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Kevin G. Robinson

Mriganka M. Dhar

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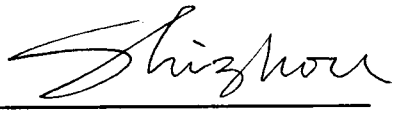
Signature 

Date April 28, 1995

IMPACT OF BIOSURFACTANT (RHAMNOLIPID R1) ON THE SOLUBILIZATION, MOBILIZATION, AND MINERALIZATION OF PCBs

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

ZHOU SHI

May, 1995

To Jun and Phebe

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ABSTRACT

Polychlorinated biphenyls (PCBs) are wildly distributed environmental contaminants which extensively bind to soil thereby limiting their bioavailability. Removal of sorbed PCBs is an essential step in enhancing the biodegradation potential of bound contaminants. Surfactants have been effectively used to desorb hydrophobic contaminants (including PCBs) from soils, however, the impact of surfactant washing on contaminant bioavailability remains unclear. The major purpose of this research is to study the treatment effectiveness of a microbially produced surfactant (biosurfactant) on the biological fate of selected PCBs.

Batch treatments were used to evaluate the impact of biosurfactant (Rhamnolipid R1) on the solubilization of non-aqueous phase 4CB and 4,4'CB and the mobilization of soil-bound 4,4'CB. Microbial utilization of 4,4'CB in the presence of biosurfactant was then evaluated by monitoring

production of $^{14}CO_2$ after addition of an acclimated bacterial culture.

Rhamnolipid R1, produced by *P. aerugenosa* (PRP652), linearly increased the apparent solubility of 4CB and 4,4'CB with increasing biosurfactant concentration (above the CMC). At a biosurfactant concentration of 4 g/L, the enhanced solubilities of mutual solutes, 4CB and 4,4'CB, were 56 and 105 fold over their respective intrinsic solubilities. Biosurfactant R1 was also an effective agent in mobilization of 4,4'CB from contaminated soils. A 4.0 g/L biosurfactant solution desorbed greater than 47% of soil-bound 4,4'CB in the first wash and greater than 85% after three washes.

Mineralization of 4,4'CB by *Alcaligenes eutrophus* (A5) was enhanced in the presence of biosurfactant. Both the mineralization rate and total amount of 4,4'CB mineralized were elevated through biosurfactant addition. For a biosurfactant concentration of 4.0 g/L, overall average mineralization rates were 1.07 and 4.43 times that measured

in controls (no biosurfactant) for solutions with and without excess 4,4'CB crystals. Mineralization enhancements were more pronounced when biosurfactant concentrations above the CMC were utilized.

The total mass of 4,4'CB mineralized in biosurfactant solutions (without excess 4,4'CB crystals) exceeded the total initial mass of 4,4'CB dissolved in biosurfactant free solutions, which confirmed that micellized 4,4'CB was biodegradable. Co-mineralization of biosurfactant was at a minimum during periods of high 4,4'CB mineralization. An enhanced mineralization of 4,4'CB in the biosurfactant soil wash fluids was also observed.

In general, solubilization and mineralization of selected PCBs were more pronounced when biosurfactant concentrations above the CMC were utilized. This fact confirmed that micelles, which only form above the CMC, played an important role in PCB solubilization and mineralization enhancement. The results of this research provide clear evidence that biosurfactant soil washing

followed by pure culture biological treatment of the wash fluid is a promising technology for PCB soil remediation.

Keywords: Biosurfactant, Rhamnolipid, Solubilization Polychlorinated biphenyl, PCB, Desorption, Mineralization

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LIST OF ABBREVIATIONS AND NOTATIONS

2CB: 2-chlorobiphenyl;

4CB: 4-chlorobiphenyl;

4,4'CB: 4,4'-dichlorobiphenyl;

^{14}C-4,4'CB: 4,4'-dichlorobiphenyl-uniformly labeled-^{14}C;

C: Aqueous biosurfactant concentration in equilibrium with soil, [mg/L];

C_{biosur}: Biosurfactant concentration, [mole/L];

CEB: Center for Environmental Biotechnology;

CMC: Critical micellar concentration, [mole/L] or [mg/L];

C_{R1}: Concentration of ^{14}C-R1 in acetone fraction, [mg/ml];

C_s: Concentration of PCBs sorbed to soil, [mg/kg_{soil}];

C_{surf}: Surfactant concentration, [mole/L];

C_w: Equilibrium concentration of PCB in solution, [mg/L];

DI: Deionized;

DOCs: Dissolved organic compounds;

DPM: Disintegrations per minute;

E: Mineralization extent, [%]

ECD: Electron capture detector;

f_{oc}: Organic carbon fraction (soil);

f_{tc}: Total carbon fraction (soil);

GC: Gas chromatography;

H: Henry's constant, [Pa*m/mole];

HOCs: Hydrophobic organic compounds;

K_m: Micelle/water partitioning coefficient, [dimesionless];

K_{mc}: Partition constant between water and micelles, [dimesionless];

K_{mn}: Partition constant between water and monomers, [dimesionless];

K_{oc}: Soil organic carbon/water partition coefficient, [L/kg];

K_{ow}: Octanol/water partition coefficient, [dimesionless];

K_p: Soil/water partition coefficient, [L/kg];

LSC: Liquid scintillation count;

LC: Liquid chromatography;

MS: Mineral salts;

MSR: Molar solubilization ratio;

MW: Molecular weight;

OD: Optical Density;

PAH: Polycyclic aromatic hydrocarbon;

PB: Phosphate buffer;

PCBs: Polychlorinated biphenyls;

PIA: *Pseudomonas* isolation agar;

PMB: Phosphate buffered mineral salt medium with biphenyl as sole carbon source;

PMG: Phosphate buffered mineral salt medium with glycerol or glucose as sole carbon source;

POE: Polyoxyethylenes

P_{R1}: Purity of biosurfactant, [%]

q_e: Equilibrium concentration of biosurfactant sorbed to soil, [mg/g];

R: Rhamnolipid (biosurfactant);

r: Average substrate utilization rate, [mg/(hr*L)];

RA'_{biosur}: Total ^{14}C activity of the ^{14}C-biosurfactant placed on the TLC plate, [DPM];

RA_{R1}: Radioactivity of the purified ^{14}C-R1 in acetone fraction, [DPM/ml];

RA'_{R1}: ^{14}C activity of the R1 placed on the TLC plate, [DPM];

R_f: Mobility of R1 on TLC plate;

S: PCB intrinsic (aqueous) solubility, [mg/L];

SA: Surface area of soil particles, [m^2/g];

SA_{R1}: Specific activity of ^{14}C-R1, [DPM/mg];

S_{cmc}: Apparent solubility of solute at surfactant concentration of CMC, [mole/L];

SDBS: Sodium dodeylbenzene-sulfonate;

S_i: Initial substrate concentration, [mg/L];

S_{mic}: Total apparent solubility of solute at surfactant concentration above CMC, [mole/L];

S_w: Intrinsic solubility of the solute in water, [M/L];

S^*_w: Apparent solubility of solute in surfactant solution, [M/L];

SR_{PCB}: Specific ratio activity of PCB, [mCi/mM];

SR_{R1}: Specific ratio activity of Rhamnolipid R1, [mCi/mM];

t: Incubation time, [hr];

t_c: Critical incubation time, [hr];

TC: Total carbon;

TLC: Thin layer chromatography;

TVA: Tennessee Valley Authority

UL: Uniformly labeled;

V_w: Molar volume of water, [L/mole];

v_o: Volume fraction of solute in octanol phase, [dimensionless];

v_s: Volume fraction of solute in micelle in dilute condition, [dimensionless];

V_s: Molar volume of surfactant, [L/mole];

v^*_w: Volume fraction of solute in octanol-saturated water phase, [dimensionless];

v_w: Volume fraction of solute in distilled water;

X_a: Molar fraction of the solute in the aqueous pseudophase, [dimensionless];

X_m: Molar fraction of the solute in the micellar pseudophase, [dimensionless];

X_{mn}: Concentration fraction of surfactant as monomers, [dimensionless];

X_{mc}: Concentration fraction of surfactant as micelles, [dimensionless];

YEPG: Yeast extract, polypeptone, and glucose medium.

CHAPTER 1 INTRODUCTION

Polychlorinated biphenyls (PCBs) are a class of chlorinated aromatic compounds consisting of 209 congeners which differ in the number and position of chlorine atoms on the biphenyl rings. Due to the chlorinated ring structure, PCBs are very hydrophobic and chemically stable (Sawhney, 1986; Hutzinger et al., 1974). As a result, they tend to extensively partition onto soil and resist chemical and biological degradation thereby presenting a difficult remediation problem. Their toxicity, potential carcinogencity, as well as ubiquitous distribution in the environment are of great concern.

Studies concerning the remediation of PCBs in the environment have shown that their low intrinsic aqueous solubilities limit many restoration efforts. It has been demonstrated that increasing chlorination of the PCB molecule results in a reduction in aqueous solubility (Sawhney, 1986; Shiu and Mackay, 1986; Hutzinger et al., 1974) and biodegradation potential (Bedard et al., 1986). In addition, PCBs with higher chlorine content bind more extensively to soil and were more difficult to remove due to their stronger association with soil materials (Haque

and Schmedding, 1979). Because mobilization of sorbed PCBs is an essential step in many soil remediation processes, enhanced release from contaminated soil via an increase in apparent aqueous solubility is a critical step in any treatment scenario.

Use of surfactant as a potential tool in environment restoration of sparingly soluble xenobiotics has become a subject of increasing study. Because surfactants have both hydrophobic and hydrophilic moieties in their structure, they can dramatically increase the apparent solubility of hydrophobic organic compounds (HOCs), including PCBs, by facilitating HOC partitioning into the hydrophobic core of surfactant micelles (Kile and Chiou, 1989; Chiou, et al., 1986). Therefore, surfactants can be effective agents in enhancing desorption of PCBs from soil (McDermott, 1989). Synthetic surfactants, while effective at increasing the aqueous solubility of HOCs, have not always enhanced HOC biodegradation (Lahu, 1991). In addition, the recalcitrant nature of many synthetic surfactants may lead to additional environmental pollution problems if used for in-situ remediation.

Biosurfactants, produced during bacterial utilization of sparingly water soluble HOCs (Itoh and Suzuki, 1972),

are excreted into the environment as a natural component of the microbial life cycle (Hommel, 1990). Though little is known about the biodegradation of biosurfactants, it was recently confirmed that biosurfactants do enhance the aqueous solubility and biodegradation of certain HOCs (Falatko and Novak, 1992; Dyke, et al., 1993; Zhang and Miller, 1992). Use of biosurfactants to enhance biodegradation of PCBs by increasing their apparent aqueous solubility is convincible. Biosurfactants provide an alternative to the potentially recalcitrant synthetic surfactants for the treatment of sparingly soluble organic contaminants.

The overall goal of this study was to evaluate the impact of a biologically produced surfactant (Rhamnolipid R1) on the solubilization, mobilization, and mineralization of selected PCB congeners. The specific objectives of the research were:

1. Determine the apparent aqueous solubility of target PCBs in biosurfactant solutions above and below the CMC and evaluate the role of micelles in PCB solubility enhancement;

2. Evaluate the impact of biosurfactant in the mineralization of 4,4'CB using a substrate specific bacteria [*A. eutrophus* (A5)];

3. Determine the effectiveness of biosurfactant in desorption of soil-bound PCB as well as bioavailability of PCB in the soil wash fluid.

CHAPTER 2 LITERATURE REVIEW

2.1 INTRODUCTION

The extensive use of polychlorinated biphenyls (PCBs) has resulted in their wide distribution in the environment. The negative impacts of PCBs on environmental quality and public health have been well documented. Due to their hydrophobicity and stability, desorption and degradation processes which naturally occur at contaminated sites are very slow. However, biosurfactants have the potential to accelerate the rate of desorption thereby enhancing biodegradation of PCBs at contaminated sites. Though researchers have addressed accelerated desorption of PCBs from soil using synthetic surfactants (McDermott, et al., 1989) and enhanced biodegradation of aliphatic hydrocarbon using biosurfactants (Zhang and Miller, 1992, 1994; Oberbremer, 1990; Jain et al., 1992), the impact of biosurfactant on PCB solubility, mobilization and biodegradation has not been well documented. This

literature review will summarize and discuss information and research results relating to the main goals of the study.

2.2 GENERAL BACKGROUND OF POLYCHLORINATED BIPHENYLS (PCBs)

Polychlorinated biphenyls (PCBs) consist of 209 congeners with the general formula $C_{12}H_{10-n}Cl_n$ (n=1-10). These compounds are systematically named according to the number and position of chlorine atoms on the biphenyl ring (Figure 2.1)

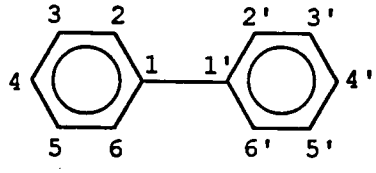


Figure 2.1 General Structure of PCBs

Although synthesis of PCBs was first described by Schmidt and Schultz in 1881, PCBs were not industrially produced until 1929 (Chairns et al., 1986). Since that

time, PCBs have been widely used as dielectric and heat transfer liquids due to their outstanding thermal stability, oxidation inertness, as well as dielectric properties (Hutzinger, et al., 1974). It has been estimated that $\approx 2.0 \times 10^6$ tons of PCBs have been produced world-wide, and a total of 5.0×10^5 tons of PCBs were sold in the USA between 1930 and 1975. Only 4% of the PCBs in the USA were destroyed while 12 % of the PCBs produced were released to the environment (ITFP, 1972)

Upon introduction to the environment, PCBs tend to partition to nonpolar phases, such as soil, sediment, and other organic containing materials due to their highly hydrophobic nature. Frank et al. (1977) reported that PCB levels in sediments of Lake Erie and Lake St. Clair were 0.252 and 0.019 mg/kg, respectively. In the sediment of Lake Superior, the average concentration was reported as 0.17 mg/kg (Eisereich et al., 1979). A U.S. Geological Survey study found up to 0.05 mg/kg PCB in some Pennsylvania lake sediments and from 0.01 to 3.2 mg/kg in seven Florida lake sediments. Also, the Tennessee Valley

Authority (TVA) has reported PCB levels as high as 7.0 mg/kg in the sediments collected from Ft. Loudon Reservoir, an impoundment of the Tennessee River (Pettigrew, 1989).

Beyond partitioning into abiotic systems, PCBs have also been shown to accumulate in biotic systems such as green algae (Urey et al., 1976), zooplankton species (Clayton et al., 1977), and bacterial strains (Furukawa, et al., 1978). PCBs can further accumulate in fish and bivalves through food chain interactions (Amico et al., 1979). Toxic effects of PCBs in human beings have also been reported. They include generalized chlorine, melanin deposition in gums and nails and liver necrosis (Kikuchi and Masuda, 1976). Children born to individuals who were exposed to PCBs have a generalized disturbance of ectodermal tissue (Rogan et al., 1988) and abnormalities of the teeth, lungs, and skin. In addition, the exposed children were developmentally delayed, lighter, and shorter than the control group.

As a result of widespread application and chemical stability, PCBs have become a ubiquitous environmental contaminant.

2.3 CHARACTERISTICS OF SURFACTANTS

Materials that possess the ability to modify interfacial interactions are referred to as surface active agents or surfactants (Myers, 1992). All surfactants have a amphiphilic or amphipathic structure which consists of at least one lyophobic group and one lyophilic group. The lyophobic group has little attraction for a polar solvent while the lyophilic group has a strong attraction for a polar solvent. For an aqueous/gas phase system, the interfacial interaction is defined as surface tension, and the lyophobic group and lyophilic group are referred as the hydrophobic group (or "head") and hydrophilic group (or "tail"), respectively. Based on the ionization state of hydrophilic group in water, surfactants are commonly classified into four groups: anionic, cationic, nonionic,

and amphoteric (molecule contains or potentially contains both negative and positive charges).

The presence of surfactants in aqueous solution will increase the free energy of the system, however, surfactant molecules tent to concentrate at interfaces to minimize the free energy increase in the system. So at the interface of water and air, the surface tension (which is thermodynamically defined as the surface excess free energy per unit area of interface) will decrease. When all interfaces are saturated, the overall free energy reduction for the system goes through a special phenomena, that is, the surfactant molecules aggregate together forming micelles which are stable in the solution. The surfactant concentration at which the micelles begin to form is termed the critical micellar concentration (CMC).

In general, most surfactants in aqueous solution can aggregate into micelles with aggregation numbers (average number of monomers per micelle) of 30-200. In the micelles, hydrophobic portions of the molecules are associated and

mutually protected from extensive contact with the polar bulk aqueous phase, forming a hydrophobic core. The shapes of commonly encountered micelles are sphere, ellipsoidal, disk-shaped, and rod-like structures.

Micelles are very important in solubility enhancement of hydrophobic compounds such as PCBs. In aqueous solutions, nonpolar additives such as hydrocarbons are intimately associated with the core of the micelle, while slightly polar materials such as fatty acids, alcohols, and esters are usually located between the core and surface of the micelles. The solutes can also be found at the surface of micelles for some nonionic surfactants with larger polar head groups such as polyoxyethylenes (POE) (Myers, 1992).

There are many internal factors (hydrophobic structure, head group type of surfactants) and external factors (temperature, pH) which can affect the solubilization characteristics of surfactants. It is generally observed that the longer the hydrophobic chain, or the less hydrophilic the head group of a surfactant, the

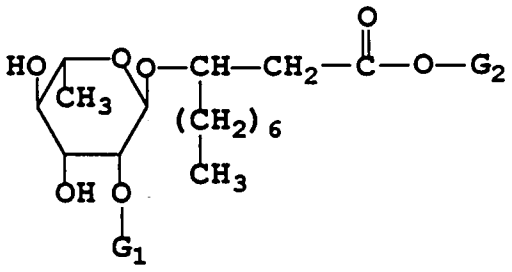
more capable the surfactant becomes in solubilization of hydrophobic compounds. Branching of hydrocarbon chains in the surfactant molecule usually results in a decrease in its solubilization power. For different types of surfactants with a same hydrophobic tail, the order of the solubilization power becomes: nonionics > cationics > anionics. Higher temperatures will increase the solubilization ability of nonionic surfactants by increasing the aggregation number of micelles. Changing of pH will greatly affect the solubility power of ionic surfactants but will have little impact on nonionic surfactants. Further, addition of polar solutes such as phenols and long-chain alcohols can greatly increase the solubility of nonpolar solutes in ionic surfactant solutions because the insertion of polar solutes between adjacent head groups in the micelle increases the space available for additional solubilized molecules. In summary, the solubilization power of surfactants depends on the whole system - solvent, surfactant, and solute.

2.4 CHARACTERISTICS AND PRODUCTION OF BIOSURFACTANTS

Biologically produced surface active compounds are termed biosurfactants. Biosurfactants can be produced by bacteria, yeast, and fungi during cultivation on various carbon sources, in particular during growth on sparingly soluble hydrocarbons. The biosurfactants produced by bacteria are either nonionic or anionic. No bacteria-produced cationic biosurfactant has been reported in the literature to date (Lang and Wagner, 1987; Pajarron et al., 1993; Shaw, 1970, 1974). Based on their biochemical structure, biosurfactants can be grouped into six general classes: hydroxylated and crosslinked fatty acids; glycolipid; polysaccharide-lipid complexes; lipoproteins-lipopetides; phospholipids; and complete cell surface (bacterial cells have demonstrated surface activity because their surface is composed of a mosaic of hydrophobic and hydrophilic moieties) (Zajic and Seffens, 1984). All biosurfactants have both hydrophobic and hydrophilic moieties as part of their chemical structure. The common hydrophobic moiety is a hydrocarbon chain of fatty acid.

The hydrophilic group is derived from either one or more of the following groups: ester or alcohol functional groups of neutral lipids; carboxylate groups of fatty acids or amino acids; phosphate-containing portions of phospholipids; or carbohydrates of glycolipids. The amphipathic structure of biosurfactants enhances their surface active properties. Due to their surface activity, biosurfactants have been widely used in agriculture, food, health care, petrochemicals and mining and mineral processing (Kosaric, et al., 1987) and recently in the remediation of environmental contamination.

Rhamnolipids are anionic biosurfactants which are a sub-group of glycolipids. The basic structure of rhamnolipids is shown in Figure 2.2. They possess one or two sugar rhamnose units and one or two fatty acid residues. There are four possible structures named as R1, R2, R3, and R4, respectively (Lang and Wagner, 1987, Pajarron, et al., 1993, Shaw, 1970, 1974).



R1: G_1=L-α-Rhamnopyranosyl-

G_2=β-Hydroxydecanoic acid

R2: G_1=H

G_2=β-Hydroxydecanoic acid

R3: G_1=L-α-Rhamnopryanosyl-

G_2=H

R4: G_1=H

G_2=H

Figure 2.2 Chemical Structure of Four Rhamnolipids Produced by Various *Pseudomonas* Species (adopted from Lang and Wagner, 1989).

The surface activity of the four rhamnolipids have been summarized in Table 2.1 (Lang and Wagner, 1987). Though their CMCs vary, these rhamnolipids appear to be equally effective in reducing surface tension. Ishigami et al. (1987) investigated the effect of pH on the aggregate structure of the rhamnolipids at concentrations above their CMC. It was found that for pH ranges of 4.3-5.8, 6.0-6.6, and >6.8, the aggregate of rhamnolipid was vesicle, lamella, and micelle, respectively. It was also reported that rhamnolipids with two fatty acids are soluble in methanol, chloroform, ethyl ether and $NaHCO_3$ solutions but insoluble in n-hexane (Jarvis and Johnson, 1949, Itoh et al., 1971, Lang and Wagner, 1987).

Rhamnolipids are produced by *P. aeruginosa* and other *Pseudomonas* species. Production can be regulated by carbon substrates, nitrogen source, multivalent cations, EDTA, temperature and pH. The best yield of rhamnolipids by *Pseudomonas aeruginosa* was observed when n-alkane, the least water soluble compound studied, was used as the carbon source rather than glycerol, glucose, or ethanol

Table 2.1 Characteristics of Rhamnolipids.

Rhamno-lipid	Minimum surface tension (dynes/cm)	CMC* (mg/L)	Minimum interfacial tension (dynes/cm)	CMC** (mg/L)
R1	27	10	<1	5
R2	26	20	<1	40
R3	30	200	<1	200
R4	25	200	<1	200

* in solution of NaCl 100 g/L, $CuCl_2$ 28 g/L, $MgCl_2$ 10 g/L,

** against n-hexadecane.

(Yamaguchi, et al. 1976, Hisatsuka, et al., 1971). When glycerol was used as the substrate, rhamnolipid production by *P. aeruginosa* was sharply reduced by adding glucose, acetate, succinate, or citrate to culture medium because these added organic substrates inhibited the formation of adaptive enzymes for the synthesis of rhamnolipid using glycerol (Hauser and Karnovsky, 1954).

The N or P source can also be important keys to regulate biosurfactant synthesis. It was observed that a decrease of surface tension by *P. aeruginosa* after N-limitation (Syldatk and Wagner, 1987) and the production of rhamnolipid began only at low nitrogen levels. The maximum yield of rhamnolipid was reported at C/N ratios of 6.6-38 (Manresa, et al., 1991, Guerra-santos, et al., 1984, Ramana and Karanth, 1989, and Hommel, 1990) and C/P ratios under 16 (Guerra-santos, et al., 1984).

A concentration reduction of multivalent cations (such as Fe^{2+}, Mg^{2+}, or Ca^{2+}) in culture media can result in enhanced production of rhamnolipid (Guerra-santos, et al.,

1984, 1986, Syldatk and Wagner, 1987, and Ramana and Karantu, 1989), while the presence of EDTA strongly inhibited rhamnolipid production by *P. aeruginosa* (Syldatk and Wagner, 1987, Goswami and Singh, 1991). Researches have also measured improved biosurfactant yields by *P. aeruginosa* at pH 6.2-6.6 and temperatures between 32-34 °C (Guerra-santos, et al., 1986). Rhamnolipids were also successfully produced by resting cells (Syldatk and Wagner, 1987), pilot-scale continuous culture methods (Reiling et al., 1986), and genetically constructed bacteria (Koch, et al., 1988).

