

In organic rich soils, microorganisms tend to be metabolically stimulated (Brady and Weil, 1999). As bacterial metabolism is increased, so must be those compounds such as biosurfactants that aid in transporting various nutrients across cell membranes in order to support this growth (Lin, 1996; Banat, 1995a; Banat,1995b). This may explain why the RST and Tulsa Rose Garden soils have a higher percentage of biosurfactant producers over the Ft. Lupton and Tall Grass Prairie soils. However, it does not explain the discrepancy between the Tulsa Rose Garden and RST soils.

The fraction of biosurfactant producers was three times the level in the RST soil than in the Tulsa Rose Garden soil (Table A2.1). One of the obvious differences between the two soils is the presence of pesticides at the Tulsa Rose Garden site. Koehler (1994) analyzed the effects of the pesticide Aldicard on various soil mesofauna and microorganisms, finding that application of the pesticide had long-term effects the microbial population within the test site, including fungi.

It was found that many classes of fungi actually increased in numbers after pesticide application. This has implications for the Tulsa Rose Garden site because many biosurfactants are produced as fungicides, and the increase in numbers of biosurfactant producers in comparison to the Ft. Lupton and Tall Grass Prairie soils (which were not treated with pesticides) may be a response to the increased competition for nutrients by such fungi.

However, this increase due to fungicidal activity may be overshadowed by the biosurfactant's role as a nutrient transporter when microbial growth is as

competitive as it may be at the RST site. Where as at the Tulsa Rose Garden a high percentage of the soil organic material has been manually added in easily degradable forms to enhance flower blooms, the soil organic material at the RST site is primarily in the original form of natural or indigenous plant matter. The high amounts of soil nutrients, including nitrogen and phosphorus, removed from the soil by grasses (which predominated on the site) can easily result in soils depleted of these nutrients (Brady and Weil, 1999; Salisbury and Ross, 1992). This nutrient depletion, even if slight, can decrease the numbers of microbes in a soil. It has been documented that biosurfactant production is actually stimulated when certain nutrients are limited, specifically nitrogen and phosphorus (Shafi and Khanna, 1995). Thus, with non-biosurfactant producing bacterial populations decreasing in conjunction with selection towards biosurfactant producing ones, the ratio of producers to non-producers might increase. Therefore, this may explain why the RST soil has a much higher percentage of biosurfactant producers than the Tulsa Rose Garden soil.

Identification of Biosurfactant Producing Bacteria

The initial isolation of suspected biosurfactant producers was done on Blood Agar plates, utilizing the ability of many biosurfactants to lyse erythrocytes which results in a band of beta hemolysis surrounding biosurfactant producing bacterial colonies (Bernheimer and Avigad, 1970; Banat, 1995a; Banat, 1995b; Lin, 1996). Such colonies were isolated and then maintained on PCA plates. Colony morphologies, growth patterns on various media, and microscopic

analyses indicated that although there were many colonies isolated, there were similarities among many of them. When the isolates were grouped according to these similarities, select colonies were chosen to represent each of these groups.

Of the different colony types analyzed, a total of four different species were identified: *Pseudomonas fluorescens*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus sphaericus.* These results were expected for two main reasons: first, both genera are common soil organisms, and second, the ability for members of both genera to produce biosurfactants is well documented (Banat, 1995a; Banat, 1995b; Georgiou, 1992; Rouse et al., 1994; Shafi and Khanna, 1995).

Conclusions

In conclusion, biosurfactant producing bacteria appear to be found in soils which have not been exposed to hydrocarbon contamination, and they seem to predominately be members of the *Bacillus* and *Pseudomonas* genera. In addition, these bacteria appear to be a significant proportion of the aerobic heterotroph population. Finally, the amount of organic matter present in the soil may effect this proportion significantly as may the availability of the organic matter and other required soil nutrients.

Potential future study of this phenomenon includes the utilization of antibody probes to search not for those bacteria with the potential to produce biosurfactants, but for the biosurfactants themselves within the soil matrix. In

addition, further investigations into the effects of soil organic matter as well as pesticide and herbicide application are being considered.

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