2.5 PCB AQUEOUS SOLUBILITY AND ENHANCEMENT

2.5.1 Aqueous Solubility

PCBs are a group of xenobiotic chemicals with low intrinsic water solubilities. Important characteristic properties (including solubilities) for PCBs were reviewed by Shiu and Mackay (1986), and Chou and Griffin (1986). For all the 209 pure congeners, solubilities at 25 °C ranged

from 0.0000012 ± 0.0000005 mg/L to 7.0 ± 0.5 mg/L. In general, solubility decreased with increasing chlorination of the molecule. The aqueous solubility of commercial Aroclor (mixtures of different PCB congeners) are summarized in Table 2.2.

Solubility of PCBs can be affected by many factors such as temperature, co-solutes, and extent of chlorination of PCB congener. Changing temperature of the solvent will change the solubility of PCBs. For example, the solubility of Aroclor 1254 increased from 0.037 mg/L to 0.119 mg/L when the temperature changed from 22 to 65 °C (Opperhuizen, 1986). It was also found that the aqueous concentration of individual congeners in the commercial PCB mixture was much lower than the solubility when individual congeners were the sole contaminant in solution (Opperhuizen, 1986). The reduction in congener solubility in the mixture was believed due to co-solute effects. This result contradicted what was reported by Li (1993) which showed that there was no significant effect of co-solutes on solubility of the tested PCB congeners. The chlorination extent of PCB

Table 2.2 Solubility of Commercial PCBs (Aroclor).

Aroclor	Approx.* Cl Wt.,%	Average* Mol. Wt	Solubility (mg/L)	Tempture (°C)	Water	Reference
1221	21	193.7	3.5			Chou (1986)
			5.0		fresh	Zita (1970)
			3.8		sea	
1242	40-42	257.5	0.2			Hutzinger (1974)
			0.288			Chou (1986)
1248	48	291.9	0.1			Hutzinger (1974)
			0.054			Chou (1986)
1254	52-54	326.4	0.04			Hutzinger (1974)
			0.042			Chou (1986)
			2-3		fresh	Zitka (1970)
			1-1.5		sea	Zitka (1970)
			0.0001		fresh	Schoor (1975)
			0.00004		NaCl(30g/L)	Schoor (1975)
			0.012-0.3		Deionized	Opperhuizen (1986)
			0.037±0.004	22	Deionized	Opperhuizen (1986)
			0.068±14	45	Deionized	Opperhuizen (1986)
			119±12	65	Deionized	Opperhuizen (1986)
1260	62	388.4	0.025			Hutzinger (1974)
			0.0027			Chou (1986)

* Data adopted from Erickson (1992)

congeners can also affect solubility. The higher chlorinated congeners usually have lower aqueous solubilities. This effect leads to a decreasing percentage of highly chlorinated congeners in a PCB mixture after dissolution in water (Schoor, 1975, and Zitko, 1970). However, Opperhuizen (1986) reported that the aqueous distribution (percent) of a PCB mixture (Aroclor 1254) dissolved in water was close to the content of original Aroclor 1254, and this result was independent of the solvent temperature.

2.5.2 Solubility Enhancement by Dissolved Organic Compounds

The addition of dissolved organic compounds (DOCs), such as fulvic acids and surfactants, can enhance the solubility of PCBs. Chiou and his co-workers (1986, 1987) found that adding humic and fulvic acids from soil and aquatic origins increased the aqueous solubility of 2,2',4,5,5'CB and 2,4,4'CB. Their data showed that 1) a high degree of linearity existed between the apparent aqueous solubility of test solutes and the concentration of

DOCs; 2) the solubility enhancement of a solute with a given DOC was inversely related to the intrinsic solubility of the solute; 3) no competitive interference was found for individual solutes in the binary solute system; 4) efficiencies in enhancing solute solubility were a function of the polarity configuration and confirmation of the DOCs; 5) the solubility enhancement decreased at high pH value; 6) solubility enhancement of various nonionic organic solutes by DOCs was significant only for solutes whose intrinsic aqueous solubility were at least 2 orders of magnitudes lower than the concentration of DOCs; 7) carboxyl and carbohydrate contents (polar groups) were lower in commercial humics than in the natural humics, so the commercial humics were more effective in enhancing the solubility of the sparingly soluble solutes, and 8) increasing solubility by DOCs can be accomplished either by changing the overall solvency of the solution or through a direct solute interaction by adsorption or partitioning. It was further confirmed that the solubility enhancement of the solute resulted from a partitioning-like interaction between the solute and DOCs (regard as microscopic or

pseudo phase), and this interaction was governed primarily by van der Waals force (Chiou, et al., 1986). Zitko (1970) investigated the solubilization of commercial PCBs using a nonionic surfactant and found that the solubility of Aroclor 1221 and 1254 in the presence of surfactant was enhanced 2000 and 4000 times, respectively, compared to fresh water alone.

The solubility of three PCBs, 4CB, 2,4,6CB, and 2,2',4,4',6,6'CB, in a mixture of water/alcohol, was examined by Li and Andren (1994). They found that the completely water-miscible alcohols (methanol, ethanol, and 1-propanol) and the partial miscible alcohols (1-butanol, 1-pentanol, and 1-hexanol) significantly increased the solubility of the tested PCBs. The impact of cosolvency was inversely related to polarity. The solubility enhancement by non water-mixable alcohols (1-heptanol and 1-octanol) were negligible. The cosolvency effect of alcohols depended on the degree of chlorination of PCBs. The highly chlorinated congener tended to be solubilized to a greater extent.

2.5.3 Modeling Solubility Enhancement of PCBs

The aqueous solubility of PCBs can be enhanced in the presence of surfactants. This enhancement results from a "partitioning-like" interaction with the non-polar part of the surfactant molecule (Kile and Chiou, 1989b). Hence, the total dissolved PCB concentration (apparent solubility) included PCB intrinsic solubility and PCB associated with surfactant (monomers and micelles). The ratio of PCB apparent solubility to its intrinsic water solubility at a given temperature was described by Kile and Chiou (1989b) as following:

$$S^*_w/S_w = (1+X_{mn}K_{mn}+X_{mc}K_{mc}) \qquad (2.1)$$

where S^*_w: apparent solubility of the solute in the surfactant solution;

S_w: intrinsic solubility of the solute in pure water;

X_{mn}: concentration of the surfactant as monomers;

X_{mc}: concentration of the surfactant as micelles;

K_{mn}: partition constant for the solute between water and monomers;

K_{mc}: partition constant for the solute between water and micelles.

Edwards et al. (1991) developed an approach to evaluate the effectiveness of a surfactant in solubilizing a given solute. These researches defined the molar solubilization ratio (MSR) and micelle-phase/aqueous-phase partitioning coefficient(K_m) as:

$$MSR=(S_{mic}-S_{cmc})/(C_{surf}-CMC) \qquad (2.2)$$

$$K_m=X_m/X_a$$
$$=MSR/((1+MSR)S_{mic}V_w) \qquad (2.3)$$

where S_{mic}: total apparent solubility of solute at surfactant concentration above CMC, M;

S_{cmc}: apparent solubility of solute at surfactant concentration of CMC, M;

C_{surf}: surfactant concentration at which S_{mic} is

evaluated, M;

X_m: molar fraction of the solute in the micellar pseudo phase;

X_a: mole fraction of the solute in the aqueous pseudo phase;

V_w: molar volume of water, 0.0185 L/mole at 25 °C.

Jafvert, et al. (1994) further correlated the partitioning coefficient (K_m) to the octanol/water partitioning coefficient (K_{ow}):

$$K_m = K_{ow} V_s V_s v^*_w / (v_o v_w) \qquad (2.4)$$

where v_o: volume fraction of chemical in octanol phase;

v_s: volume fraction of solute in micelles in dilute conditions;

v^*_w: volume fraction of solute in octanol-saturated water phase;

v_w: volume fraction of solute in distilled water;

V_s: molar volume of surfactant;

K_{ow}: octanol/water partitioning coefficient.

2.6 PCB-SOIL INTERACTIONS

2.6.1 Sorption

Sorption of PCBs by soils is the major nondestructive process affecting PCB concentration after introduction into the aquatic environment. Sorption has been suggested to be hydrophobic, which is the partitioning of the nonpolar solute (PCBs) out of the polar solvent (water) onto hydrophobic sites on the soil surface. The hydrophobic sites are primarily associated with the soil organic mater. The main driving forces for sorption are the repulsion between the water and PCB molecules, as well as the attraction between soil organic matter and PCBs. This mechanism can well explain why the higher chlorinated (more hydrophobic) PCB congeners not only sorb to a greater extent but are also held more tightly by the soil than the less chlorinated congeners (Sawhney, 1986, and Chou and Griffin, 1986).

The amount of PCBs sorbed by soils at equilibrium has been described by the following simple linear relation (Chou and Griffin, 1986):

$$C_s = K_p C_w \qquad (2.5)$$

where C_s: amount of PCBs sorbed to soil, mg/kg_{soil};

C_w: equilibrium concentration of PCBs in solution, mg/L;

K_p: sorption constant, L/kg.

K_p values of selected soil materials used in PCB sorption studies are listed in Table 2.3 (Chou and Griffin, 1986). Sorption of PCBs followed the series: Medium Temperature Coal Char > High Temperature Coal Char > Catlin Soil > Montmorillonite Clay > Ottawa Silica Sand (Chou and Griffin, 1986).

A three-variable regression analysis of the PCB sorption constant (K_p), total organic fraction (f_{oc}), and surface area (SA_1) was developed by Lee et al. (1979):

Table 2.3 Sorption Constant (K_p), Total Organic Carbon Fraction (f_{oc}), and Surface Area (SA) of Selected Soil Materials Used in PCB Sorption Studies.

Sorbent	K_p (L/kg)	f_{oc} (%)	SA (m^2/g)
Ottawa silica sand	22	<0.1	0.4
Montmorillonite clay	172	0.93	20.1
Montmorillonite clay (LTA*)	145	0.13	20.2
Catlin silt loam	532	4.73	26.5
Catlin, 6 hr (LTA*)	472	4.37	25.4
Catlin, 12 hr (LTA*)	310	3.64	24.5
Catlin, 336 hr (LTA*)	239	1.84	23.8
Medium temp. coal char 650 °C	1938	74.04	253
Medium temp. coal char 650 °C (LTA*)	1432	64.04	214
High temp. coal char 980 °C	1220	76.62	44
High temp. coal char 980 °C (LTA*)	1174	32.14	120

* TLA: low temperature ashed sample

$$K_p = 188 + 3.36\ SA_1 + 11.4\ f_{oc} \qquad (2.6)$$

The magnitude of the coefficients for SA and f_{oc} indicates that f_{oc} is the dominant factor in sorption. If only f_{oc} is considerated, equation 2.6 can be simplified as:

$$K_p = 255 + 18.5\ f_{oc} \qquad (2.7)$$

Because sorption of PCBs to soils is primarily due to hydrophobic interactions, a second relationship was developed in which K_p was correlated with the organic carbon partitioning coefficient, K_{oc}, and the total organic fraction of the sorbent, f_{oc} (Mackay, D., 1991; Chou and Griffin, 1986):

$$K_p = K_{oc}\ x\ (f_{oc})^{-1} \qquad (2.8)$$

K_{oc} values are compound properties, not soil properties which have been related to other compound properties such as water solubility (S):

$$\log K_{oc} = 5.85 - 0.64 \log S \text{ (mg/L)} \qquad (2.9)$$

This relationship suggests a very useful way to estimate the sorption of PCBs under many circumstances of soil conditions using only the solubility of PCBs.

2.6.2 Soil Washing

Once sorbed to soil, PCB are difficult to desorb. The use of surfactants to enhance PCB desorption from contaminated soils is a growing research area. McDermott, et al. (1989) reported that a bench scale process, with four countercurrent stages, extracted PCBs (Aroclor 1260) from a laboratory contaminated soil (1 g PCB/kg soil) using a 1% surfactant (Sodium dodeylbenzene-sulfonate, SDBS) solution. Greater than 95% of the soil bound PCB could be extracted from the soil under the test conditions. The PCBs in the extracting effluent were further removed by precipitation to a concentration level of 1.8 µg/L, approximately the solubility of Aroclor 1260. Abdul and co-

workers (1991 and 1992) evaluated the effectiveness of an alcohol ethoxylate surfactant (Witconol SN70) for washing PCB from soil. Using a laboratory column apparatus, surfactant concentration of 5,000, 10,000, and 20,000 mg/L effectively removed 66, 86, and 50 % of the Aroclor 1248 (1728 g/kg) from contaminated soil after 20 washings. A surfactant concentration of 10,000 mg/L was found to be most effective. Lower concentrations resulted in fewer micelles in solution into which soil-bound PCBs could partition and higher concentrations resulted in elevated adsorption of surfactant on the soil which led to pore clogging and changes in soil surface chemistry thereby decrease PCB mobility.

Based on their laboratory experiment, Abdul and co-workers (1992) conducted a field test of their PCB surfactant-washing processes. During 70 days of washing, 5,375 gallons of a 0.75% aqueous surfactant solution were applied at the site at an average rate of 77 gal/day. A total of 10,981 gallons of leachate were recovered at an average rate of 157 gal/day. A total of 1.6 kg of PCBs (10%

of the initial mass) with a maximum concentration of 65 mg/L was extracted from the site. The test results indicate that in-situ surfactant washing is a promising candidate for the remediation of PCB contaminated soil systems.

In addition to synthetic surfactants, biosurfactants can successfully enhance removal of hydrophobic pollutants from soils. Harvey et al. (1990) reported that biosurfactants produced by *P. aeruginosa* enhanced the release of Exxon Valdez spilled oil from Alaskan gravel. In the temperature range of 10-80 °C and a contact time of 1 minute, a 1% surfactant solution increased oil recovery by 2-3.5 times above that found for water alone. Falatko and Novak (1992) studied the effect of biosurfactants on the mobility of gasoline from a sandy soil. Two biosurfactant mixtures were produced by growing bacteria on glucose and vegetable oil, as well as on gasoline. It was found that both biosurfactants increased the solubility of the test components in gasoline over their intrinsic solubility. In general, compounds with lower solubilities had larger percent increases in solubility. Dyke et al. (1993) used

partially purified rhamnolipid produced by *P. aeruginosa* UG2 to enhance desorption of 2,2',5,5'CB, 3,3',4,4',5,5'CB, and other PAHs sorbed to soil. They found that removal of PCBs depended upon the type of soil, equilibrium time, and concentration of biosurfactant. Using a 5 g/L biosurfactant solution for soil washing, recoveries of 43 and 69% for 2,2',5,5'CB and 3,3',4,4',5,5'CB were achieved in silty loam soil. Approximately 20% of the biosurfactant was bound to this soil. For a sandy loam soil, recoveries for the two tested PCB congeners were about 55% in each case. Recovery of 2,2',5,5'CB from the silty loam soil using biosurfactant was 7.6 times higher than that recovered using the anionic surfactant SDS, but only 0.8 times higher than that recovered using a polyoxyethylene glycol surfactant (Witconol SN70).

2.7 PCB AEROBIC BIODEGRADATION AND ENHANCEMENT

Numerous studies investigating the microbial breakdown of PCBs have been reported since 1973 (Boyle, et al. 1992). Both aerobic and anaerobic microorganisms can transform

PCBs. Under anaerobic conditions, removal of chlorine atoms from the biphenyl ring was accomplished via reductive dechlorination. Under aerobic conditions, oxidative dechlorination or hydrolytic dehalogenation was described (Boyle, et al., 1992). A review of studies dealing with aerobic PCB biodegradation follows since this mechanism is germane to this research.

2.7.1 Aerobic Microbial Utilizers of PCBs

Microorganisms, such as *Alcaligenes* group (*A. eutrophus, A. faecalis*); *Pseudomonas* group (*P. putida, P. testosteroni, P. cruciviae, P. cepacia, P. paucimobilis, P. pseudoalcaligenes*); *Corynebacterium, Arthrobacter, Acinetobacter, Achromobacter, Klebsiella pneumoniae,* and *Bacillas brevis,* can aerobically biodegraded PCBs (Table 2.4). These bacteria have been isolated from soils, sediments, wastewater, or engineered treatment plant sludge.

Table 2.4 Summary of PCB Biodegradation Studies.

Bacteria	PCB substrate	Reference
Alcaligenes eutrophus H850	2,6CB; 4,4'CB; 2,4,4'CB; 2,4,6CB; 2,3,2',5CB 2,4,6,4'CB; 2,4,6,2',4'CB; 2,4,6,3,'5,'CB; 2,4,6,2',4',6'CB; 2,5CB; 2,4,3',4'CB; 2,5,2',6'CB; 2,6,2',6'CB; 3,5,3',5'CB; 2,4,5,2',5'CB; 2,4,6,2',5'CB; 2,3,6,2',3',6'CB; 3,4,2'CB; 2,5,2',5'CB; 2,3,5,6CB; 2,3,2',3'CB; 2,5,3',4'CB; 3,4,3',4'CB. Aroclor 1248	Bedard, D. L. et al. (1986)
	Aroclor 1254; Aroclor 1242 2,3,CB; 2,4CB; 4,4'CB; 2,4,4',CB; 2,3,2',3'CB; 2,4,5,2',3'CB; 2,4,3',4'CB; 3,4,3',4'CB; 2,2'CB; 2,5,2'CB; 2,5,4'CB; 2,3,2',5'CB, 2,5,2',5,CB; 2,5,3',4'CB; 2,4,5,2',5'CB; 2,4,2',4'CB; 2,4,5,2',4',5'CB	Bedard, D. L. et al. (1987a,b)
	Aroclor 1242	McDermott, J.B et al. (1989)
Alcaligenes sp LPS 3B	4CB; 4,4'CB	Pelligrew, C.A.,Jr (1989)
Alcaligenes sp y42	4CB	Furukawa, K. (1976)
Alcaligenes sp	4CB	Sayler, G. S. et al. (1984)

Table 2.4 (continued)

Bacteria	PCB substrate	Reference
Alcaligenes sp A2 and A5	4CB	Shields, M. S. et al. (1985)
Alcaligenes faecalis Pi434	PCB	Abramowicz, D.A. (1990)
Pseudomonas sp LB400	2,4'CB; 4,4'CB; 2,4,4'CB; 2,5,2',5'CB; 2,3,2',5'CB; 2,4,6,2',4'CB; 2,3,2',3'CB; 2,4,3',4'CB; 2,4,5,2',3'CB; 3,4,3',4'CB; 2,4,5,2',4',5'CB; 2,2'CB; 2,3CB; 2,5,2'CB; 2,5,4'CB; 2,4,2',4'CB; 2,4,6,2',4'CB; 2,5,3',4'CB; 2,4,5',2',5'CB; 2,3,4,2', 5'CB; 2,4,5,2',4',5'CB	Bopp, L. H. (1986)
Pseudomonas	2,4'CB; Aroclor 1242	Baxter, R. M. et al. (1984)
Pseudomonas sp 1008	2,4,5,2',4',5'CB Aroclor 1254	Sayler, G. S. et al. (1977)
Pseudomonas sp 7509	Aroclor 1254; Aroclor 1221	Liu, D. (1989)
Pseudomonas putuda LB400	Aroclor 1242	McDermott, J.B et al. (1989)
Pseudomonas putida LPS 10c *testosteroni* LPS 10a	4CB; 4,4'CB	Pelligrew, C. A.,Jr (1989)

Table 2.4 (continued)

Bacteria	PCB substrate	Reference
Pseudomonas *cepacia* RJB H201,Pi704 *paucimobilis* Q1 *pseudoalcaligenes* KF707	PCB	Abramowicz, D. A. (1990)
Pseudomonas sp MB86	4CB	Barton, M. R. et al. (1989)
Pseudomonas *cruciviae*	4CB	Takase, I. et al. (1986)
Corynebacterium sp MB1	the same as for *A. eutrophus* H850	Bedard, D. L. et al. (1986, 1987b)
	Aroclor 1242	McDermott, J.B et al. (1989)
Arthrobacter sp LPS 10B	4CB, 4,4'CB	Pelligrew, C.A. Jr (1989)
sp M5	4CB	Furukawa, K. et al. (1982)
Acinetobacter sp p6 and B1B	Aroclor 1254	Kohler, H-P. et al. (1988)
Acinetobacter sp p6	4CB	Furukawa, K. et al. (1978, 1982)

Table 2.4 (continued)

Bacteria	PCB substrate	Reference
Acinetobacter sp A8	4CB	Shields, M. S. et al. (1985)
Achromobacter sp	4CB	Ahmed, M. et al. (1975)
Klebsiella pneumoniae	4CB	Kamp, P. F. (1979)
Bacillas brevis	4CB	Tasse, R.F. et al., (1984)

2.7.2 Metabolic Pathway

The metabolic pathways involved in the aerobic biodegradation of PCBs have been well reviewed (Abramoicz, 1990; Chaudhry and Chapalamadugu, 1991; Adriaens, et al. 1991; Boyle, et al., 1992; Mohn and Tiedje, 1992). In general, the aerobic breakdown of PCBs involves initial addition of O_2 at the 2,3 position of the biphenyl ring by a dioxyenase enzyme, with subsequent dehydrogenation to the catechol followed by ring cleavage (Abramowicz, 1990).

Besides the proposed 2,3-dioxygenase degradative pathway, PCBs can be metabolized through other routes. It is known that attack of 2,5,2',5'CB by *A. eutrophus* H850 and *Pseudomonase* SP LB400 involves a novel 3,4-dioxigenase degradation pathway (Bedard, et al., 1986, 1987, Abramowicz, 1990). It is currently unknown whether the 2,3 and 3,4-dioxigenase activities originated from the same enzyme or not.

2.7.3 PCB Biodegradation Enhancement by Increasing the Production of Specific Enzyme

Production of specific enzymes in a catabolic pathway is critic for bacterial utilization of substrate. Modification of the culture media and genetically engineering bacteria are two important ways which can improve bacterial production of the required enzymes.

It was reported that growth of *Pseudomonas* LB400 in media containing biphenyl as the sole carbon source can enhance PCB degrative activity (Mondello, 1989, Abramowicz, 1990). One possible explanation is that biphenyl as the sole carbon source can induce the Biphenyl/PCB degrading enzyme(s). It was also observed that the degradation of PCB in soil was enhanced upon addition of biphenyl as a carbon source, but the rate decreased compared to the assays without soil (Abramowicz, 1990). It is possible that the presence of biphenyl not only induced the production of PCB utilization enzymes, but helped to release soil-bound PCB. In addition, the PCB-degrading activity of the growing

cells was significantly greater than that of the resting cells for *Acinetobacter* sp. P6 and *Arthrobacter* sp. BIB (Kohler, et al., 1988) because the initially induced enzymes in the culture media were washed out in the resting cell suspensions.

Genetically engineering bacteria are another important tool for PCB biodegradation enhancement. Khan and Walia (1990) cloned the PCB-degraditive genes from *Pseudomonas putida* into an *E. coli* strain, and found that target gene production increased by 20 fold over that measured in the parent strain. Mondello (1989) cloned PCB-degradative genes known to degrade mono- to hexa-CB from *Pseudomonas* LB400 into a broadhost-range vector, and a number of *E. coli* transformants FM4560 capable of degrading PCBs were isolated. The ability of genetic constructed strains approached that of LB400 in PCB biodegradation while this ability did not depend on the growth of organism on biphenyl. Further, when the engineered cells were grown with succinate as the carbon source, their PCB degradation ability was markedly superior to that of LB400 strain.

2.7.4 HOC Biodegradation Enhancement Using Biosurfactant:

The biodegradation of hydrophobic organic compounds (HOCs) is often limited by low water solubilities and dissolution rates. The use of biosurfactants may overcome these limitation by enhancing aqueous solubility of HOCs. As a result, the extent and rate of biodegradation can be improved.

Biosurfactant was first found to enhance the growth of microorganisms utilizing n-alkane as substrate. Hisatsuka et al. (1977) reported that a protein-like activator together with rhamnolipids stimulated growth of *P. aeruginosa* S7B1 on n-alkanes. Koch et al. (1991) used *P. aeruginosa* mutants deficient in extracellular rhamnolipid production to demonstrate that rhamnolipid was essential for uptake and growth of this strain on C_{12} to C_{19} aliphatic hydrocarbons. Zhang and Miller (1992, 1994) showed that rhamnolipid enhanced the aqueous dispersion of octandecane by more than 4 orders of magnitude, from 0.009 to 320 mg/L. The enhancement of dispersion was found to be due to the

lowering of surface tension by the rhamnolipid at low concentration, and due to the physical association within the rhamnolipid aggregates when the concentration was above the CMC. As a result of dispersion enhancement, octadecane mineralization increased from 5 to 20% when 0.3 g rhamnolipid/L was supplemented with the cultural medium. Further study found that addition of rhamnolipid can change the cell hydrophobicity, which suggested that the bioavailability of octadecane in the presence of rhamnolipid is controlled by both aqueous dispersion and cell hydrophobicity. Oberbremer et al. (1990) used rhamnolipid to enhance the rate and extent of hydrocarbon degradation by soil bacteria. It was found that the degradation extent of the tested hydrocarbons increased 12-18% for the samples with biosurfactant. The added rhamnolipids were degraded at a reasonable extent only when the target HOCs were depressed due to biodegradation. Jain et al. (1992) have also shown that addition of 0.1 mg rhamnolipid per g of soil significantly enhanced the biodegradation of aliphatic tetradecane, pristane and hexadecane but not aromatic 2-methylnaphthalene.

In summary, addition of biosurfactants can enhance the biodegradation of sparingly soluble aliphatic hydrocarbons. Though the potential exists for biosurfactant enhancement of biodegradation for aromatic compounds such as PCBs, there is no report in the literature to date documenting such an interaction.

CHAPTER 3 MATERIALS AND METHODS

3.1 POLYCHLORINATED BIPHENYLS (PCBs)

Three non ^{14}C-labeled PCB congeners [2-chlorobiphenyl (2CB), 4-chlorobiphenyl (4CB), and 4,4'-dichlorobiphenyl (4,4'CB)] and one ^{14}C uniformly labeled(UL) PCB congener [4,4'-dichlorobiphenyl-UL-^{14}C (^{14}C-4,4'CB) with specific radioactivity of 13.8 mCi/mMole] were used in this study. In addition, biphenyl was used as the sole carbon source in the culture medium to enhance *Alcaligenes eutrophus* production of PCB degrading enzymes (see discussion in 4.3.1). 4CB and 4,4'CB were used to evaluate the effect of biosurfactant on their solubility and biodegradation while ^{14}C-4,4'CB was chosen to evaluate only mineralization of the test compound (that is complete oxidation to CO_2 and H_2O). 2CB was used as an internal standard during analysis of PCB containing samples. The molecular weight, purity, and other information of the above compounds are listed in

Table 3.1. The structure of each test compound is shown in Figure 3.1.

3.2 SOILS

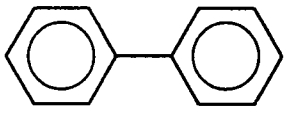
3.2.1 Collection

Two natural and uncontaminated surface soils were used in the study. They were collected by shoveling soil from a depth between a few inches and a few feet below the soil surface at a site in Memphis, Tennessee. The first soil was collected from an area containing no plant growth while the second soil was collected from an area containing dense plant growth at the surface. General characteristics of the two soils were expected to be similar due to the close proximity of the collection sites. However, organic carbon content would vary due to the presence or absence of organic litter at the soil surface. The collected raw soils were stored in plastic containers at 5 °C before further treatment.

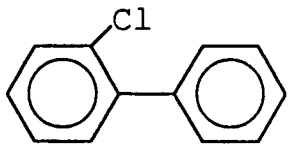
Table 3.1 Properties of Test PCB Congeners.

PCB Congener	MW	Purity (%)	Specific Radioactivity (mCi/mmole)	Solubility* (mg/L)	Log K_{ow}*	H* (Pa M/mol)
Biphenyl	154.2	98		7.0±0.5	3.9	46.0
2-Chlorobiphenyl (2CB)	188.7	99		5.5±0.5	4.3	74.6
4-Chlorobiphenyl (4CB)	188.7	99		1.2±0.3	4.5	58.1
4,4'-Dichlorobiphenyl (4,4'CB)	223.1	99		0.06±0.02	5.3	62.8
4,4'-Dichlorobiphenyl-UL-^{14}C (^{14}C-4,4'CB)	223.1	99	13.8	0.06±0.02	5.3	62.8

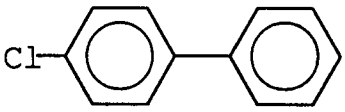
* Shiu and Mackay (1986)



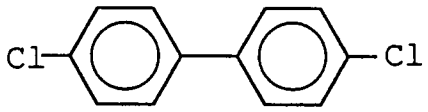
Biphenyl



2-Chlorobiphenyl



4-Chlorobiphenyl



4,4'-Dichlorobiphenyl

Figure 3.1 Chemical Structures of Test Compounds.

3.2.2 Pre-treatment of Soil

Both raw soils contained large size particles, such as gravel, sand, and roots. If these soils were used without pretreatment, large errors could result due to difficulties in replicating small test samples. To improve sample homogeneity and therefore reproducibility, both soils were pre-treated in the study. After removing large roots and gravel, raw soils were homogenized using a mortar and pestle, then screened using a No. 60 sieve with a pore size of 0.25 mm. Particles with a size $\leq$0.25 mm were collected while the soil particles >0.25 mm were replaced in the mortar to be further homogenized and screened again. The final discarded soil materials were sand and gravel, which accounted for about 15% of the raw (untreated) soil weight.

The screened soil was mixed with deionized (DI) water and allowed to settle overnight. The water was then decanted to remove remaining roots, leaves, and fine suspended particles. After decanting, the soil was washed with DI water then centrifuged at 2,000 rpm for 30 minutes

to remove non-setting fines. This soil wash procedure was repeated three times. After autoclaving three times to eliminate soil bacteria, the soils were covered with aluminum foil, air dried and stored at ambient temperature before use. The total carbon content (TC) of the soils, before and after treatment, was analyzed.

3.2.3 Soil Total Carbon (TC) Content Analysis

Soil total carbon (TC) was determined using a carbon analyzer (DC-80, Rosemount Analytical Inc.). Soil samples were dried in oven at 105 °C overnight, then cooled to room temperature in a desiccator before total carbon analysis. 5-8 mg of the dried soil was weighted and place into a metal boat for sample delivery into the analyzer. After the sample was pushed into combustion furnace, the soil sample was oxidized at 800 °C in an oxygen atmosphere. The total carbon (in mg) of the soil sample was detected and referenced against a carbon standard. Based on the measured TC value and the weight of the soil sample, the total carbon fraction (f_{tc}) of the soil was determined.

3.3 BACTERIA

Pseudomonas aeruginosa was reported in the literature to be a good producer of Rhamnolipid R1, a very efficient and well characterized biosurfactant. The culture conditions for the production of biosurfactants were also well documented (see section 2.4). In this study, a strain of *Pseudomonas aeruginosa*, PRP652 which was initially isolated from a gasoline contaminated soil was cultured to produce Rhamnolipid R1.

Alcaligenes eutrophus, a PCB specific microorganism, has been widely used for PCB biodegradation (Table 2.4). In this research, a strain of *Alcaligenes eutrophus* A5 which was initially isolated from a PCB contaminated sediment in Ft. Loudon Reservoir, Knoxville, Tennessee, was chosen for the PCB mineralization test.

Both the *P. aeruginosa* and *A. eutrophus* strains were supplied by the Center for Environmental Biotechnology

(CEB) at the University of Tennessee, Knoxville, and stored at -80°C.

3.4 PRODUCTION, PURIFICATION AND CHARACTERIZATION OF BIOSURFACTANT (RHAMNOLIPID R1)

3.4.1 Production

Rhamnolipids are synthesized and excreted by *Pseudomonas aeruginosa* or other *Pseudomonas* sp. in response to environment stresses such as depressed substrate concentration, pH changes, or nutrient limitation. Research has demonstrated that nitrogen limitation resulted in the production of rhamnolipids by *P. aeruginosa* (Syldatk and Wagner, 1987; Ramana and Karanth, 1989). In this work, a nitrogen exhaustion method was used to enhance production of biosurfactant (R1).

Pseudomonas aeruginosa (PRP652) in frozen stock was streaked on a *Pseudomonas* isolation agar (PIA) plate (Table 3.2). After the plate was incubated for 2 to 3 days, one of

Table 3.2 YEPG Media, YEPG Plate, and PIA Plate for Maintaining and Isolation of *P. aeruginosa*.

Media/Plate	Composition	Concentration
YEPG Medium	Dextrose	1.0 g/L
	Polypeptone	2.0 g/L
	Yeast Extract	0.2 g/L
	NH_4NO_3	0.2 g/L
	DI water	1000 ml
	pH	adjusted to 7 with NaOH
YEPG Plate	Agar	16 g/L in YEPG medium
PIA PLate	*Pseudomonas* Isolation Ager	16 g/L in YEPG medium

the colonies on the plate was inoculated into a 250 ml Erlenmeyer flask which contained 100 ml of phosphate buffered mineral salt medium (PMG) with glucose or glycerol as the sole carbon source (Table 3.3). The flask was incubated at 31°C with mixing (150 rpm) for 2 days. An aliquot of the culture media (10 ml) was then transferred into each of four 4-liter flasks containing 1 liter PMG media. The 4-liter flasks, plugged with cotton pushings, were incubated at 31 °C with mixing (150 rpm) until the cultures reached a late stationary phase. It was reported that maximum accumulation of biosurfactant occurs in this phase (Ramana, K. V. and N. G. Karanth, 1989). Under the test conditions, 6 to 7 days were required for the culture to reach this phase.

Radiolabeled biosurfactant was also produced through the addition of ^{14}C-glucose as the primary substrate. The *P. aeruginosa* was first inoculated into a 250 ml flask containing 100 ml PMG media and incubated (31 °C and 150 rpm) for 2 days. Then 1.5 ml of the cultural media in the 250 ml flasks was transferred into a 500 ml flask

Table 3.3 PMG Medium for *Pseudomonas aeruginosa* PRP652.

Composition	Weigh
KH_2PO_4	3.0 g/L
K_2HPO_4	7.0 g/L
$(NH_4)_2SO_4$	1.0 g/L
$MgSO_4$ $7H_2O$	0.1 g/L
D-Glucose or Glycerol	20 g/L 30 g/L
DI water	1,000 ml
pH	7.0

containing 150 ml of PMG media. Once the cells in the 500ml flasks reached the stationary phase (about 40 hours), 1 ml of the uniformly labeled ^{14}C-glucose (98%, 288.0 mCi/mmole, 0.1 mCi/ml) was added to the 500 ml flasks and the culture was further incubated for another 4 to 5 days in order to yield the ^{14}C-biosurfactant.

3.4.2 Purification

Biosurfactants produced by *P. aeruginosa* include four possible rhamnolipids with different numbers of rhamnose or carboxyl groups. Each of the four biosurfactants are soluble in ether, acetone, and chloroform. Furthermore, they have different mobilities on silicic acid so they can be separated using silicic acid liquid chromatography. In general, purification of biosurfactant R1 includes the following procedures: harvesting by extraction, purifying by liquid chromatography (LC), and confirming by thin layer chromatography (TLC).

After reaching stationary phase, the culture media containing *P. aeruginosa* was centrifuged at 10,000 rpm for 20 minutes using a Beckman Centrifuge (Model J2-21) to remove cells. The remaining cultural media (supernatant) was then acidified to pH 2.5-3.0 by addition of a few drops of concentrated HCl (10 N) to precipitate the biosurfactant. After acidification, the supernatant was volumetrically added to a separator funnel and extracted with an equal volume of ether (Reagent grade) by shaking for 10 seconds. After extraction, the ether portion was decanted and evaporated using a Rotavapor (Buchi Inc.). Each sample was extracted twice.

The residual in the rota-evaporation flask was re-dissolved in 50 ml chloroform (Reagent grade) by gently shaking. The chloroform was then poured into a glass chromatography column (2.5 cm x 20 cm) containing ≈ 30 ml of 100 mesh silicic acid (1/3 of total volume). Biosurfactant was eluted from the column (≈ 3 ml/min) using a progressively higher volume ratio of acetone to chloroform. Acetone to chloroform ratios of 0, 1:9, 3:7,

5:5, and 10:0 (v/v) were used to elute different fraction from the column. Each fraction (≈ 60 ml) was collected separately.

Eluted samples were analyzed using thin layer chromatography (TLC) to identify fractions containing the rhamnolipid biosurfactant. An aliquot (50 µl) of each fraction was carefully placed on a thin layer plate (silicic gel, thickness: 250 mm, 60A K6, Whatman Inc.) using a syringe. After air drying in a hood for 30 minutes, the plate was placed into a covered glass tank and developed in 455 ml of organic solvent with a volume ratio of chloroform : methanol : acetic acid = 80 : 10 : 1 for about 1 hour. The plate was then removed and air dried again. An anthrone regent (mixture of 0.1 g of 98% Anthrone; 50 ml 98% H_2SO_4 and 2 ml DI water) was applied to the plate before heating (105 °C for 5 to 10 minutes). The anthrone positive species (rhamnolipids) identified by the presence of a blue color (complexes of anthrone and rhamnose moiety) were then isolated. Four rhamnolipids, R1, R2, R3 and R4, were found in the fractions. The one with

the lowest mobility was identified as the biosurfactant R1. The pure R1 was always found in the 100% acetone fraction (10:0) while small amounts of R1, R2, R3, R4 and impurities were found in other fractions. The same procedures as above were used to purify the ^{14}C-biosurfactant.

3.4.3 Quantification

Quantification of biosurfactant (both ^{14}C and non-^{14}C labeled) in the acetone solution was performed using a standard weighing method. Aluminum weigh boats were washed three times with acetone, dried in an oven at 105 °C overnight, then cooled to ambient temperature in a desiccator before weighting. Drying and weighing were repeated until two consecutive weightings were within 5% of each other. Aliquots of the acetone fractions (10-15 ml) containing biosurfactant R1 were quantitatively added to each weigh boat. After the acetone was evaporated in a hood, the boats were again heated in an oven at 105 °C overnight. The weigh boats were weighed repeatedly until

the error of two consecutive readings was less than 5%. The weight of biosurfactant in the boats was determined as the difference of the average dry weight of the boats before biosurfactant solvent addition and after the solvent evaporated. Based on this weight and the loaded volume of acetone fraction in the boats, the concentration of biosurfactant in the acetone eluent was determined. Once the concentration was determined, the biosurfactant solution was stored in a screw-capped bottle in the dark and refrigerated (5°C) for later use.

3.4.4 Specific Activity of ^{14}C-Biosurfactant

Specific activity is an important parameter for quantification of radioactive compounds. It is the radioactivity (DPM) per unit mass or volume of the compound. The specific activity of ^{14}C-biosurfactant was determined as following:

One ml of ^{14}C-biosurfactant acetone solution was placed into each of three liquid scintillation counter

(LSC) vials and purged with air to strip the acetone. Then 0.5 ml K_2HPO_4 (30 mM) and 1.5 ml deionized (DI) water were volumetrically added to each of the vials. After the vials were shaken reciprocally for 2 hours, 10 ml LSC cocktails (Readysafe™) was added to each vial. After vortex mixing for 20 seconds, the vials were stored at ambient temperature for about 24 hours in order to stabilize the samples (quench chemilluminecence) before measuring. All DPM values were measured using a Beckman LSC (Model LS-3801). According to the DPM value and sample volume, the radioactivity of the ^{14}C-R1 acetone solution was determined. Utilizing the ^{14}C-R1 concentration determined previously, the specific activity (SA) of R1 was then calculated.

3.4.5 Purity of R1 in Biosurfactant Solution

The previously discussed biosurfactant purification procedure confirmed the presence of biosurfactant R1, but not the purity. The purity (%) of biosurfactant R1 (^{14}C- and non-^{14}C-R1) in the ^{14}C-biosurfactant acetone fraction

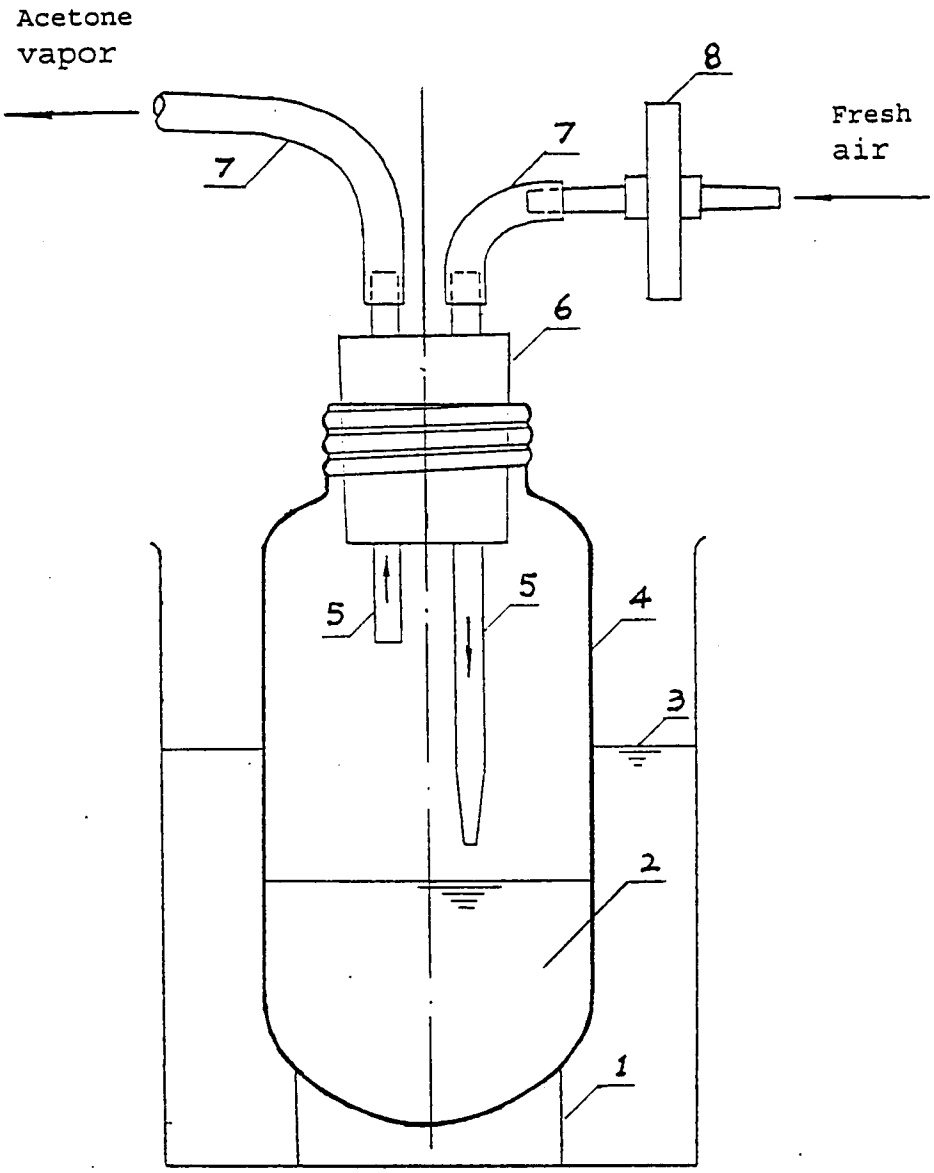
was further determined as follows. Six aliquots (100 µl) of ^{14}C-biosurfactant acetone solution were placed at six points on the TLC plate and allowed to dry in a hood. The first two of the points on the plate along with the silicic gel surrounding the points were scrapped from the plate and placed into two LSC vials before the TLC plate was developed. After the TLC plate was developed, the biosurfactant positive reagent (anthrone regent) was spread along the developing line of two of the four remaining points, while the lines for the other two points were covered with aluminum foil during spreading. According to the position of biosurfactant R1 in the spread lines, the areas of biosurfactant R1 in the two lines without spreading anthrone reagent were identified and scrapped from the plate and placed into another two LSC vials. The rest of the area in the two lines without reagent spread were also scrapped into the other two LSC vials. After placing 0.5 ml of 30 mM K_2HPO_4 and 1.5 ml DI water into all the LSC vials, the vials were shaken for 2 hrs then 10 ml of LSC cocktail were added for the measurement of DPM. The purity of biosurfactant was determined as the percentage of

the measured DPM for biosurfactant R1 (scrapped from the unspread lines on the TLC plate) to the total measured DPM for both R1 and the non-R1 compounds (scrapped from the same unspread lines on the TLC plate).

The purity of R1 for the non-^{14}C-biosurfactant solution was assumed to be the same as the ^{14}C-biosurfactant solution because the same production and purification procedures were used.

3.4.6 Preparation of Biosurfactant Solution

In this study, all biosurfactant solutions used in solubility and mineralization tests were prepared free of bacteria and pH equal to 7. The device shown in Fig 3.2 was used avoid bacterial contamination during the preparation of biosurfactant solutions. After the device was washed and autoclaved, a known volume of biosurfactant in acetone was volumetrically added to a 150 ml glass bottle. The bottle was stoppered and a vacuum applied to remove acetone. Fresh air entered the bottle through an air filter (pore size,



1. Support Pad;
2. Biosurfactant Solution;
3. Hot Water;
4. Centrifuge Bottle (150 ml);
5. Glass Tubes;
6. Rubber Plug;
7. Plastic Tubing;
8. Air Filter (pore size: 0.22 um).

Figure 3.2 Device for Preparation of Biosurfactant Solution Which Minimized Bacterial Contamination.

0.22 ml) which prevented bacteria from entering the sample. A hot water bath was used to accelerate the evaporation of acetone. After the acetone was removed, a known volume of sterilized K_2HPO_4 (30 mM) was added to the bottle to prepare a stock biosurfactant solution. Additional biosurfactant concentrations were obtained by diluting this stock solution using the same K_2HPO_4 buffer. Further, all biosurfactant solutions except one series used for surface tension measurement were adjusted to pH 7 with 8.5% (w/w) H_3PO_4. Because the volume change of biosurfactant solutions due to addition of H_3PO_4 was less than 0.7%, the change in the concentration was assumed to be negligible.

After biosurfactant solutions were prepared, one inoculation loop of each solution was streaked on YEPG plates. If no growth appeared on the plates after incubation, the solution was confirmed to be free of bacterial contamination and ready for use. Otherwise, the solution was re-purified before use.

3.4.7 Measurement of Surface Tension

Surface tension was defined as the surface excess free energy per unit area of air/liquid interface (Myers, 1992). It is a measure of surface activity by a surfactant. The lower the surface tension of a surfactant solution, the higher the surface activity of a surfactant at a given concentration. Further, the critical micellar concentration (CMC), an important parameter of a surfactant, was determined by varying the biosurfactant concentration and measurement of the corresponding surface tension at each concentration. Surface tension was measured using a surface tension meter.

Before all surface tension measurements, glassware was washed with detergent, rinsed at least three times with hot tap water, then rinsed an additional three times with DI water before oven drying at 105 °C. Biosurfactant solutions, ranging from 0.0 to 4.0 g/L, were prepared both with and without a pH adjustment as discussed previously. 10 ml of each solution was placed into a glass beaker

(diameter x depth = 5.5 x 1.1 cm), and the surface tension measured using a Tensiomat (Model 21, Fisher Co.) at ambient temperature (21.8 ± 0.4 °C). Prior to the measurement, a 3-point calibration of the Tensiomat was performed by adjusting the arm length of the torsion balance. Between measurements, the Platinum ring of the Tensiomat was cleaned with acetone then heated in a gas flame until it glowed red to thermally oxidize any residual organic matter on the surface of the ring. For each biosurfactant solution, the measurement was repeated three times, and the average of the readings was taken as the measured surface tension.

3.5 APPARENT AQUEOUS SOLUBILITY OF PCBs

The apparent aqueous solubility of PCB was evaluated to determine the effectiveness of the biosurfactant in PCB solubility enhancement. PCB crystals were added to the biosurfactant solutions, and the solution allowed to come to equilibrium. Excess crystals were then removed by centrifugation. PCB dissolved in the biosurfactant solution

was determined using both Gas chromatography (GC) and Liquid Scintillation Counting (LSC).

For solubility enhancement analysis using GC, PCB/ether solutions were prepared by weighing and dissolving PCB crystals into ether (reagent grade) to achieve a concentration range between 0.2-2.0 g/L. The tubes containing the solutions were wrapped with alum foil to avoid photooxidation then stored at 5 °C. 0.010-0.0115 ml of the PCB/ether solution was volumetrically transferred into 25 ml Corex centrifuge tubes. The volume used was based on the total required PCB in each tube (≈ 5 times that required to saturate the biosurfactant solution with PCBs). Ether was evaporated by purging with house air which had been filtered (pore size 0.22 μm) to avoid bacterial contamination. 10 to 15 ml of the biosurfactant solution, ranging in concentration from 0 to 4.0 g/L, were added to the remaining PCB crystals in each of the tubes. The tubes were then sealed using Teflon lined screw-caps. Solutions containing PCB crystals and biosurfactant were mixed for 48 hours on an end over end mixer, then centrifuged (30

minutes, 2000 rpm) to remove any remaining PCB crystals from solution. 1 ml of the supernatant was transferred to a 15 ml glass tube that had been pre-loaded with 0.36 gm of conditioned Accell™ QMA anionic exchange resin (500 A, Waters Co.). 4 ml of hexane was then added to extract PCBs from the aqueous phase. The QMA resin was added to minimize emulsion formation during extraction. Biosurfactant was bonded to the QMA surface by ionic exchange, resulting in reduced emulsion formation. Extraction consisted of reciprocal shaking for 30 minutes followed by centrifuged at 2,000 rpm for 15 minutes. 1 ml of the hexane phase from each tube was placed in a glass vial for PCB analysis using a GC (Model GC-144, Shimadzu). The GC was equipped with an electron capture detector (ECD), a capillary column (SPB-1 or SPB-5, Supelco, Inc., inside diameter x length = 0.25 mm x 30m), and an automatic sampler (AOC-14). Nitrogen was used as the carrier gas (0.88 ml/min) as well as the make-up gas (35 ml/min). The column temperature was held at 40 °C for 2 minutes, raised to 80 °C at a rate of 10 °C/min then raised to 225 °C at the rate of 6 °C/min. A final temperature of 225 °C was maintained for 2 minutes. The

injector and detector temperatures were maintained at 300 °C. To normalize the PCB GC-peaks, an internal standard, 10 µl of 2CB in ether solution (5 mg/L) was mixed with each 1 ml sample. 1 ml sample volume was analyzed using a splitless technique.

The ^{14}C-4,4'CB in biosurfactant solutions was determined using LSC as follows. ^{14}C-4,4'CB solutions were prepared in centrifuge tubes using the same procedures discussed above. After centrifugation, 0.5 ml of the supernatant was added to a 20 ml scintillation vial, then 1.5 ml DI water and 10 ml cocktail were added followed by vortex mixing for 20 seconds. Samples were stabilized for 24 hours at room temperature before counting ^{14}C activity. Based on the specific activity of ^{14}C-4,4'CB, the measured ^{14}C activity, and the sample size, the solubility of PCB was determined.

3.6 MINERALIZATION OF 4,4'CB IN PRESENCE OF BIOSURFACTANT

3.6.1 Preparation of Culture Media

According to the literature, *A. eutrophus* can grow in phosphate buffered mineral salt medium (PMB) using biphenyl crystals and yeast extract as the carbon substrate (Bedard, et al., 1986, 1987). In this study, the PMB media (Table 3.4) containing only dissolved biphenyl as the sole carbon source was used to culture *A. eutrophus*. This medium was prepared by mixing 78 ml of filter sterilized (0.22 mm) phosphate buffer (PB) (Table 3.4) and 912 ml of DI water in a 2 liter flask. This solution was then magnetically stirred for 1 hour followed by autoclaving for 25 minutes. 2 gm of biphenyl crystals (Sigma, Inc.) were added to the hot solution with mixing until the liquid cooled to ambient temperature and the dissolved biphenyl recrystallized. 10 ml of filter sterilized (0.22 μm) mineral salts (MS) (Table 3.4) were added to the flask which was then shaken (150 rpm) for 2 days in an incubator (31 °C). After confirming sterilization (no growth of

Table 3.4 PMB medium for *Alcaligenes eutrophus* A5.

Medium	Composition	Weight
PB	K_2HPO_4	56.8 g/L
	KH_2PO_4	22.0 g/L
	NH_4Cl	27.7 g/L
MS	$MgSO_4$	19.5 g/L
	$MnSO_4$ H_2O	5.0 g/L
	$FeSO_4$ $7H_2O$	1.0 g/L
	$CaCl_2$ $2H_2O$	0.3 g/L
	H_2SO_4	Acidify MS to pH 2
PMB	PB medium	78 ml
	MS medium	10 ml
	DI water	912 ml
	Biphenyl	2 g
		pH adjust to 7

bacteria on the YEPG plates after streaking), the media was transferred into 150 ml pre-autoclaved glass centrifuge bottles and centrifuged at 10,000 rpm for 30 minutes (Model J2-21, Beckman centrifuge) to separate biphenyl crystals from solution. 100 ml of the supernatant (PMB media) was added to each of 250 ml pre-sterilized flasks for culture of *A. eutrophus* (A5).

Because of the low solubility of biphenyl in the PMB media (≈ 7 mg/L), organism growth was limited. A continuous supply of biphenyl substrate was required to stimulate cell growth to the relatively high density levels required for subsequent experiments while avoiding biphenyl crystals in the PMB solution. This was accomplished by placing biphenyl crystals into dialysis tubing (molecular weight cut-off 50,000, diameter 22 mm, Spectrum medical industries, Inc.) before addition to the 250 ml flasks containing the PMB medium. Because the dialysis tubing was preserved in 0.1% sodium azide, it was washed with sterilized DI water and then conditioned in 30 mM K_2HPO_4 buffer (pH adjusted to 7) before addition of the biphenyl crystals. All the above

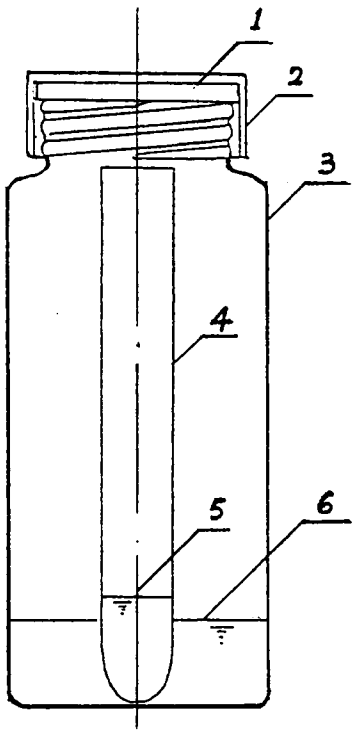
procedures were conducted under a gas flame to avoid bacteria contamination.

3.6.2 Preparation of Resting Cell

Resting cells are commonly used to evaluate the time course of biodegradation. Preparation of resting cells usually include culture, wash, and resuspension of the bacteria. In this study, strains of *A. eutrophus* were inoculated from frozen stocks (-80°C) to 250 ml flasks containing 100 ml PMB media. The strains were then incubated at 30°C with mixing (150 rpm). After growing to log phase (about 2 days), cells were harvested by centrifuging the culture media at 10,000 rpm for 25 minutes. The cells were then washed twice with 30 mM K_2HPO_4 buffer (pH=7) to remove any residual biphenyl or other medium compounds. Next, the cells were suspended in 30 mM K_2HPO_4 buffer to an optical density of 2.0 at 600 nm resulting in a final cell density of 3.6×10^{10} cells/ml (as determined by a standard plate count method).

3.6.3 Mineralization of 4,4'CB (without 4,4'CB Crystal Substrate)

In this study, a $^{14}CO_2$-trap (NaOH) replacement method was used to allow for continuous sampling over time and reduce biosurfactant consumption. The mineralization reactors (Figure 3.3) consisted of 22 ml glass vials with Teflon-lined caps containing a 2 ml culture tube as the $^{14}CO_2$ trap. Reactors were soaked in 10% HNO_3, washed with DI water, then autoclaved before use. ^{14}C-4,4'CB saturated biosurfactant solutions (0-4 g/L) were prepared as previously discussed. After 1 ml of ^{14}C-4,4'CB solution was placed into each of the vials (reactors), and 0.3 ml of 1 N NaOH was added to the 2 ml culture tube (center well), 50 µl of the prepared *A. eutrophus* resting cell suspension was inoculated into the each of the vials. Final cell density in each vial was about 1.7×10^9 cells/ml which was calculated based on the initial cell density (3.6×10^{10} cells/ml) and the final sample volume in the vial. Vials were capped, shaken (150 rpm) and incubated at room temperature ($21 \pm 1^{\circ}C$). The evolved $^{14}CO_2$ resulting from



1. TEFLON/Rubber Septum;
2. Cap;
3. Glass Vial (22 ml);
4. Culture Tube (2 ml);
5. NaOH (1 N, 0.3 ml);
6. Sample for Mineralization.

Figure 3.3 Micro-reactor Used for Mineralization of PCBs.

mineralization of ^{14}C-4,4'CB was trapped in the 1 N NaOH stored in the center well. The NaOH in the center well, as well as 0.7 ml of sterilized DI water used to rinse the well, was transferred to scintillation vials at various incubation time intervals and analyzed for ^{14}C activity. NaOH (0.3 ml, 1N) was replenished in the center well for subsequent sampling. These steps were repeated throughout the time course of the experiment. Abiotic controls were established by heat killing cells in a 80°C water bath for 40 minutes before inoculation. The inactivity of the controls was confirmed by the absence of cellular growth after incubation on YEPG medium plates. Both samples and controls were run and analyzed in triplicate.

To establish the reliability of the NaOH replacement method, some samples were acidified with H_2SO_4 and shaken for 30 minutes to evolve the dissolved $^{14}CO_2$ in solution before sampling the NaOH for ^{14}C activity of trapped $^{14}CO_2$. A comparison of the data showed no significant difference between the two methods. Because of the ease of the method, the NaOH replacement method was subsequently used. Further,

the replacement method can also avoid oxygen limitation in the reactor by allowing air to enter the vial during NaOH replacement, though there may be some 4,4'CB loss due to the opening of the cap during NaOH replacement.

In order to investigate whether the biosurfactant was co-mineralized as a carbon source with 4,4'CB by the *A. eutrophus* (A5), the same experiment was repeated but substituting the ^{14}C-biosurfactant and 4,4'CB for the biosurfactant and ^{14}C-4,4'CB, respectively. In this experiment, ^{14}C activity in the trap could only result from mineralization of the biosurfactant.

3.6.4 Mineralization of 4,4' CB (with 4,4'CB Crystal Substrate)

Low biosurfactant concentration results in limited enhancement of aqueous PCB. The role of the biosurfactant in mineralization enhancement of PCB at low biosurfactant concentrations is limited due to the exceedingly low mass of PCB present. Mineralization data may not reflect the

true impact of biosurfactant on PCB mineralization enhancement. To provide a continuous source of PCB substrate at low levels, a mineralization experiment was performed in which excess PCB crystals were present in the test solutions. The 4,4'CB saturated solution with excess crystals was prepared as follows:

0.026 mg of 4,4' CB in an ether solution was placed into each of the 22 ml vials. The amount of 4,4' CB in each vial was enough to maintain the presence of 4,4'CB crystals during mineralization experiments even at the highest biosurfactant concentration. After evaporating the ether by purging with filter sterilized air, 1 ml of biosurfactant (0-4 g/L) was placed into each vial. Vials were then sealed and shaken (150 rpm) for 2 days at room temperature to equilibrate. Other procedures were the same as in the mineralization of the 4,4'CB solution without the presence of crystals. To confirm whether biosurfactant R1 was co-mineralized by the A5 during mineralization of 4,4'CB, the same experimental procedures were repeated by using the ^{14}C-biosurfactant and non-labeled 4,4'CB.

3.7 DESORPTION AND MINERALIZATION OF 4,4' CB FROM CONTAMINATED SOILS

3.7.1 Soil Contamination

Contamination of soil was accomplished by quantitatively adding 1.0 gm of the pre-treated soil to 15 ml glass tubes with Teflon-lined caps. After 3 ml of 30 mM K_2PO_4 sterilized solution was added to each tube, the soil slurry was vortex-mixed for 10 seconds and then shaken on an end-over-end rotor for 2 days. At the end of shaking, the tubes were centrifuged at 2,000 rpm for 30 minutes, the supernatant decanted, and the soil containing tubes autoclaved. After washing and autoclaving three times, 3 ml of ^{14}C-4,4'CB solution, prepared in the same way as for the 4,4 CB solubility test, were added to each tube. The tubes were then shaken on an end-over-end rotor for 2 days, followed by centrifuging at 2,000 rpm for 30 minutes. The supernatant was then decanted. The above contamination

procedures were repeated (13 time) until the desired level of soil contamination was achieved.

In the batch contamination, the loss of 4,4'CB due to volatilization and sorption to the tube was estimated using controls which contained no soil. ^{14}C-activity in the control solutions before and after each batch contamination was taken as the loss of 4,4'CB due to volatilization into the head space and/or sorption to the surface of the tubes.

The amount of soil pore water remaining after sample centrifugation and soil losses due to the contamination procedure were determined by weighing tubes containing wet and dried (105 $^{\circ}C$) soils before and after 13-batch contamination treatments. The change of soil f_{tc} was also determined using the TC analyzer as discussed section 3.2.

3.7.2 Desorption of 4,4'CB from Soil with Biosurfactant

Though PCBs strongly bind to soils due to their hydrophobic nature, biosurfactant may enhance desorption

from soil through facilitating PCB partitioning into the hydrophobic core of the biosurfactant micelles. Biosurfactant solutions (pH=7) were prepared as discussed previously. Because the average volume of pore water remaining in the soils (both high and low f_{tc} soil) after contamination was 3.19 ml (the weight of the remaining water in the soils was determined as the weight difference of the supernatant-decanted soil samples before and after drying in an oven at 105 °C), the biosurfactant solutions were prepared at concentrations of 0, 0.025, 0.25, 1.25, and 5.0 g/L respectively to allow for pore water dilution. 2.5 ml of the each solution was mixed with the soil pore water, resulting in final biosurfactant concentrations of 0, 0.02, 0.2, 1.0, and 4.0 g/L, respectively.

Soil washing was achieved by adding 2.5 ml of each biosurfactant solution to 15 ml glass tubes containing the contaminated soils. After vortex-mixing for 10 seconds, the tubes were shaken for 2 days on a end-over-end rotor. The tubes were then centrifuged at 2,000 rpm for 30 minutes. Subsequently, 0.5 ml of supernatant from each tube, plus

1.5 ml of DI water and 10 ml of LSC cocktail, were added to scintillation vials to measure ^{14}C activity.

The procedures to determine the loss of biosurfactant sorbed to the soils during the soil washing were the same as above, except that non-labeled 4,4'CB solution was used for soil contamination, and the ^{14}C-biosurfactant solution was used for soil washing.

3.7.3 Mineralization of 4,4' CB in Soil Wash Solution

1 ml of the soil wash supernatant was added to each vial for mineralization. The other procedures were the same as discussed in the Section 3.6.3.

CHAPTER 4 RESULTS AND DISCUSSIONS

4.1 CHARACTERISTICS OF BIOSURFACTANT R1

4.1.1 Yield

A nitrogen exhaustion method (Ramana and Karanth, 1989) was used to culture *P. aeruginosa* PRP652 to produce the biosurfactants (rhamnolipids) used in this study. The growth of *P. aeruginosa* PRP652 in PMG medium is characterized in Figure 4.1. After a short lag phase (10 hours), the bacteria rapidly reached the stationary phase (after 40 hours' growth). It was reported by Ramana and Karanth (1989) that rhamnolipid production by *P. aeruginosa* began at the onset of the stationary phase (40 hr) due to nitrogen exhaustion, and maximum accumulation occurred during the later stages of this phase (> 100 hr). In this study, biosurfactant was harvested from the culture after 144-168 hours of incubation.

Based on the methods used to produce and purify R1 in this study, 918 mg of rhamnolipid R1 were recovered from 4

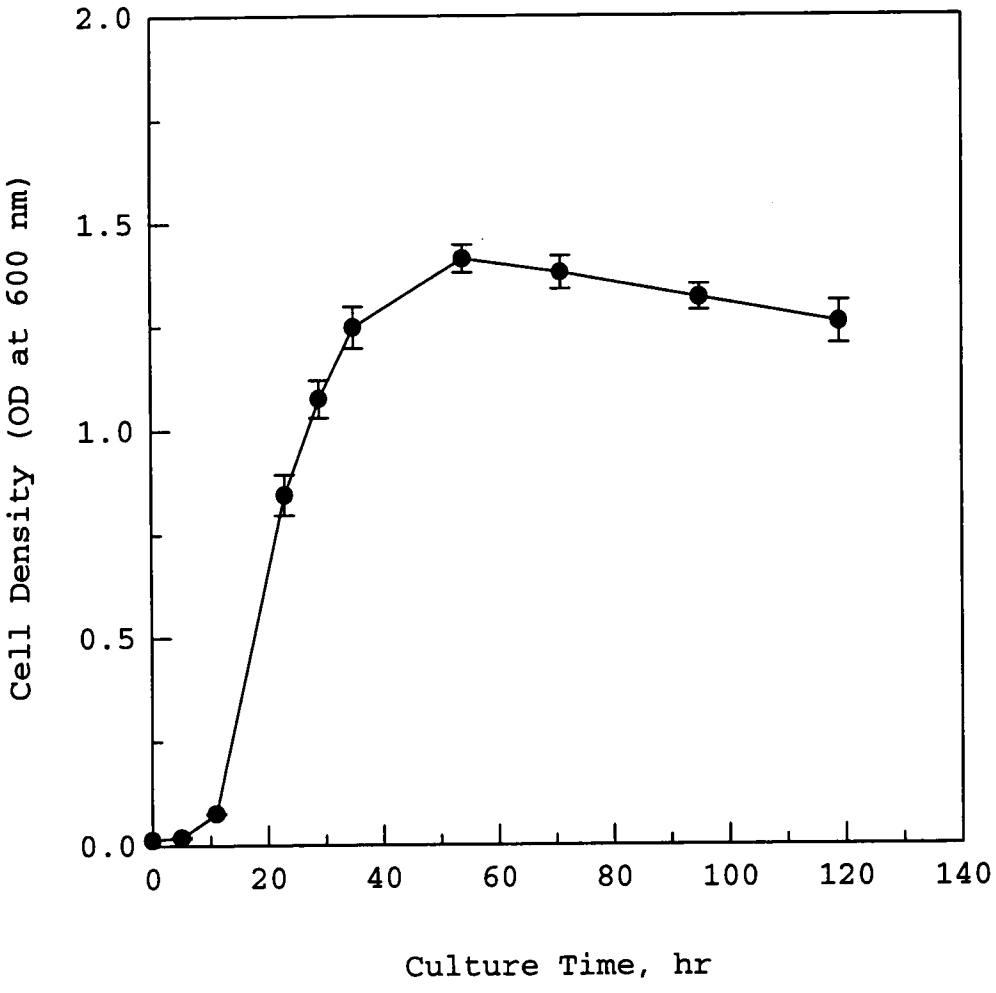


Figure 4.1 Growth Curve of *P. aeruginosa* PRP652 in PMG Medium with 2% D-Glucose at 31 °C.

liters of culture medium containing 30 g/L glycerol. The yield of rhamnolipid R1 by the *Pseudomonas aeruginosa* PRP652 was 7.7 mg per g of glycerol. Other researchers (Itoh, et al., 1971; and Ramana and Karath, 1989) have reported maximum yields between 31-43.7 mg rhamnolipid R1 per g of glycerol. Since this study focused on the role of biosurfactant in the enhancement of solubility and biodegradation of PCBs, the reasons for the lower yield of biosurfactant were not investigated further.

4.1.2 Specific Activity

The following equation was used to calculate the specific activity (SA) of the purified ^{14}C-biosurfactant R1 in the acetone solution (fraction) in this study:

$$SA_{R1}\,(DPM/mg) = \frac{RA_{R1}\ (DPM/ml)}{C_{R1}\ (mg/ml)} \qquad (4.1)$$

where:

RA_{R1}: Radioactivity of the purified ^{14}C-biosurfactant R1 in acetone fraction, DPM/ml;

C_{R1}: Concentration of the purified ^{14}C-biosurfactant R1 in acetone fraction, mg/ml.

The radioactivity and concentration of the R1 in the acetone fraction were measured as 8772 DPM/ml and 0.726 mg/ml, respectively, resulting in a specific activity of 1.21×10^4 DPM/mg. Based on this specific activity, the total ^{14}C radioactivity of the 190.4 mg generated ^{14}C-biosurfactant was calculated as 1.755×10^6 DPM. Since the ^{14}C-biosurfactant radioactivity was synthesized from ^{14}C-glucose (5 ml, 2.22×10^8 DPM/ml), the conversion efficiency (the ^{14}C activity ratio of the biosurfactant to the glucose) was 0.32%.

4.1.3 Purity

Based on the method (section 3.4.5) and the results (section 4.1.4) of thin layer chromatography (TLC), the ^{14}C-biosurfactant in the acetone fraction included rhamnolipid R1 and a small amount of impurities. The purity of the ^{14}C-biosurfactant was determined using the following equation:

$$P_{R1}(\%) = RA'_{R1}/RA'_{biosur} \times 100\% \qquad (4.2)$$

where RA'_{R1}: ^{14}C activity of the rhamnolipid R1 (in ^{14}C-biosurfactant solution) placed on the TLC

plate, DPM;

RA'_{biosur} : Total ^{14}C activity of the ^{14}C-biosurfactant acetone solution on the TLC plate, DPM.

The measured ^{14}C activity for the rhamnolipid R1 (in the ^{14}C-biosurfactant acetone solution) and for the whole ^{14}C-biosurfactant acetone solution placed on the TLC plate were 991 and 1037 DPM, respectively. The purity of the generated R1 in ^{14}C-biosurfactant acetone solution was determined as 96%.

Because both the ^{14}C- and non-^{14}C biosurfactant R1 were produced and purified under the same conditions, the purity of non-^{14}C biosurfactant was assumed to be the same as the ^{14}C-biosurfactant.

4.1.4 Structure

A typical thin layer chromatogram (TLC) result of all fractions (1 to 5) collected from the liquid chromatography separation procedure appears in Figure 4.2. The separation of compounds by TLC is based upon differences in partitioning between the mobile phase (organic solvent) and the stationary phase (silisic gel). The driving force from the mobile phase (based on hydrophobicity) varies for

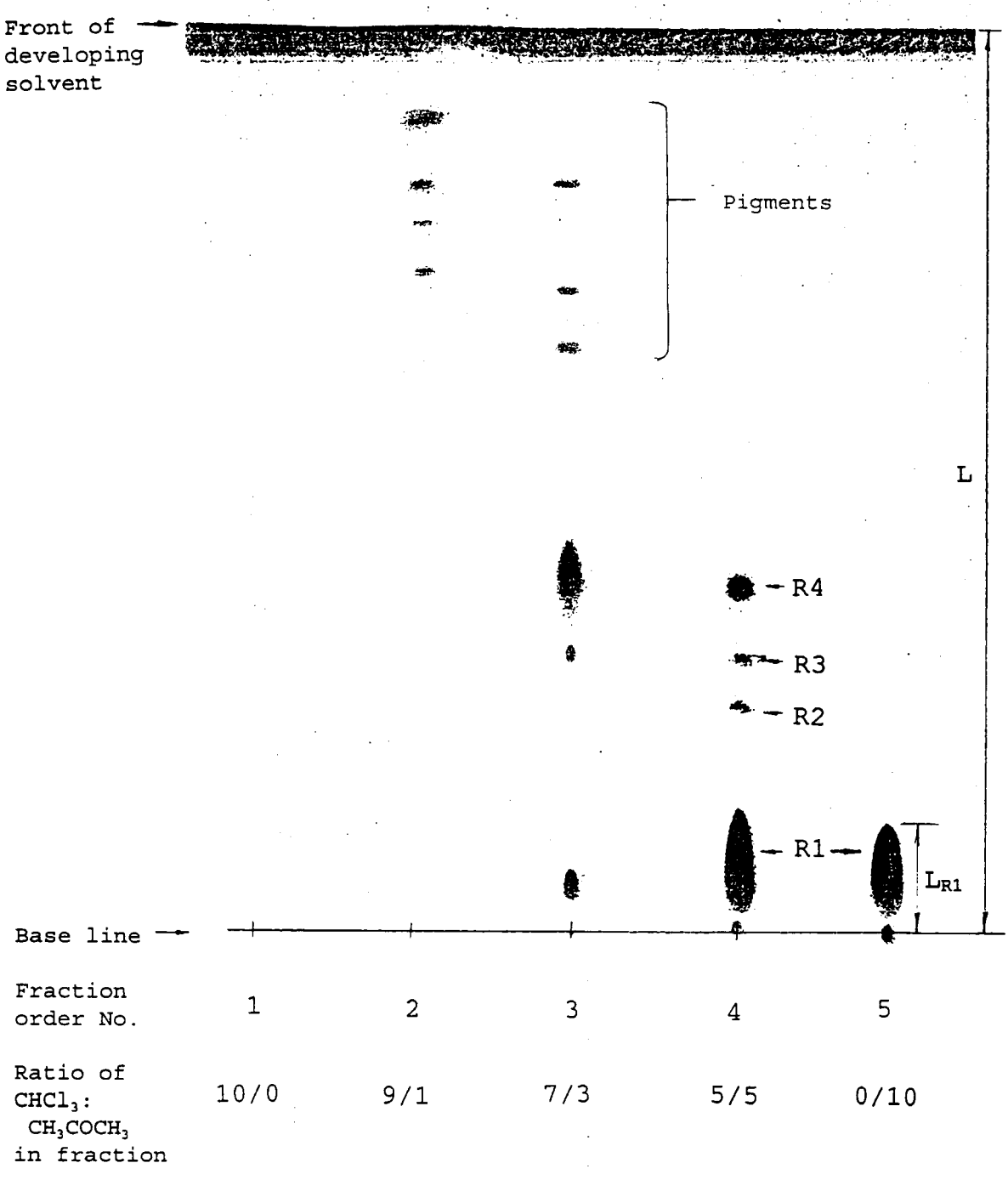
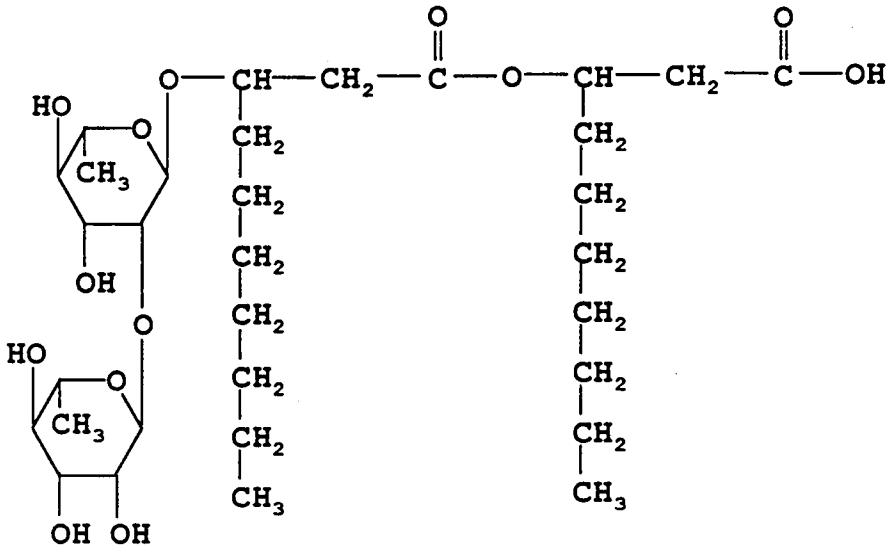


Figure 4.2 Thin Layer Chromatogram (TLC) of Fractions from Liquid Chromatography (LC) Separation. (R1, R2, R3, and R4: Rhamnolipids; Base line: Start point of TLC for all fractions).

different compounds which results in mobility differences along the TLC plate.

There were four constituents, R1, R2, R3, and R4, which were positive (blue color) to the anthrone reagent, indicating that these compounds have at least one sugar moiety in their structure (Ramana and Karanth, 1989, and Itoh, et al., 1971). Of the four biosurfactants isolated by TLC, R1 had the lowest mobility ($R_f=L_{R1}/L=0.12$) due to the fact that this molecule had the greatest number of sugar residues and C=O groups of the four rhamnolipids identified. In comparing these results, as well as the production conditions of R1, with those found by other researchers studying biosurfactants (Ramana and Karanth, 1989, Long and Wagner, 1987), the structure of R1 was tentatively identified as: L-rhamnopyranosyl-L-rhamnopyranosyl-β-hydroxydeconyl-β-hydroxydeconate (Figure 4.3). This compound has a molecular weight of 650. Based on its structure, R1 could be classified as an anionic biosurfactant containing sugar residues as the polar (hydrophilic) units and hydrocarbon chains in fatty acid residues as the non-polar (hydrophobic) units.



MW=650

Figure 4.3 Molecular Structure of Biosurfactant Rhamnolipid R1 Produced by *Pseudomonas Aeruginosa* (adopted from Ramana and Karanth, 1989).

4.1.5 Surface Tension and Critical Micellar Concentration

Surface tension as a function of biosurfactant concentration is plotted in Figure 4.4. Surface tension decreased with increasing biosurfactant concentration down to a point where further increases of biosurfactant no longer significantly changed surface tension. This point was defined as the critical micellar concentration (CMC) of the biosurfactant. The CMC is a very important parameter to evaluate surface activity and effectiveness of the biosurfactant in solubility enhancement of hydrophobic compounds. When the concentration of the biosurfactant is above its CMC, micelles begin to form in solution which can facilitate contaminant partitioning into the micelle hydrophobic cores, resulting in solubility enhancement of contaminant. In this study, the relationship between biosurfactant concentration (log) and surface tension can be linearly represented by two straight lines; the first in the range of biosurfactant concentration above the CMC and the second in the range below the CMC. CMC was determined as the biosurfactant concentration corresponding to the intersection of the two lines. The CMC for the R1 biosurfactant in 30 mM K_2HPO_4 with and without pH adjustment were determined as 54 mg/L and 40 mg/L, respectively. This is in agreement with the CMC values of

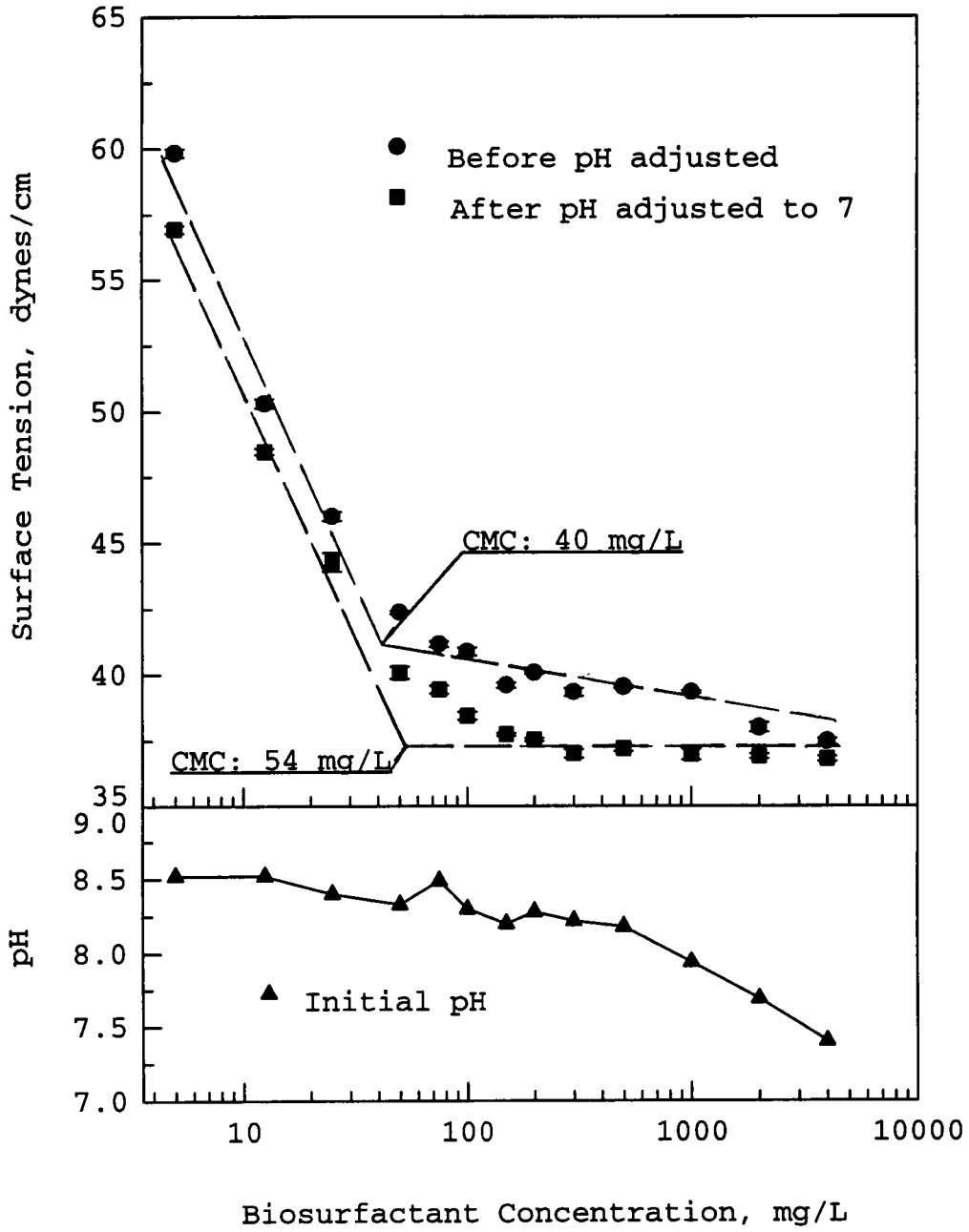


Figure 4.4 Determination of CMC of Biosurfactant (Rhamnolipid R1) in Aqueous Solutions ($t=21.8\pm0.4^{\circ}C$).

30-40 mg/L for rhamnolipid R1 reported in the literature (Zhang and Miller, 1992; Dyke, et al., 1993; and Ishigami, et al., 1987).

As shown in Figure 4.4, adjustment of pH changed the slope of the surface tension line alone CMC, raised the CMC value by a margin of 14 mg/L, and also decreased the resulting surface tension of the solutions. Because rhamnolipid R1 is an anionic surfactant with a pK_a of 5.6 (Ishigami, et al., 1987), changes in pH can shift its ionization equilibrium resulting in a change of its aggregation number (number of surfactant molecules in an aggregate) or aggregate type. Both of these parameters impact surface activity. Ishigami, et al. (1987) reported that the aggregates of rhamnolipids were pH-sensitive. For pH values in the range of 4.3-5.8, 6.0-6.6, and >6.8, the type of aggregates formed are vesicle, lamella/lipid particle and micelle, respectively. In this study, the initial pH values (above 7.5) were only adjusted to 7 (> 6.8), so the type of biosurfactant aggregates formed (micelles) did not change. The impact on CMC and surface tension due to pH adjustment was, therefore, due to changes in the aggregation number. Using a buffer (K_2HPO_4) to decrease the pH of the biosurfactant solutions decreased the ionization extent of the biosurfactant which decreased

the static repulsion between biosurfactant molecules. As a result, the aggregation number of the biosurfactant increased, consequently, the CMC increased, the surface tension decreased and the slope of the surface tension line changed. The CMC of Rhamnolipid R1 was evaluated at pH=7 because subsequent solubilization and mineralization studies were performed at this pH.

4.2 SOLUBILITY Of 4CB AND 4,4'CB IN THE PRESENCE OF BIOSURFACTANT

Biosurfactant R1 concentrations, ranging from 0 to 4.0 g/L in 30 mM K_2HPO_4 (pH=7.0), were used to increase the apparent aqueous solubility of PCBs (Figure 4.5). When 4CB and 4,4'CB were in the same solutions (mutual co-solutes in the mixture) and the concentration of R1 was increased from 0 to 4 g/L, the apparent solubility (determined by GC) of 4CB increased from 1.05 mg/L to 59.13 mg/L and the apparent solubility of 4,4'CB increased from 0.04 mg/L to 4.21 mg/L. The solubilities of 4CB and 4,4'CB were enhanced 56 and 105 times, respectively, at a biosurfactant concentration of 4.0 g/L (74 times CMC). When biosurfactant concentrations were above the CMC, solubility of 4CB and 4,4'CB could be expressed as a linear function of the biosurfactant concentration.

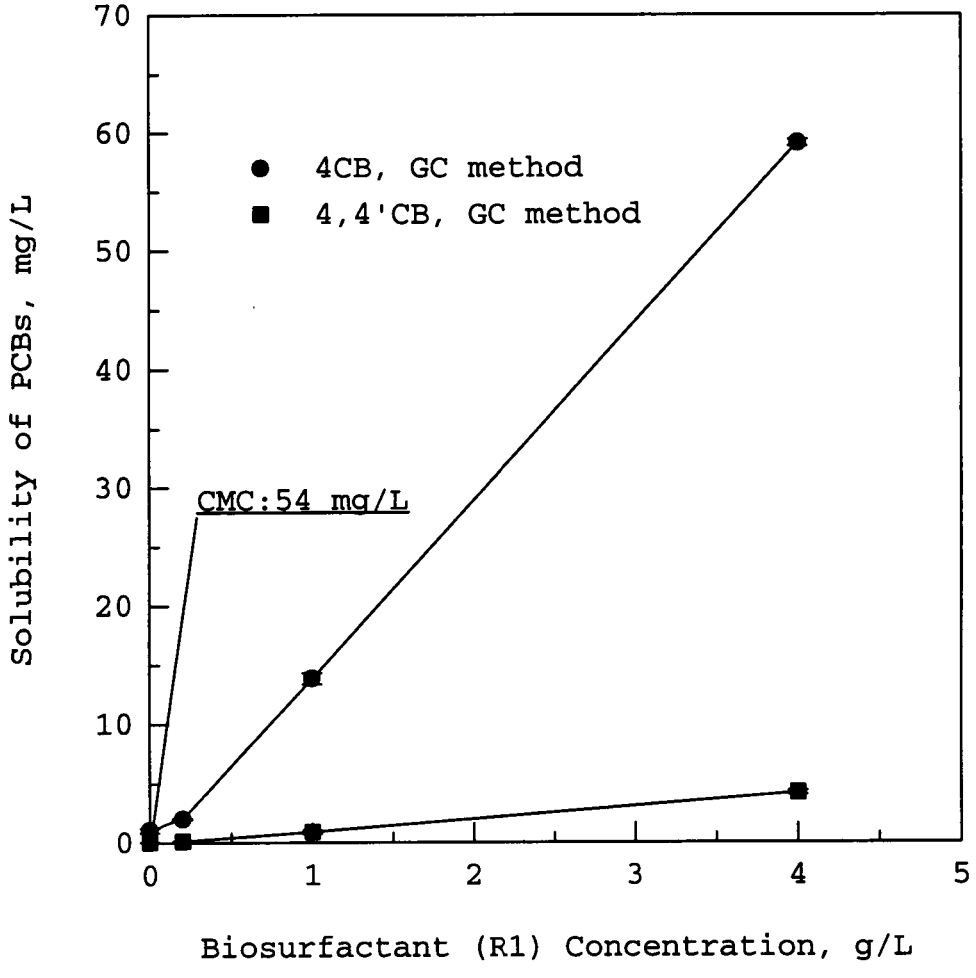


Figure 4.5 Solubility of 4CB and 4,4'CB in the Presence of Biosurfactant (4CB and 4,4'CB Co-present in Each Sample).

The effect of 4CB co-solvency on 4,4'CB's solubility can be observed in Figure 4.6. The solubility of 4,4'CB, as the sole solute, was similar to its solubility in the presence of the 4CB co-solute for biosurfactant concentrations below the CMC. When the biosurfactant concentration was above the CMC, the solubility of 4,4'CB, as the sole solute, was higher than that when 4CB was present in the sample. Apparently, the presence of 4CB decreased the apparent solubility of 4'4'CB at elevated biosurfactant concentrations. Because the impact of surfactants on solubilization of non-polar compounds is dependent upon the number and size of micelles in solution, monomers do not contribute significantly to solubilization (Harwell, 1992). For a solution with constant biosurfactant concentration, pH, and temperature, the number and size of micelles are constant, so total solubilization capacity should be constant. Based on these considerations, for biosurfactant concentrations above the CMC, the solubility enhancement of 4,4'CB mainly results from the partitioning of 4,4'CB into the micelle pseudophase. The presence of cosolute 4CB will compete with 4,4'CB for the fixed number of partitioning sites in the micelles, resulting in a decreased apparent solubility of 4,4'CB. For biosurfactant concentrations below the CMC, no micelles form in solution, therefore the biosurfactant existed only as monomers which

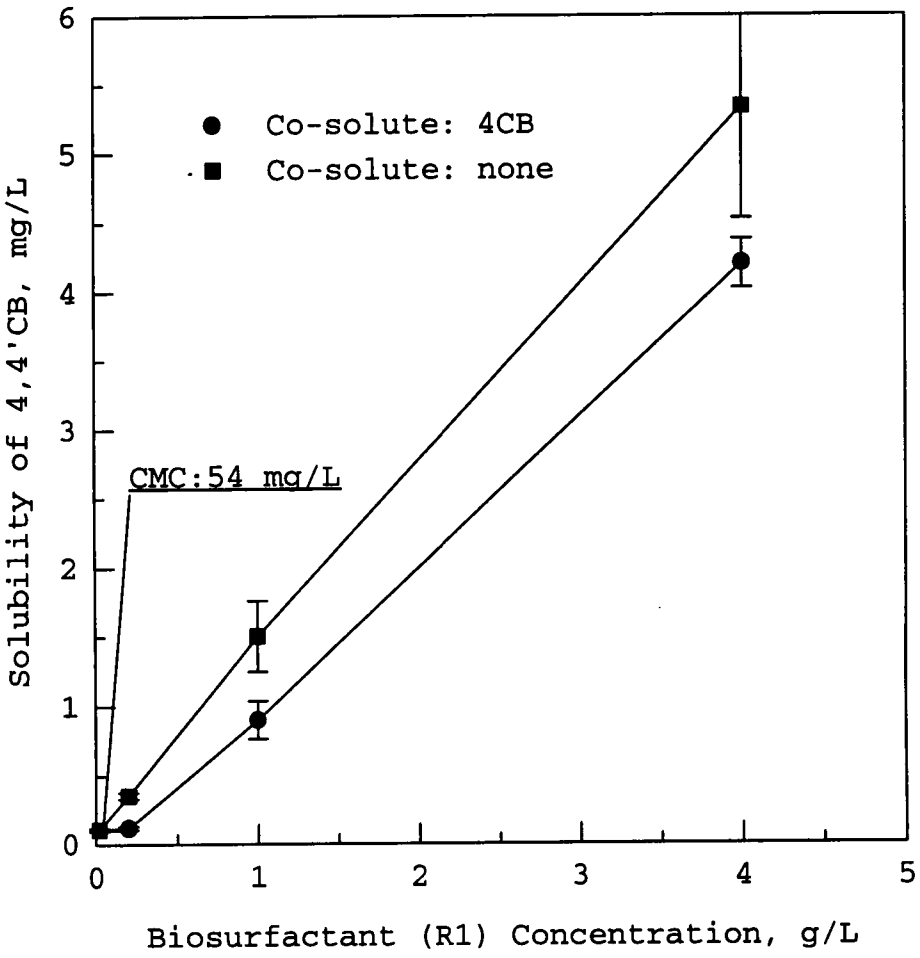


Figure 4.6 Impact of 4CB Co-solute on Apparent Solubility Enhancement of 4,4'CB.

have less effect on solubility enhancement. This explains the similar results for the solubility of 4,4'CB in the presence and absence of 4CB at low biosurfactant concentrations. Since most environmental PCB contaminants exist as mixtures, the co-solute effect may be important when estimating the solubility of PCB congeners.

The molar fractions of 4,4'CB in biosurfactant solutions are listed in Table 4.1. An increase in biosurfactant concentration increased the molar fraction of 4,4'CB; however, the presence of 4CB (as a co-solute) reduced the overall extent of 4,4'CB molar fraction of 4,4'CB enhancement in solution. Based on Henry's law, a decrease in molar fraction will result in a decrease of vapor pressure. This may be important when partitioning of PCB between liquid and air is considered in multi-phase system.

The molar solubilization ratio (MSR) and the micelle/aqueous pseudophase partitioning coefficient (K_m) were used to further evaluate the effectiveness of biosurfactant R1 in the enhancement of PCB solubility. The MSR indicates the number of moles of solute which can be solubilized by one mole of surfactant in the micelle form, whereas the K_m is the ratio of solute in the micellar

Table 4.1 Molar Fraction of 4,4'CB in Biosurfactant Solutions

Biosurfactant Concentration		Solubility (M)		Molar Fraction	
(g/L)	(M)	Without 4CB in Solution (M)	With 4CB in Solution (M)	Without 4CB in Solution (M)	With 4CB in Solution (M)
0	0	$2.06X10^{-7}$	$1.79x10^{-7}$	$3.71x10^{-9}$	$3.22x10^{-9}$
0.02	$3.07x10^{-5}$	$4.93x10^{-7}$	-	$8.87x10^{-9}$	-
0.2	$3.07x10^{-4}$	$1.57x10^{-6}$	$5.38x10^{-7}$	$2.82x10^{-8}$	$9.68x10^{-9}$
1.0	$1.54x10^{-3}$	$6.72x10^{-6}$	$4.03x10^{-6}$	$1.21x10^{-7}$	$7.25x10^{-8}$
4.0	$6.15x10^{-3}$	$2.39x10^{-5}$	$1.89x10^{-5}$	$4.30x10^{-7}$	$3.40x10^{-7}$

pseudophase to the solute in the aqueous pseudophase. It is a function of MSR. The greater the MSR, the more solute can be solubilized by a unit mass of biosurfactant, therefore the more effective the surfactant. The MSR and K_m values presented in Table 4.2. were calculated using equations 4.3 and 4.4 (Hayase and Hayano, 1977; Atwood and Florence, 1983; and Edward, et al., 1991)

$$MSR=(S_{mic}-S_{CMC})/(C_{biosur}-CMC) \qquad (4.3)$$

$$K_m=X_m/X_a$$
$$=MSR/[(MSR+1)(S_{CMC}V_w)] \qquad (4.4)$$

where

S_{mic}: Solubility of PCB at $C_{biosur}>CMC$, (M);

S_{CMC}: Solubility of PCB at $C_{biosur}=CMC$, (M);

C_{biosur}: Biosurfactant concentration at which S_{mic} was measured, (M);

V_w: Molar volume of water, $V_w=0.0180$ L/mole at 22 °C;

X_m: Molar fraction of PCB in micellar pseudophase;

X_a: Molar fraction of PCB in aqueous pseudophase.

The values of log K_m for all tested PCBs were above 5, indicating that the ratio of PCBs partitioning in micelle pseudophase to the PCB partitioning in aqueous pseudophase

Table 4.2 Molar Solubility Ratio (MSR) and Micelle/Aqueous Partitioning Coefficient (K_m) of PCB for Biosurfactant R1.

PCB Solute	S_{mic} (M)	S_{CMC} (M)	C_{biosur} (M)	MSR	log K_m
4CB*	3.13×10^{-4}	5.56×10^{-6}	6.15×10^{-3}	5.07×10^{-2}	5.68
4,4'CB**	1.88×10^{-5}	1.79×10^{-7}	6.15×10^{-3}	3.70×10^{-3}	5.98
4,4'CB	2.39×10^{-5}	9.33×10^{-7}	6.15×10^{-3}	3.79×10^{-3}	5.35

* 4,4'CB cosolute presents at 1.88×10^{-5} M.
** 4CB cosolute presents at 3.13×10^{-4} M.

was $\geq 10^5$ to 1. This further confirms that micelles of biosurfactant R1 play a critical role in PCB solubility enhancement. The MSR values varied somewhat with 4CB which showed the largest MSR value. Though one mole of R1 can solubilize more 4CB than 4,4'CB (MSR of 4CB was greater than that of 4,4'CB), the micelle/aqueous partitioning ratio (K_m) for 4,4'CB was slightly greater than that for 4CB. The greater K_m for 4,4'CB indicated that the biosurfactant was comparatively effective in enhancing the solubility of the more hydrophobic congener (4,4'CB) than the less hydrophobic congener (4CB) when considering their intrinsic solubilities in water. In the presence of the 4CB co-solute, the K_m of 4,4'CB was slightly higher than its K_m without the 4CB co-solute, while the apparent solubility of 4,4'CB was lower than that without the co-solute. This indicates that the comparatively hydrophilic co-solute (4CB) competed with the comparatively hydrophobic solute (4,4'CB) for partitioning sites in the biosurfactant micelles. This competition resulted in 1) a decrease in the total apparent aqueous solubility of 4,4'CB due to the fact that some partitioning sites within the micelles were occupied by 4CB at a given biosurfactant concentration, and 2) an increase of the K_m value of 4,4'CB. For the more hydrophobic solute, 4,4'CB, there is a greater tendency to partition into micellar (hydrophobic) phase and a less

tendency to partition into water (hydrophilic) phase. This results in an increased ratio (K_m) of 4,4'CB though the total amount and molar fraction (Table 4.1) of 4,4'CB dissolved in the micelle and water phases decreased.

4.3 MINERALIZATION OF 4,4'CB IN THE PRESENCE OF BIOSURFACTANT

4.3.1 Evaluation of Growth Media on PCB Biodegradation by *Alcaligenes eutrophus* A5

Growth of *Alcaligenes eutrophus* in different culture media may impact cell yield and PCB biodegradation potential. Two culture media, YEPG and PMB (PMB with three sub-modifications: without biphenyl crystals in solution, with biphenyl crystals in solution, and with dialysis bag containing biphenyl crystals), were evaluated to determine cell yield and PCB biodegradation potential.

The growth of *A. eutrophus* on the YEPG and PMB media is characterized in Figure 4.7. The cell density was monitored spectrophotometrically using a 600 nm wavelength. It was found that PMB media with biphenyl crystals present in solution yielded the highest cell density while the PMB media with dissolved biphenyl (no biphenyl crystals) as the

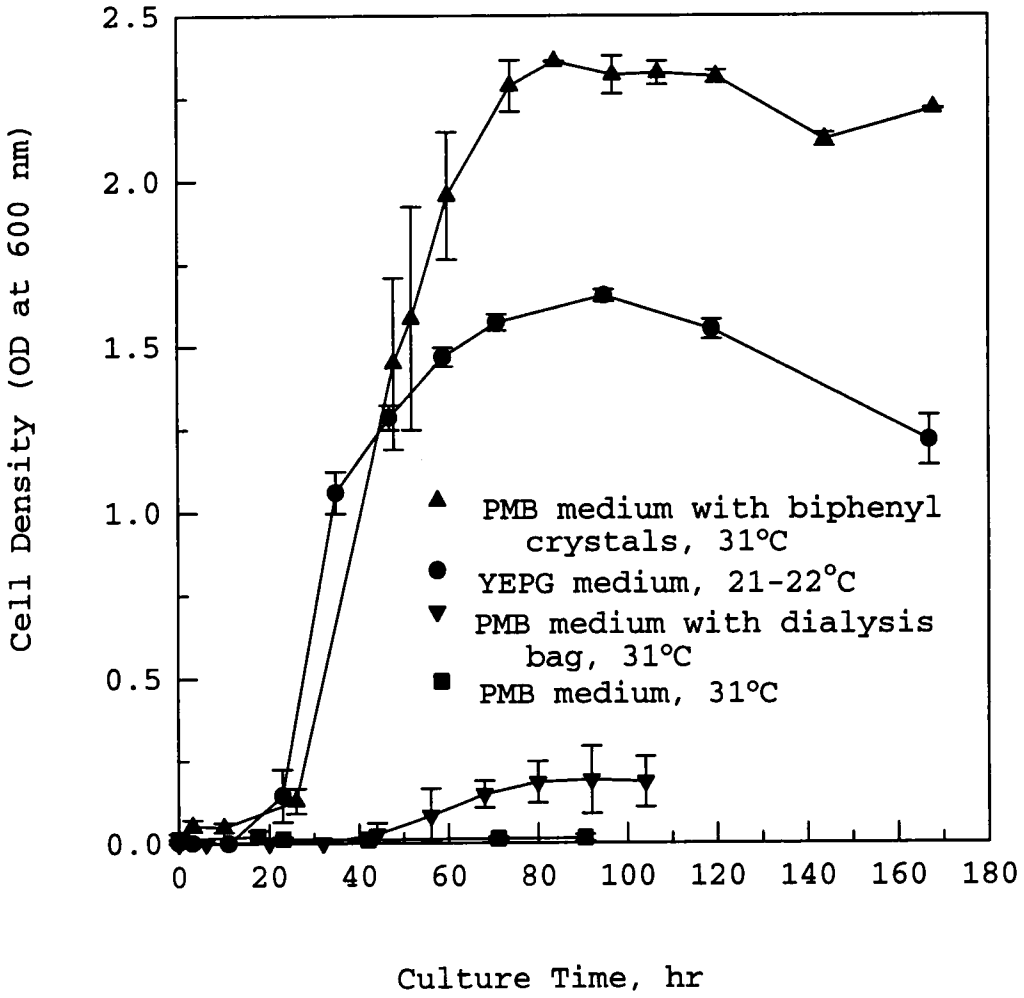


Figure 4.7 Growth Curves for *A. eutrophus* A5 under Different Culture Conditions.

carbon source yielded the lowest cell density. The cell density in the YEPG media and PMB media containing biphenyl crystals in a dialysis bag were ranked the second and the third, respectively.

The extent of PCB biodegradation and mineralization by the *A. eutrophus* grown in the YEPG and PMB (with dissolved biphenyl) is shown in Figures 4.8 and 4.9. Biodegradation was determined as the difference in PCB remaining in biotic controls (heat-killed bacteria) and biologically active samples. The remaining aqueous PCB was measured by GC analysis after sample extraction. Mineralization of PCB was measured as the trapped $^{14}CO_2$ evolved from the complete oxidation of ^{14}C-PCB. For the same incubation time, *A. eutrophus* cultured in PMB medium biodegraded and mineralized PCBs to a greater extent than the same bacteria cultured in YEPG medium. Biphenyl added as the only carbon source in the PMB medium but not in the YEPG medium apparently induced enzymes in the bacteria which can act upon the test PCBs. This induction has been reported in the literature for the *Pseudomonas* LB400 (Mondello, 1989 and Abramowicz, 1990). Based on the above results, pre-exposure of *A. eutrophus* to biphenyl enhances biodegradation and mineralization of 4CB and 4,4'CB. This may be due to the structural similarity of biphenyl and PCBs.

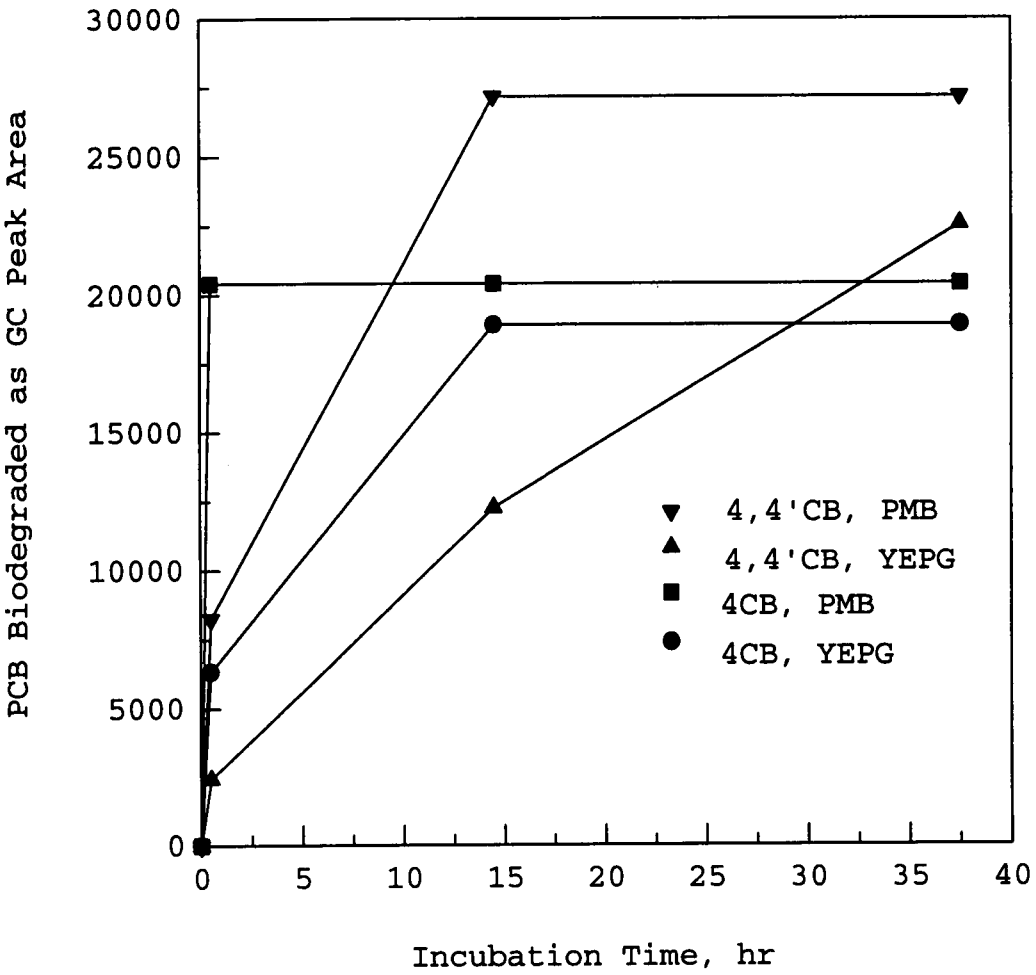


Figure 4.8 Impact of Growth Media Composition on the Biodegradation of 4CB and 4,4'CB in the Biosurfactant Solution (1 g/L) [50 ul of A5 Suspention (OD=0.2 at 600 nm) in 1 ml Sample].

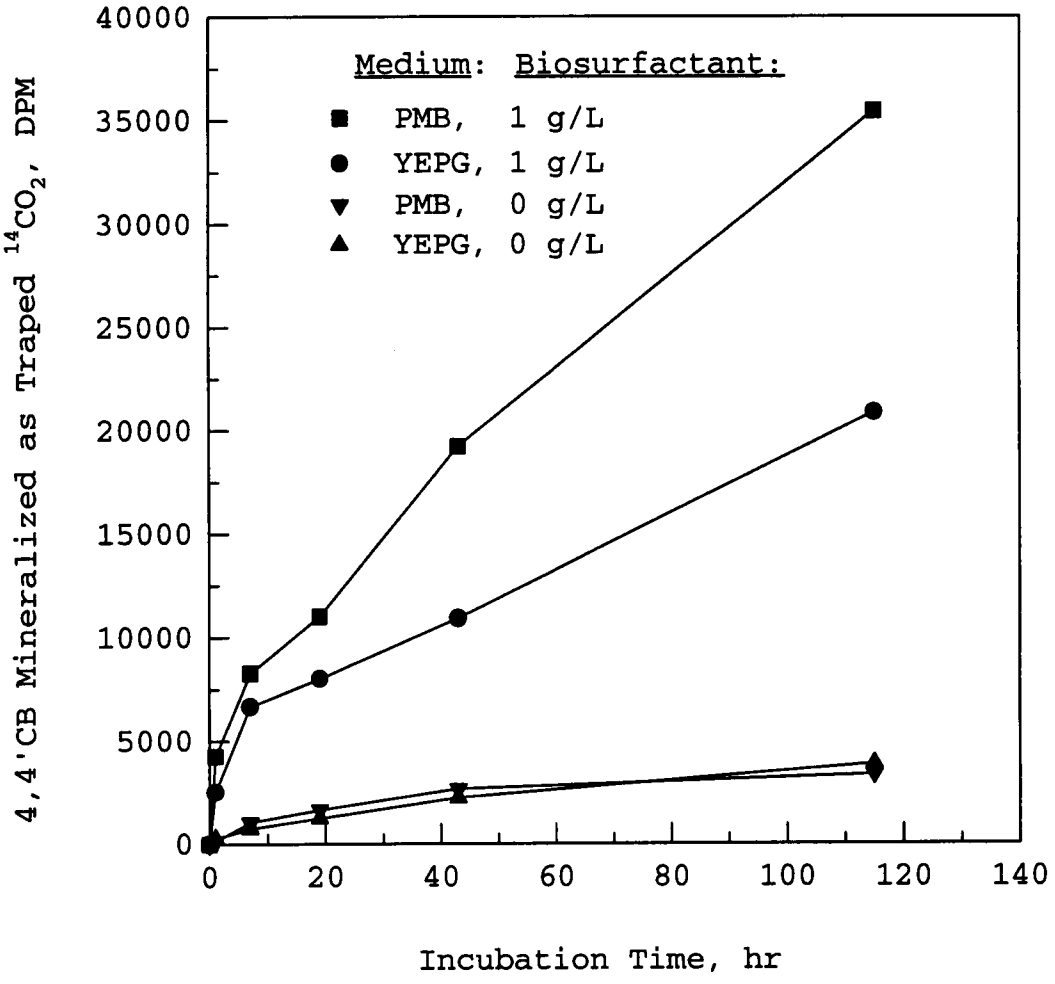


Figure 4.9 Impact of Growth Media Composition on the Mineralization of 4,4'CB in Biosurfactant of Solution (1 g/L) [50 ul of A5 Suspention (OD=2.0 at 600 nm) in 1 ml Sample)].

Based on the high PCB biodegradation ability and high cell yield, the media with biphenyl crystals contained in dialysis bags was selected as the growth media for the further PCB mineralization experiments.

4.3.2 Comparison of NaOH Replacement and Non-replacement Methods for 4,4'CB Mineralization

The results of 4,4'CB mineralized by *A. eutrophus* using NaOH ($^{14}CO_2$ trap) replacement and non-replacement methods are shown in Figure 4.10. Comparison of the results from the two methods showed little significant difference. Based on the ease of the procedure, the NaOH replacement method was used in all subsequent experiments.

4.3.3 Mineralization of 4,4'CB in the Presence of Biosurfactant

Mineralization: Results of mineralization of 4,4'CB by *Alcaligenes eutrophus* (A5) in biosurfactant solutions (0-4 g/L) without excess 4,4'CB crystals are presented in Figure of 4.11. Mineralized 4,4'CB (as $^{14}CO_2$) was determined as the trapped DPMs in the biologically active samples minus the DPM value (Figure A.1) for the biological controls (heat-killed A5). There was no obvious lag phase for any sample.

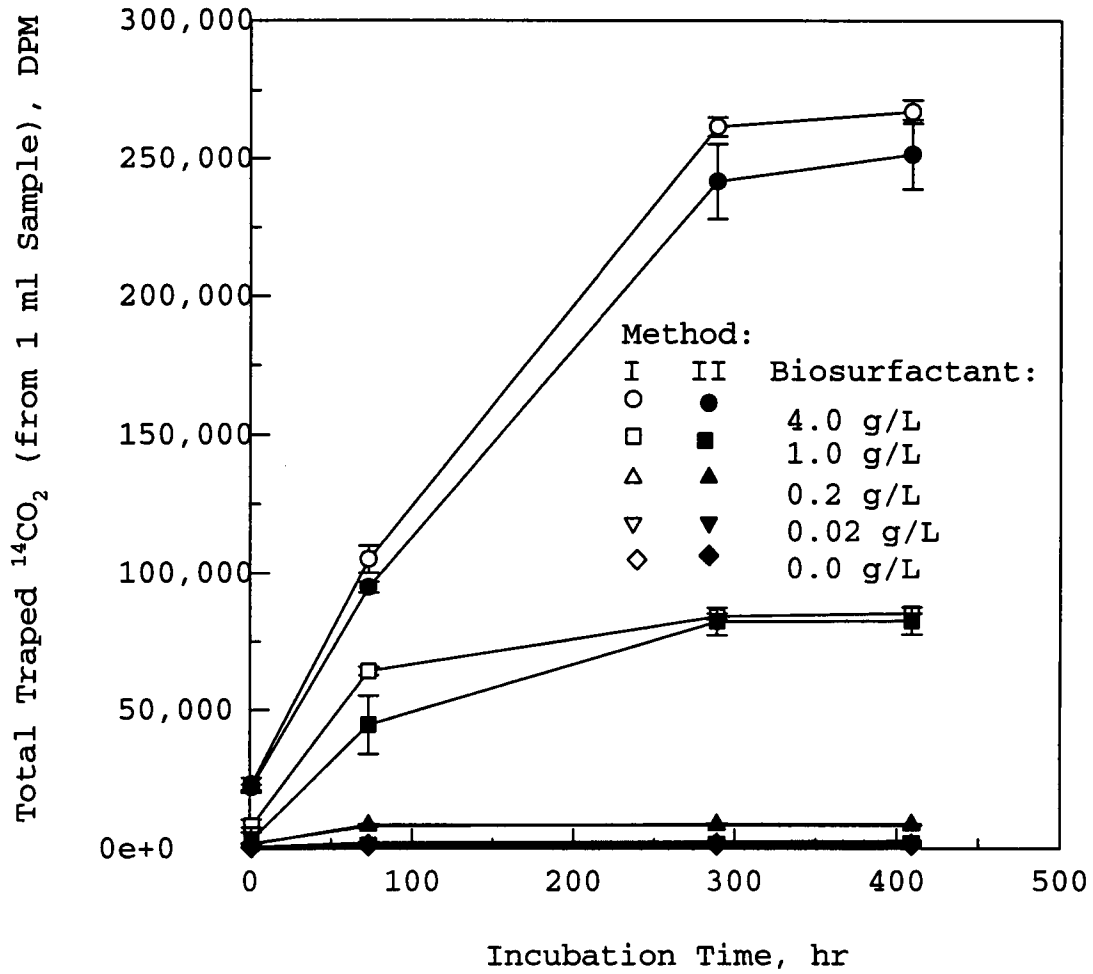


Figure 4.10 Comparison of the Two Methods used to Evaluate Mineralization of 4,4'CB (Method I: without the NaOH Replacement; Method II: with the NaOH replacement).

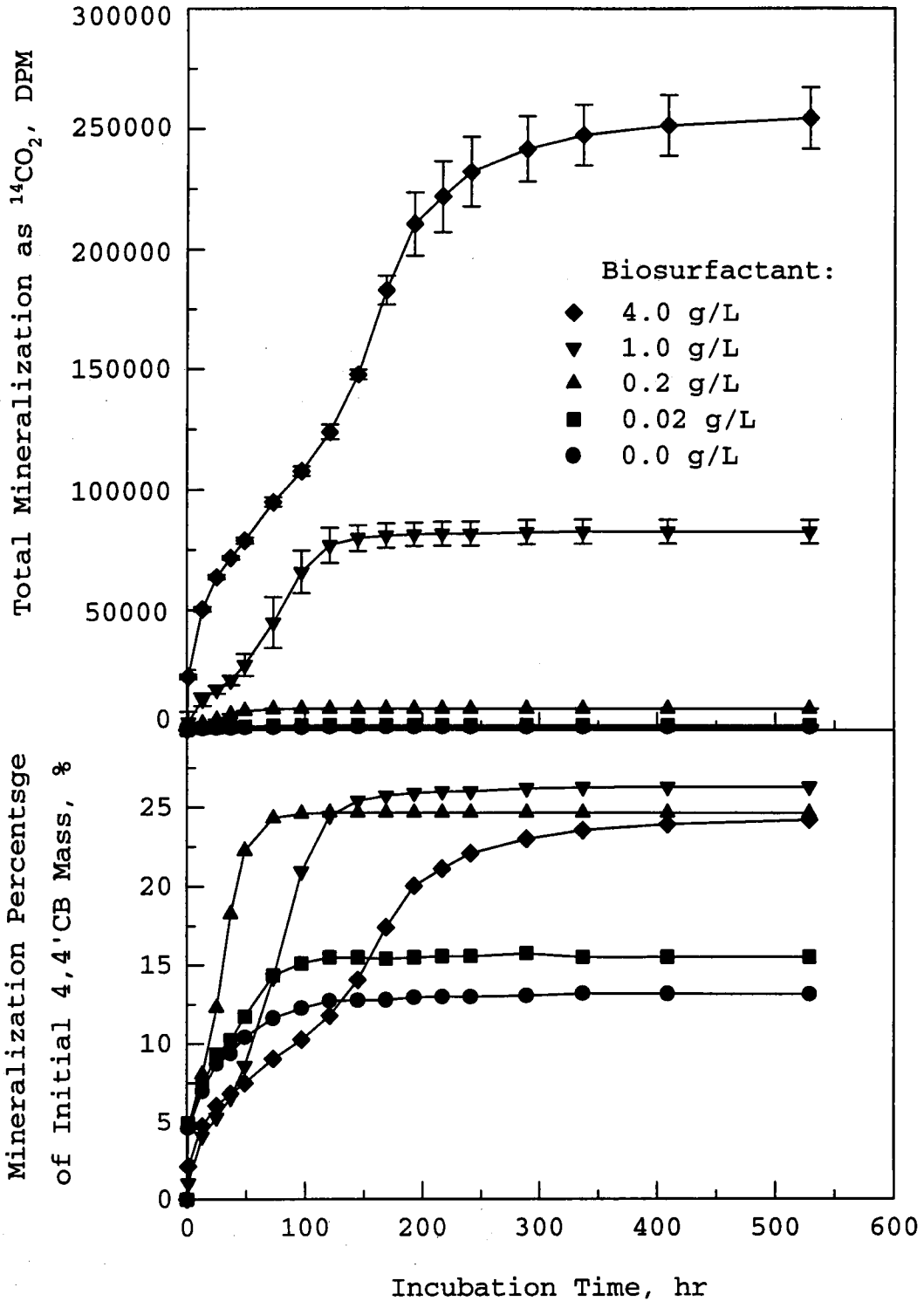


Figure 4.11 Mineralization of 4,4'CB by *A. eutrophus* in Biosurfactant Solutions without 4,4'CB Crystals.

Mineralization began in the log phase before reaching stationary phase. For higher biosurfactant concentrations, log phase mineralization continued for a longer period of time. The total amount of mineralized 4,4'CB increased with increasing biosurfactant concentration. Total mineralized 4,4'CB in the biosurfactant range of 0.2 to 4 g/L (above the CMC), exceeded the total dissolved 4,4'CB at a biosurfactant concentration of 0 g/L for the same volume of sample. This result confirmed the fact that micellized 4,4'CB was bioavailable. There are two readily apparent ways for *A. eutrophus* to mineralize micellized 4,4'CB in the system without excess 4,4'CB crystals. The first is that *A. eutrophus* can directly utilize the 4,4'CB associated with the micelles. The second is that the *A. eutrophus* can not access micellized 4,4'CB, but can only use free 4,4'CB (dissolved in the aqueous pseudophase). In this case, as the free 4,4'CB concentration in solution decreases due to mineralization, 4,4'CB is released from the micelle phase into the aqueous pseudophase. Micelles, therefore, act as a source of 4,4'CB for the bacteria.

Mineralized 4,4'CB was 13.2, 15.5, 24.7, 26.3, and 24.2% of the initial dissolved substrate for solutions containing biosurfactant concentrations of 0, 0.02, 0.2, 1.0, and 4.0 g/L at the end of incubation (529 hours). For

biosurfactant concentrations above the CMC (0.2-4.0 g/L), the percent mineralization was inversely related to biosurfactant concentration for incubation times ≤ 108 hours.

A number of potential reasons could explain the interesting results. First, the mineralization percentage of initial 4,4'CB in solutions (E) could be defined as:

$$E = \frac{r}{S_i} \times t \qquad (4.5)$$

where r: average substrate utilization rate for the incubation time period t, mg/(L*hr);

S_i: initial substrate concentration, mg/L

t: incubation time, hr.

For shorter incubation periods (≤ 108 hr), both the average substrate utilization rate (r) and initial substrate concentration (S_i) increased with increasing biosurfactant concentration, however, the increasing extent of initial substrate concentrations at elevated biosurfactant levels was greater than that of the average substrate utilization rate (Figure 4.5, 4.17, and 4.18). Therefore, solutions containing higher biosurfactant

concentrations resulting in a lower percentage (E) of PCB mineralization based on equation 4.5. Changes in substrate utilization rate (S) at high biosurfactant dose may be due to changes in cell hydrophobicity caused by biosurfactant sorption on the cell surface. Such an interaction results in a reduced mineralization rate. Zhang and Miller (1994) observed that the addition of rhamnolipid changed cell hydrophobicity and concluded that both the aqueous dispersion and cell hydrophobicit controlled bioavailablity of octadecane.

The amount of $^{14}CO_2$ generated during mineralization of 4,4'CB in 0.02 g/L biosurfactant is very similar to that generated without biosurfactant present. Because the biosurfactant concentration is below the CMC, only monomers exist in solution and the actual biosurfactant (monomers) concentration may be significantly lower due to sorption onto reactor surfaces. However, the extent of $^{14}CO_2$ generated at biosurfactant concentrations well above the CMC was much greater than for biosurfactant concentrations well below the CMC at the end of incubation confirmed that the presence of micelles clearly enhance mineralization of 4,4'CB.

The amount of $^{14}CO_2$ collected was directly impacted by the initial mass of PCB present in solution. Low biosurfactant concentrations resulted in less PCB mass in the test solutions. Therefore, total $^{14}CO_2$ data may not accurately reflect the enhancement impact of biosurfactant on PCB mineralization. When excess 4,4'CB crystals were present in solution, substrate limitation was minimized. When present, PCB crystals can serve as a substrate source if biosurfactant can continuously facilitate dissolution of PCB (crystals) into solution (micelle pseudophase). The results of 4,4'CB mineralization by *A. eutrophus* in the presence of 4,4'CB crystals are shown in Figure 4.12. Once again, no lag phase was observed and a stationary phase was reached after 600 hours of incubation. The total amount of $^{14}CO_2$ generated as well as the percent mineralized increased with increasing biosurfactant concentration. After 1012 hours of incubation, 4.6, 4.6, 5.3, 6.3, and 10.9 % of the initial 4,4'CB mass (including the excess crystals) was mineralized. Further, total mineralized 4,4'CB, compared to systems without excess 4,4'CB crystals present, increased at all biosurfactant concentrations. The presence of 4,4'CB crystals, as a continuous supply of 4,4'CB to the system, led to a increase in the total amount of mineralized 4,4'CB for a given incubation time.

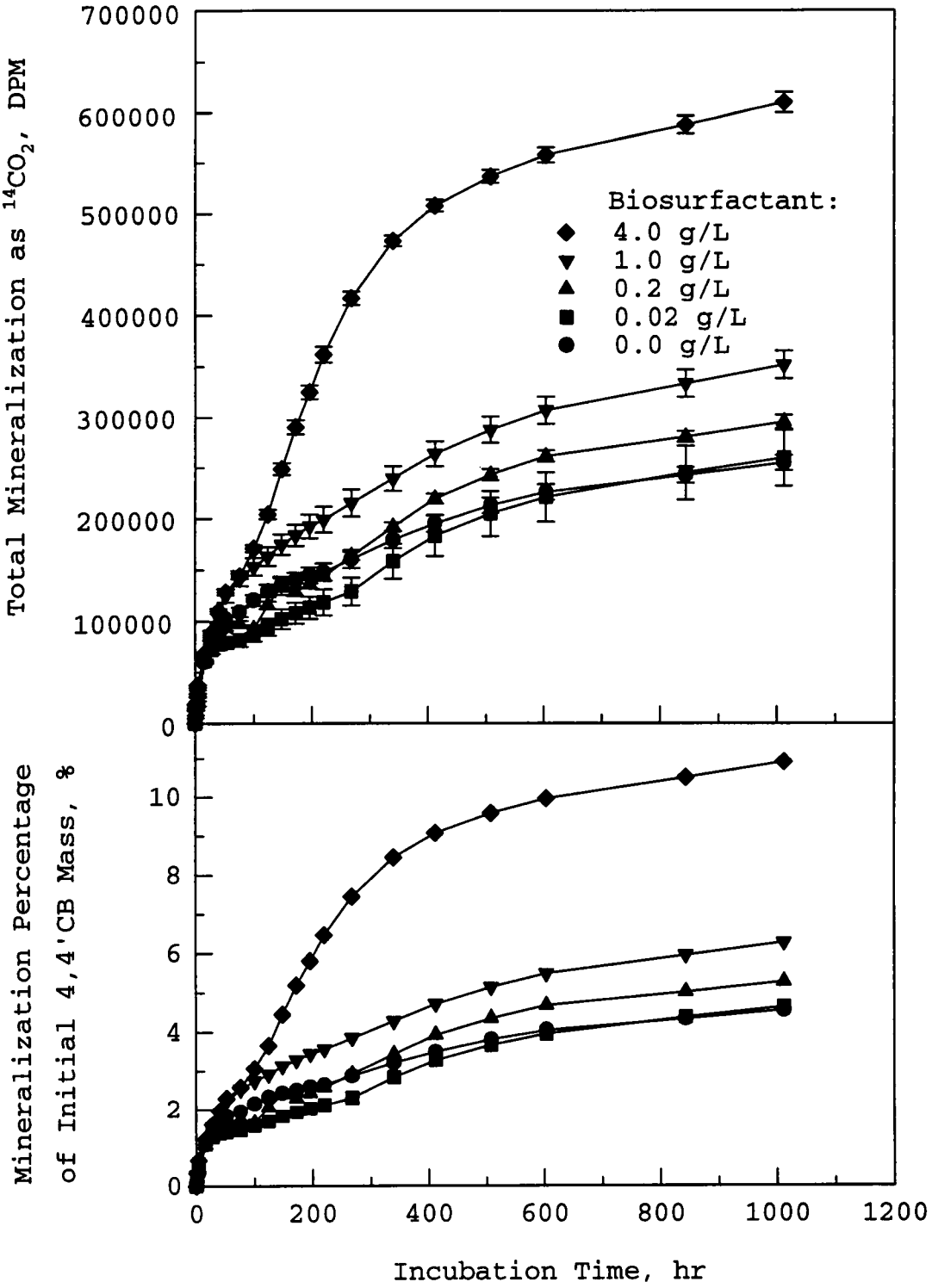


Figure 4.12 Mineralization of 4,4'CB by *A. eutrophus* in Biosurfactant Solutions with 4,4'CB Crystals.

The 4,4'CB mineralization percentage of initial 4,4'CB in solution, with and without excess 4,4'CB crystals presence, was not as high as expected. This may be due to the fact that 4,4'CB is difficult to be degraded by *A. eutrophus* (Bedard, et al., 1987a, b; Bopp, 1986). Some researchers (Rochkind, et al., 1986; Abramowicz, 1990) have reported that *Alcaligenes sp.* and many other bacteria can only mineralize 4,4'CB to chlorinated benzoic acid, that is, only about 50% of the initial PCB mass could be mineralized to CO_2. If chlorinated benzoic acid is the principle end-products of biodegradation (in addition to CO_2) then the actual 4,4'CB degradation extent in this study would be double the values (percentage) presented in Figure 4.11 and 4.12). Additional reasons for the low mineralization results observed may be that the PCB congener (as a sole carbon and energy source) did not support cellular growth, or other nutrients were deficient during mineralization because cell density dropped significantly during incubation. It was reported (Baxter, et al., 1975, Sawhney, 1986, Bedard et al., 1986) that 4,4'CB in addition to other congeners were not easily degraded by *A. eutrophus*, especially when only one congener was present in the culture medium. Modifications in the mineralization assay such as adding biphenyl to the solution may improve the mineralization extent. Build-up of

by-products in the culture medium may also inhibit the mineralization process. For example, chlorobenzoate, a common product from PCB biodegradation, was difficult to metabolized further by *A. eutrophus* and many other bacteria (Abramowicz, 1990). In this study, during the mineralization of 4,4'CB by *A. eutrophus*, there was evidence which indicated that build-up of intermediates did occur during bio-transformation (see section concerning intermediates). Low mineralization may also be due to the fact that not all the 4,4'CB loaded in the micro-reactor was bioavailable due to physicochemical changes such as vaporization to the head space and adsorption to the surface of the reactor (vial). The strong association of 4,4'CB with micelles may partially inhibit bioavailability. Lastly, a pH decrease in the culture medium due to accumulation of H^+ during mineralization of 4,4'CB may also impact mineralization processes.

Mass Balance: A mass balance of 4,4'CB at the end of incubation (529 hours) for solutions without excess 4,4'CB crystals is shown in Figure 4.13. Recovery is based on measured $^{14}CO_2$ activity (mineralization), and ^{14}C activity in solution. The solution ^{14}C activity includes non-degraded 4,4'CB, by-products and intermediates from degradation, and the ^{14}C incorporated into cellular

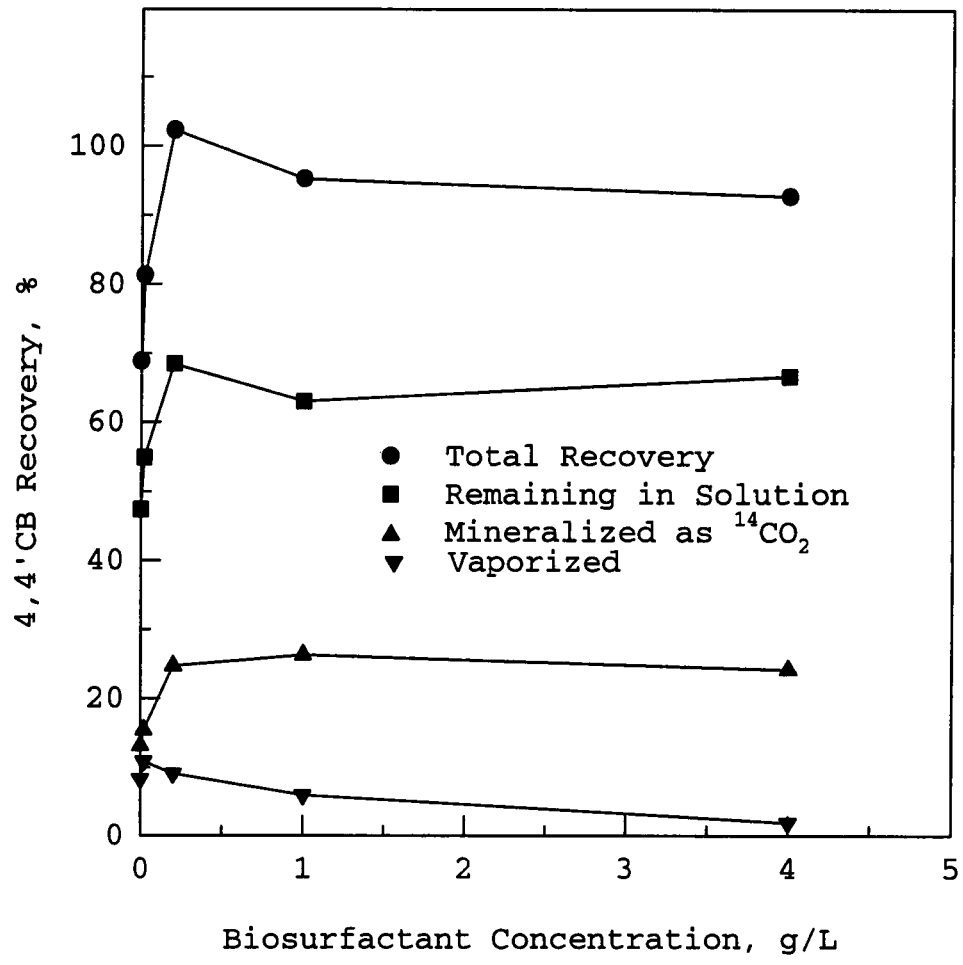


Figure 4.13 Mass Balance of 4,4'CB at the End of Incubation without Excess 4,4'CB Crystals.

biomass. Total recovery was the sum of the mineralized and solution ^{14}C activity. For high biosurfactant concentrations (0.2-4.0 g/L), recovered activity was above 90%. At low biosurfactant concentrations (0-0.02 g/L), recovery was 65-80%. The lower recoveries are believed due to losses associated with volatilization into the head space and /or sorption to vial walls.

Production of Intermediates: Researchers (Sayler et al., 1977, Furukawa and Matsumura, 1976) have reported that degradation of PCBs involved the production of a yellow-colored intermediate which degrades to chlorobenzoic acid over time. During mineralization of 4,4'CB in this study, a light lemon yellow color appeared in solutions with and without excess 4,4'CB crystals. Over time, the color become more vivid but finally disappeared. The appearance and disappearance times of the color intermediate are listed in Table 4.3. The higher the biosurfactant concentration, the more intense the color built-up and the longer the color persisted in solution. Because the color appeared only in biologically active solutions containing 4,4'CB, this material is likely an intermediate by-product resulting from the biodegradation of 4,4'CB. Further, accumulation of the color pigment indicates that biodegradation of this intermediate was initially rate limited.

Table 4.3 Appearance and Disappearance Time of Colored Intermediate during Mineralization of 4,4'CB in Biosurfactant Solutions.

	Biosurfactant Concentration (g/L)	Appearance Time (hr)	Disappearance Time (hr)
With excess 4,4'CB crystals	0	not observed	not observed
	0.02	13	25
	0.2	13	73
	1.0	25	121
	4.0	25	217
Without excess 4,4'CB crystals	0	16	268
	0.02	16	268
	0.2	16	268
	1.0	16	508
	4.0	16	844

Mineralization Rates: Mineralization rates as a function of incubation time for solutions containing no excess 4,4'CB crystals are presented in Figure 4.14. Mineralization rates were obtained by dividing the evolved ^{14}C activity of $^{14}CO_2$ in a given time period (t) by that time period. In general, initial rates were very high, dropped quickly then stabilized before finally approaching zero. The higher the biosurfactant concentration, the higher the initial rate. Further, there was a second rate increase during the stable rate period in the high biosurfactant solutions (1.0-4.0 g/L) which corresponded to the disappearance of the color intermediate. This strongly indicates that the elevated rate was due to the mineralization of the color intermediate. At low biosurfactant solutions, there was no significant secondary rate increase.

Mineralization rate as a function of incubation time for the solution containing excess crystals are shown in Figure 4.15. Rate trends are similar to those found in the no crystal system. However, color development of the intermediate was more intense and the secondary rate increase remained elevated for a longer period of time, presumably due to enhanced intermediate production in the presence of crystals. There was little secondary rate

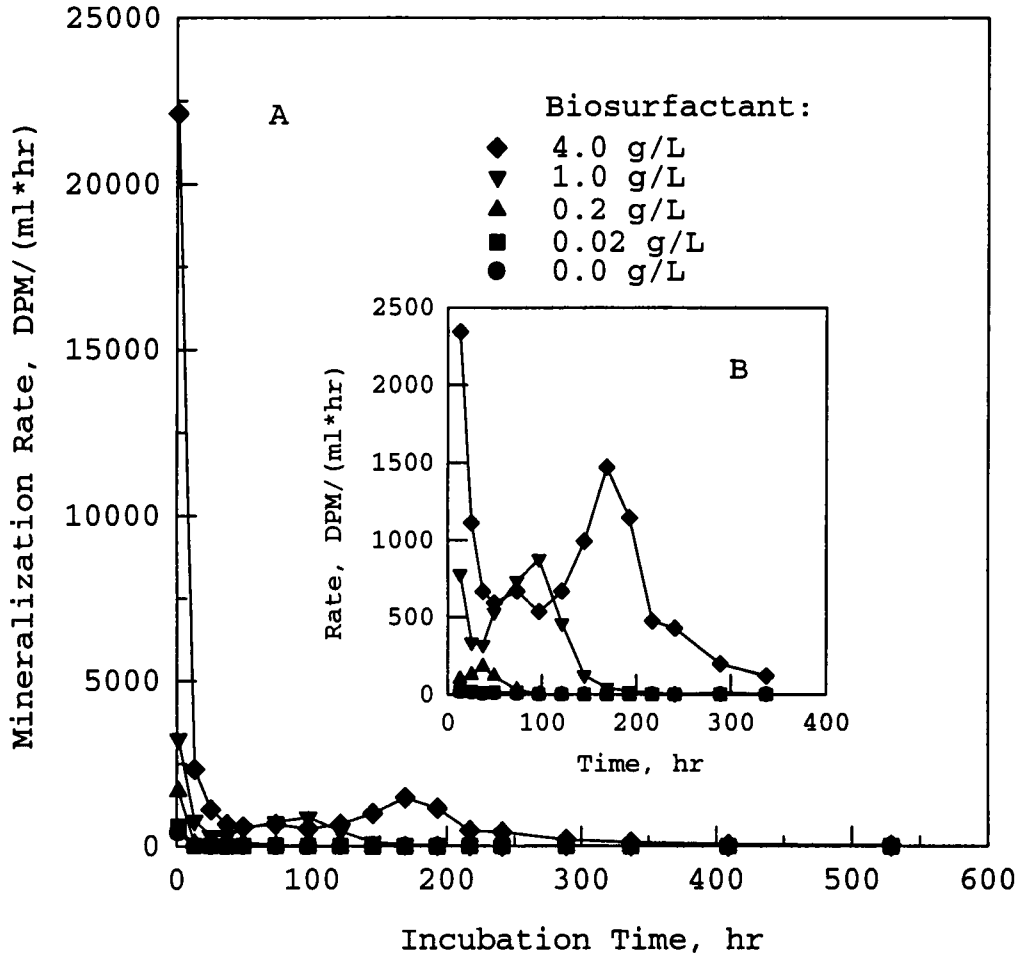


Figure 4.14 Mineralization Rate of 4,4'CB by *A.eutrophus* without Excess 4,4'CB Crystals (B is an Amplified Part of A).

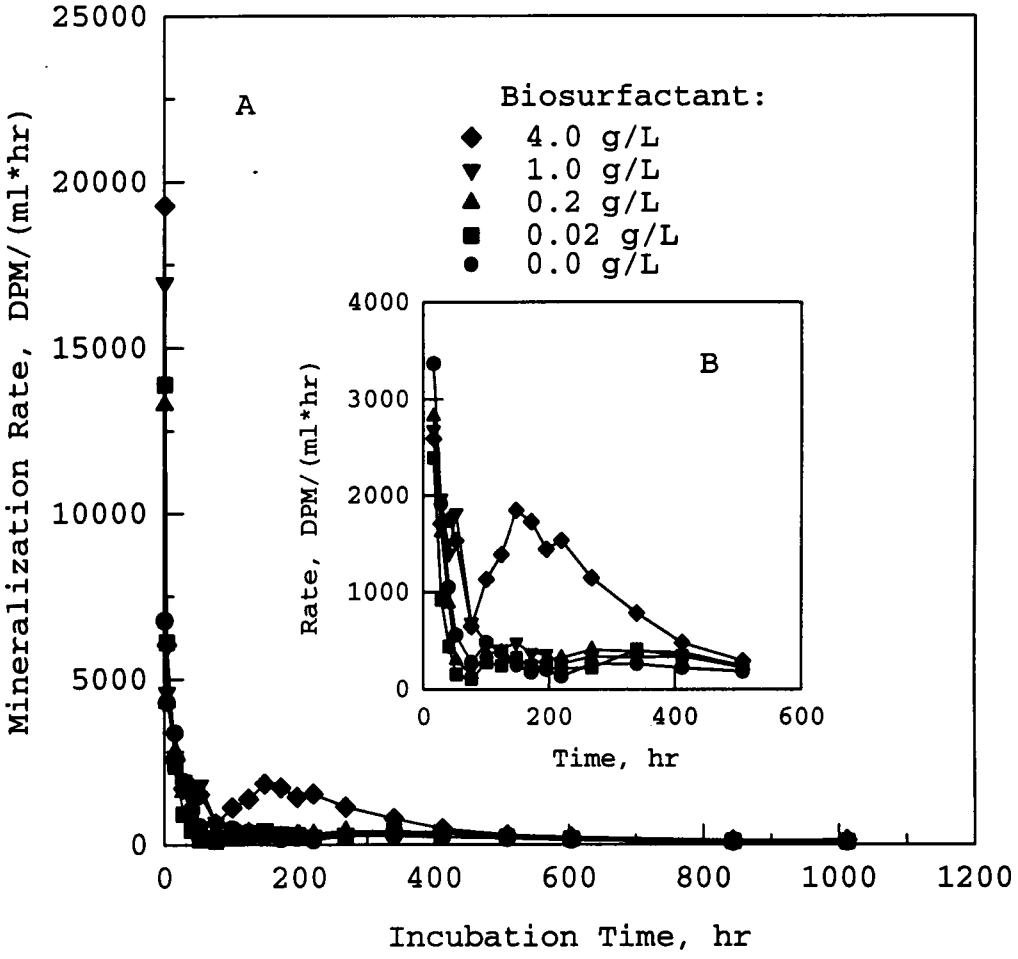


Figure 4.15 Mineralization Rate of 4,4'CB by *A. eutrophus* with Excess 4,4'CB Crystals (B is an Amplified Part of A).

elevation observed in low biosurfactant solutions (0-1.0 g/L).

Another approach was developed to describe 4,4'CB mineralization processes. Based on the data presented in Figure 4.11, a critical incubation time (t_c) which distinguishes fast (first, r_1) and slow (second, r_2) mineralization rates can be determined. The intersection point of two linear relationships defines t_c which can be used to model the time course of mineralization in this batch system (Figure 4.16). The slopes r_1 and r_2 represent average rates of the fast and slow mineralization, respectively. Similarly, for solutions containing excess4,4'CB crystals, two critical time points, t_{c1} and t_{c2}, can be determined thereby distinguishing three mineralization rates (r_1, r_2, and r_3) for the system. Rates can once again be estimated by the slope of each linear relationship (Table 4.4). This simplified approach can be helpful in designing a batch system or estimating the extent of mineralization in the system tested.

Mineralization rates (r) versus substrate (4,4'CB) and biosurfactant concentrations are shown in Figures 4.17 and 4.18, respectively. Based on these results, the following conclusions can be made: a) the first mineralization rate

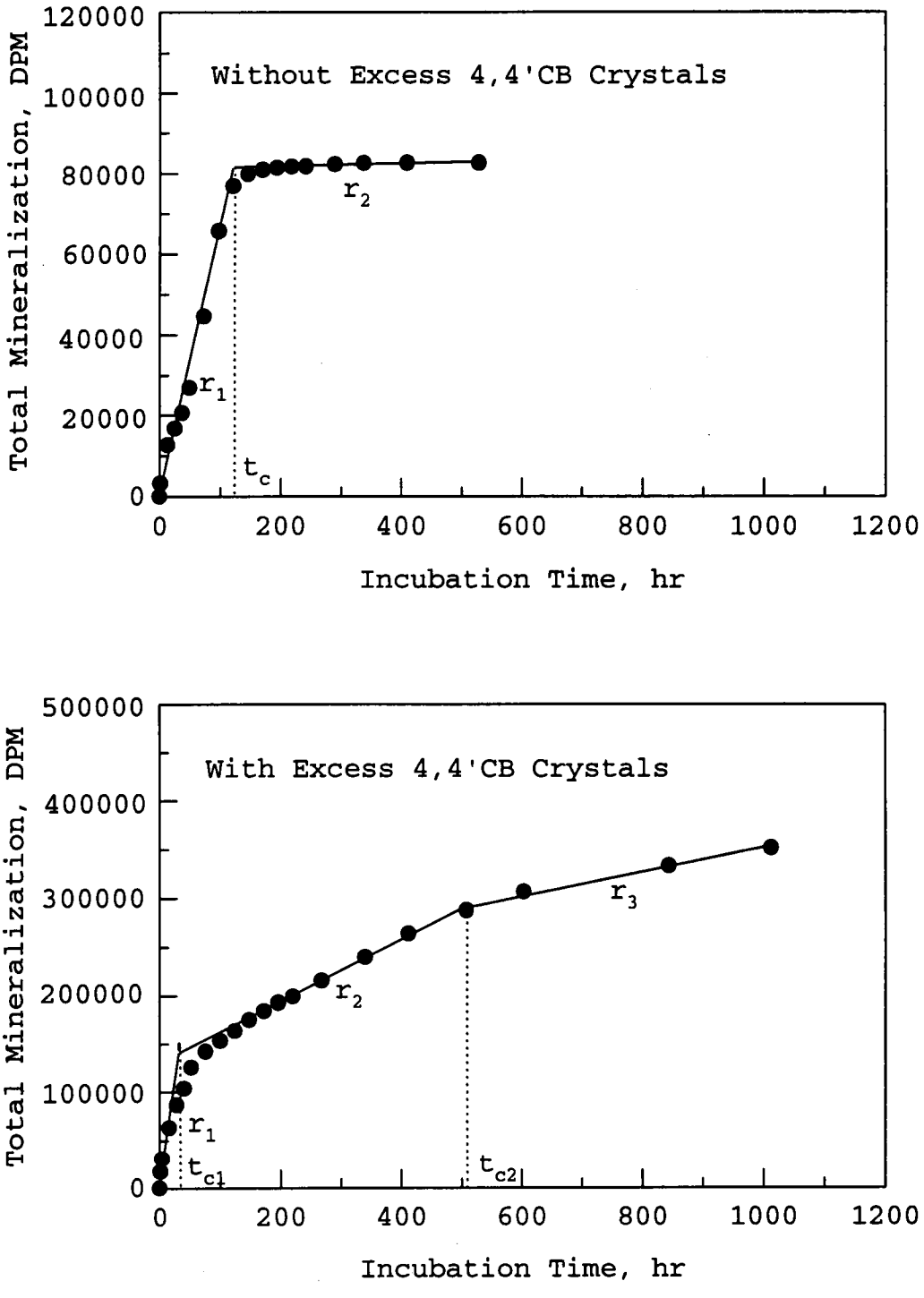


Figure 4.16 Determination of Mineralization Rate of 4,4'CB by *A. eutrophus* in the Presence of Biosurfactant (1 g/L).

Table 4.4 Critical Time (t_c) and Average Initial Mineralization Rate (r) of 4,4'CB in Solutions with and without Excess 4,4'CB Crystals.

Biosurfactant Concentration (g/L)			0.0	0.02	0.2	1.0	4.0
Solubility of 4,4'CB	(mg/L)		0.0657	0.0879	0.26	2.29	7.64
	(DPM/L)		9094	12136	35708	314002	1050523
No Excess Crystals	t_c	(hr)	49	49	49	121	217
	r_1	(DPM/(ml*hr))	22.7	37.4	163.2	634.8	1022.5
		(mg/(L*hr))	0.164	0.271	1.19	4.62	7.43
	r_2	(DPM/(ml*hr))	0.2	0.1	0	14.2	104.7
		(mg/(L*hr))	0.001	0.001	0	0.104	0.761
With Excess Crystals	t_{c1}	(hr)	28	28	28	28	28
	r_1	(DPM/(ml*hr))	2963.4	2570.9	2839.9	3094.3	3180.6
		(mg/(L*hr))	21.4	18.6	20.7	22.6	23.1
	t_{c2}	(hr)	508	508	508	508	340
	r_2	(DPM/(ml*hr))	271	277.1	340.9	418.9	800.2
		(mg/(L*hr))	1.96	2.01	2.48	3.05	5.82
	r_3	(DPM/(ml*hr))	83.1	109.1	104.1	126.7	147.3
		(mg/(L*hr))	0.600	0.790	0.758	0.924	1.07

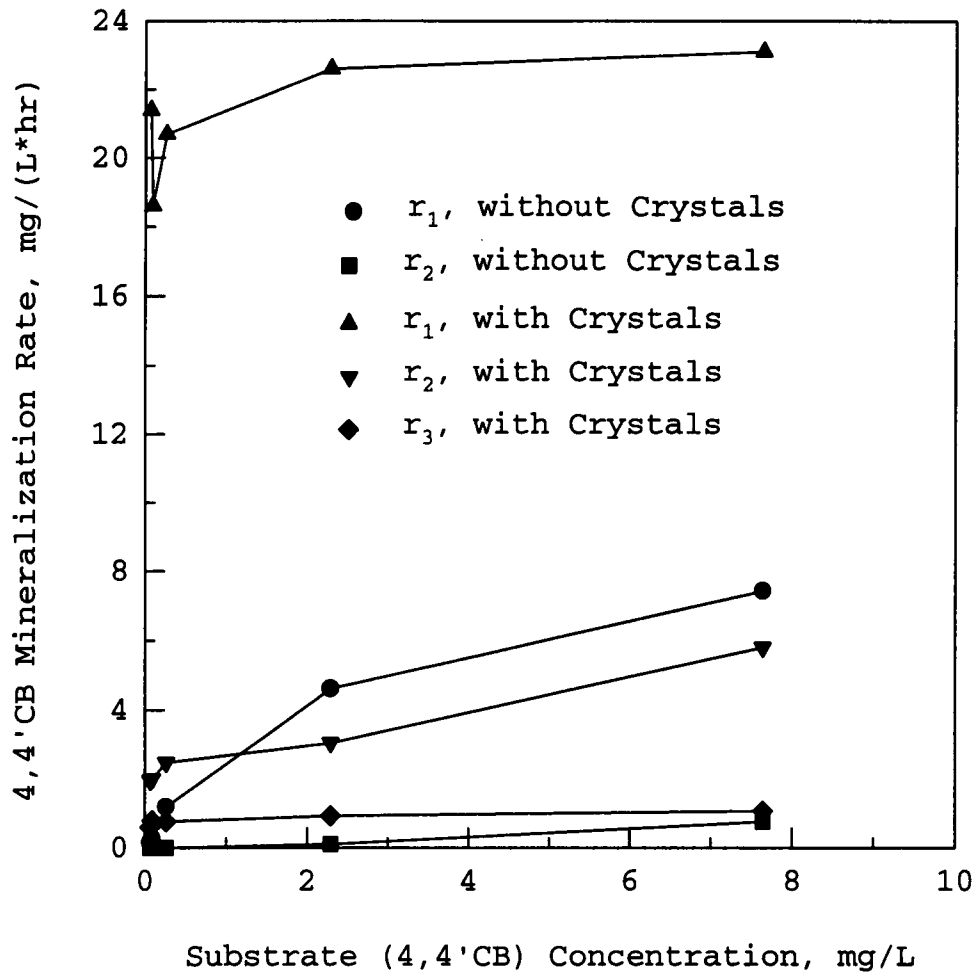


Figure 4.17 Effect of Substrate (4,4'CB) Concentration on Mineralization Rates of 4,4'CB.

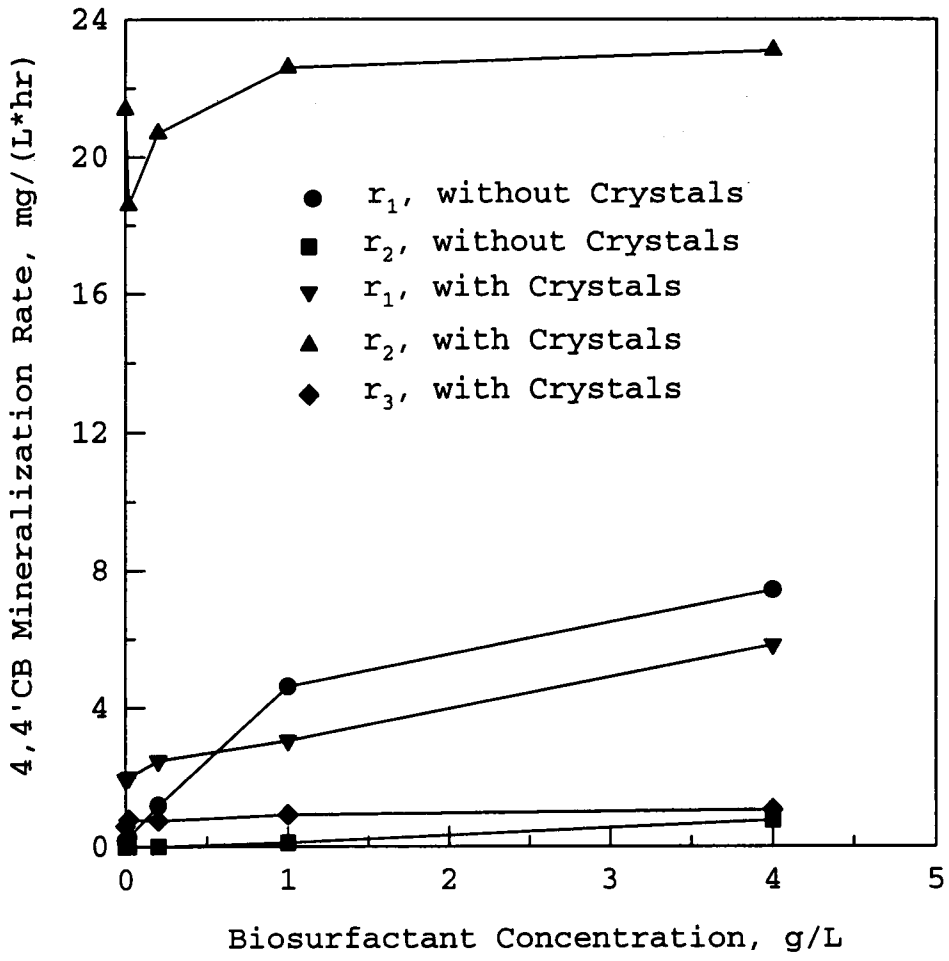


Figure 4.18 Effect of Biosurfactant Concentration on Mineralization Rates of 4,4'CB.

(r_1) increased with increasing biosurfactant concentration and initial 4,4'CB (substrate) concentration, which confirmed that biosurfactant enhanced the mineralization rate. b) the presence of 4,4'CB crystals enhance the mineralization rate (r_1). It appears that the mineralization rate r_1, in the system without 4,4'CB crystals was limited by lack of a PCB source (4,4'CB crystals) to support high mineralization rates. From figures 4.17 and 4.18, initial mineralization rates were much higher than the final mineralization rates in the same system. Additionally, when excess 4,4'CB crystals were present, bacteria density was almost constant and similar in all biosurfactant solutions (0-4 g/L) at incubation times less than 108 hours (see following section), therefore r_1 can be considered the initial specific substrate utilization rate for the system.

Variation in Cell Density During Incubation: The change in *A. eutrophus* cell density during mineralization of 4,4'CB in the presence of biosurfactant is shown in Figure 4.19. For solutions without excess 4,4'CB crystals, the cell density slightly increased from an initial level of 10^9 cells/ml to a final level of 10^9-10^{10} cells/ml at the end of incubation (529 hours). For solutions without biosurfactant, the cell density decreased to 10^6 cells/ml

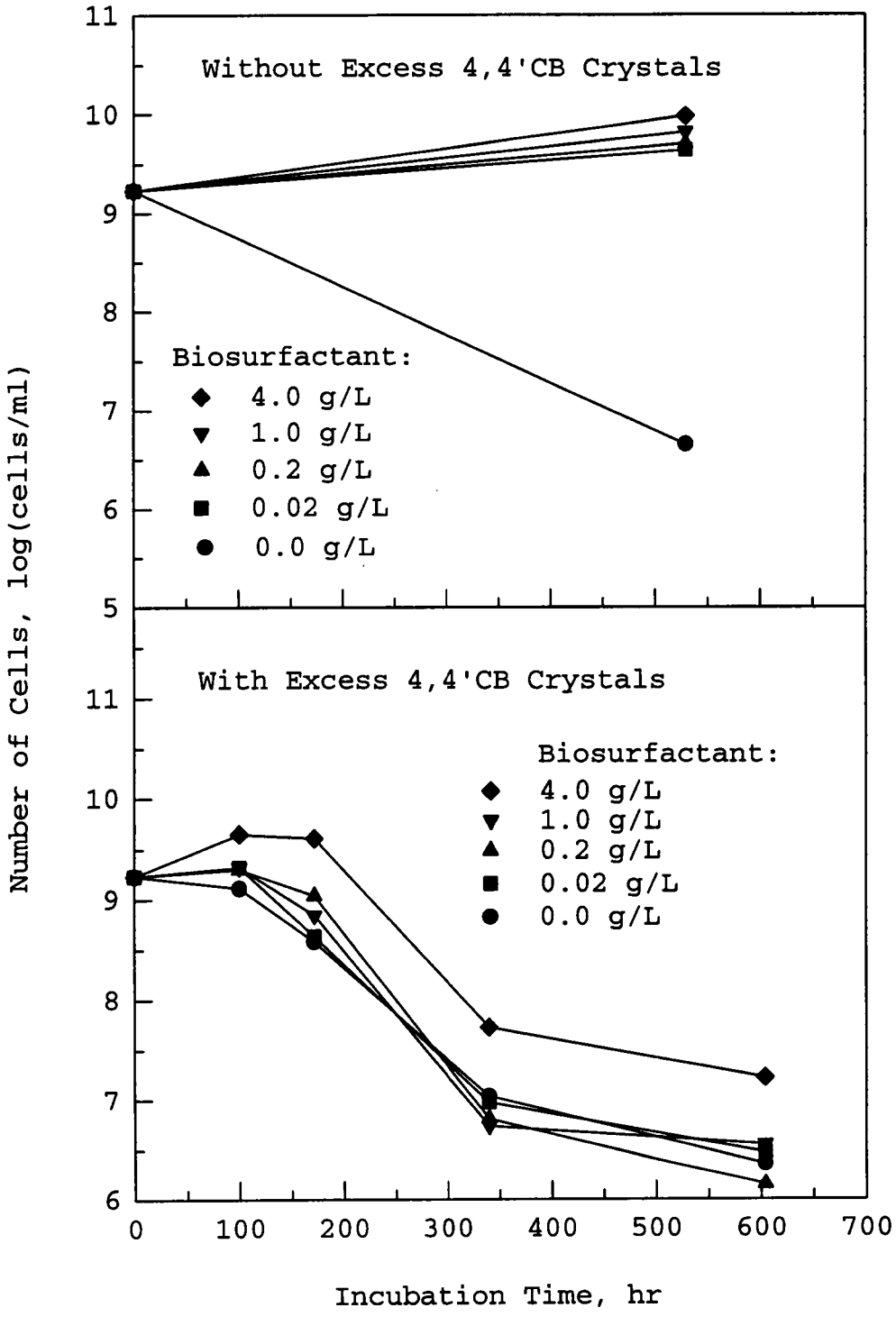


Figure 4.19 Changes in *A. eutrophus* Cell Density during Mineralization of 4,4'CB.

after 529 hours incubation. Since cell densities were only measured at the beginning and the end of the each experiment, the change during incubation was unknown.

In the presence of excess 4,4'CB crystals, cell density remained relatively constant during the first 200 hours of incubation, then decreased to a level of 10^6-10^7 cells/ml at the end of incubation (600 hr). The reason for the decrease in cell density was not clear. It was possible that the bacteria switched from PCB to biosurfactant as the carbon source once PCB levels were significantly reduced. Data to support this possibility is presented in the following section.

Co-mineralization of Biosurfactant: Co-mineralization of biosurfactant during mineralization of 4,4'CB by the *A. eutrophus* is shown in Figures 4.20 and 4.21. For solutions without excess 4,4'CB crystals (Figure 4.20), an increase in biosurfactant concentration (from 0.02 g/L to 4 g/L except for 1.0 g/L) resulted in an increase in the total amount of mineralized biosurfactant during the first 500 hours of incubation. However, the percent of initial biosurfactant mineralized decreased with increasing biosurfactant concentration. The reason for the exception of 1.0 g/L biosurfactant solution is unknown. At 500 hours,

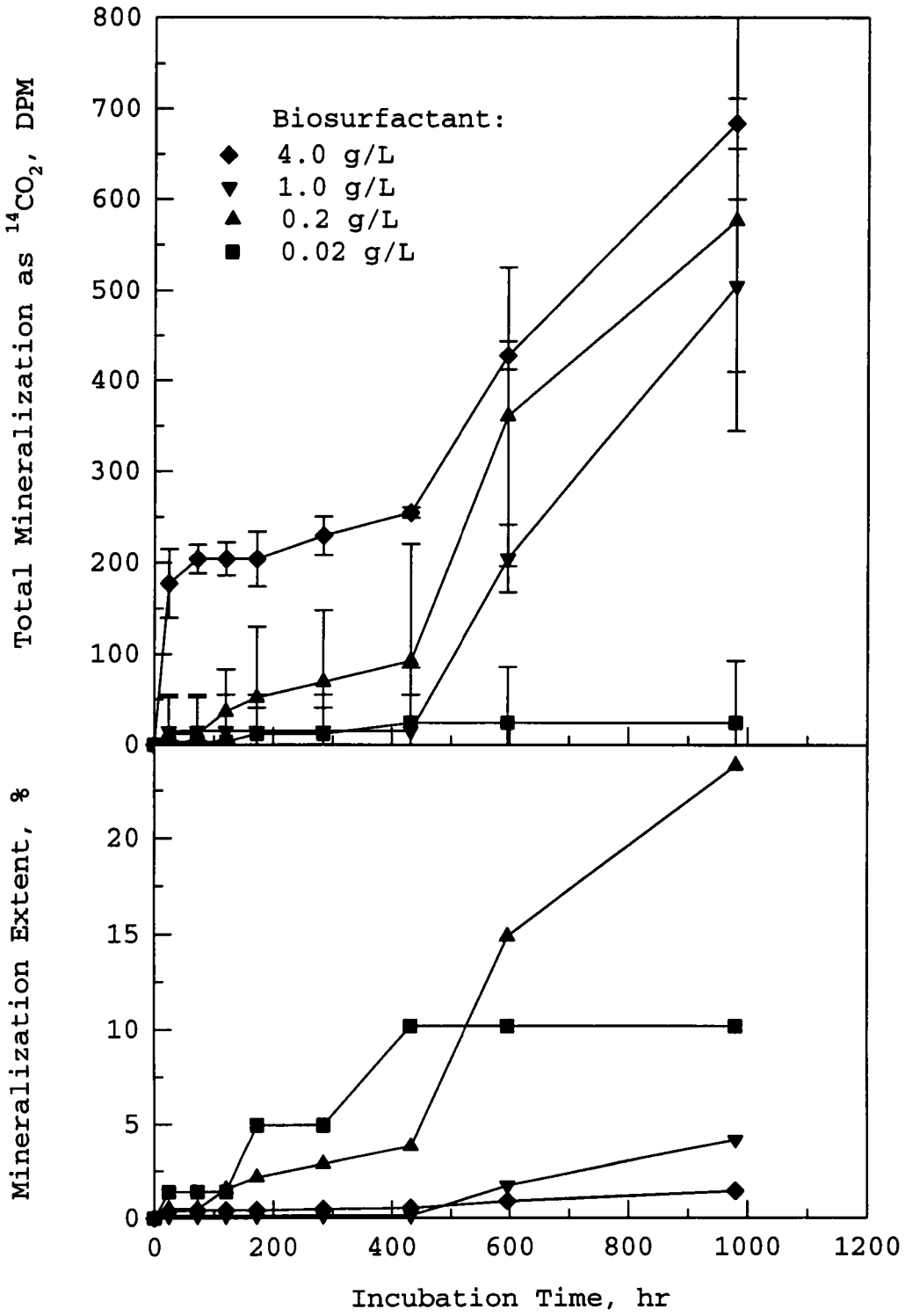


Figure 4.20 Co-mineralization of ^{14}C-biosurfactant by *A. eutrophus* in 4,4'CB Solutions without Excess 4,4'CB Crystals.

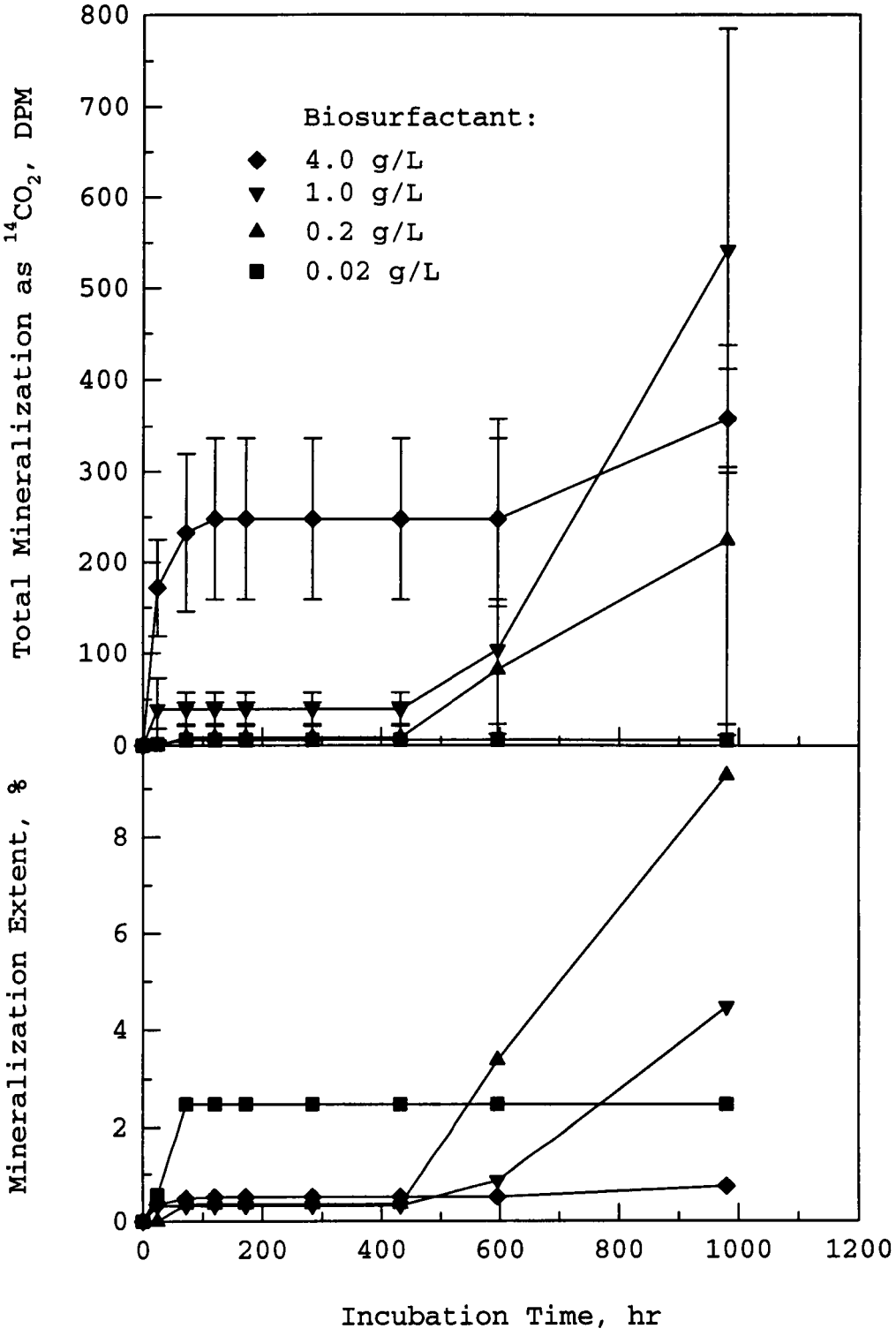


Figure 4.21 Co-mineralization of ^{14}C-biosurfactant by *A.eutrophus* in 4,4'CB Solutions Containing Excess 4,4'CB Crystals.

mineralized biosurfactant for all concentration levels (0.02-4 g/L) was less than 10% of the initial biosurfactant added. After 980 hr incubation, the maximum mineralization percentage, 24%, appeared in the 0.2 g/L biosurfactant solution. Mineralization percentages for the other biosurfactant solutions were still less than 10%. The extent of PCB utilization in the 0.2 g/L biosurfactant solution stabilized more quickly than in higher biosurfactant solutions (Figure 4.11) therefore bacteria in these micro-reactors may utilize biosurfactant for survival, resulting in a higher extent of biosurfactant mineralization.

For samples in which excess 4,4'CB crystals were present (Figure 4.21), an increase in biosurfactant concentration also resulted in an increase in the total amount of mineralized biosurfactant but decreased the mineralization percentage. After 500 and 980 hr incubation, the mineralized biosurfactant for all solutions was less than 3% and 10%, respectively.

In comparing results from the two solution systems (with and without excess 4,4'CB crystals), less biosurfactant was co-mineralized in the presence of 4,4'CB crystals. In addition, biosurfactant co-mineralization was

not significant until extensive PCB mineralization had occurred. It appears that *A. eutrophus* can shift its enzyme systems from 4,4'CB to biosurfactant over time.

A mass balance for ^{14}C-labeled biosurfactant in both systems (with and without excess crystals) is shown in Figure 4.22. Data was normalized to the initial mass of biosurfactant added to each solution. Total recovery was determined as the sum of three components: soluble ^{14}C (non-degraded biosurfactant and intermediates); trapped (degraded) $^{14}CO_2$, and ^{14}C associated with cells. The solution phase ^{14}C was the predominant phase of activity in either system. At low biosurfactant concentrations (0.02 g/L), ^{14}C recoveries, with and without excess 4,4'CB crystals, were 93.2 and 88.8%, respectively. In the biosurfactant range of 0.2-4.0 g/L, recoveries were 59-68%. Biosurfactant losses were believed due to sorption on the wall of the vials. Further study showed that there was little difference (<3.0%) of ^{14}C activity in solutions before and after removal of cells by high speed centrifugation. That result indicates only a small amount of biosurfactant sorbed to the cell surface and/or was synthesized as a cellular component. Synthesis of biosurfactant as cell material in which its DPM could not be counted was not significant.

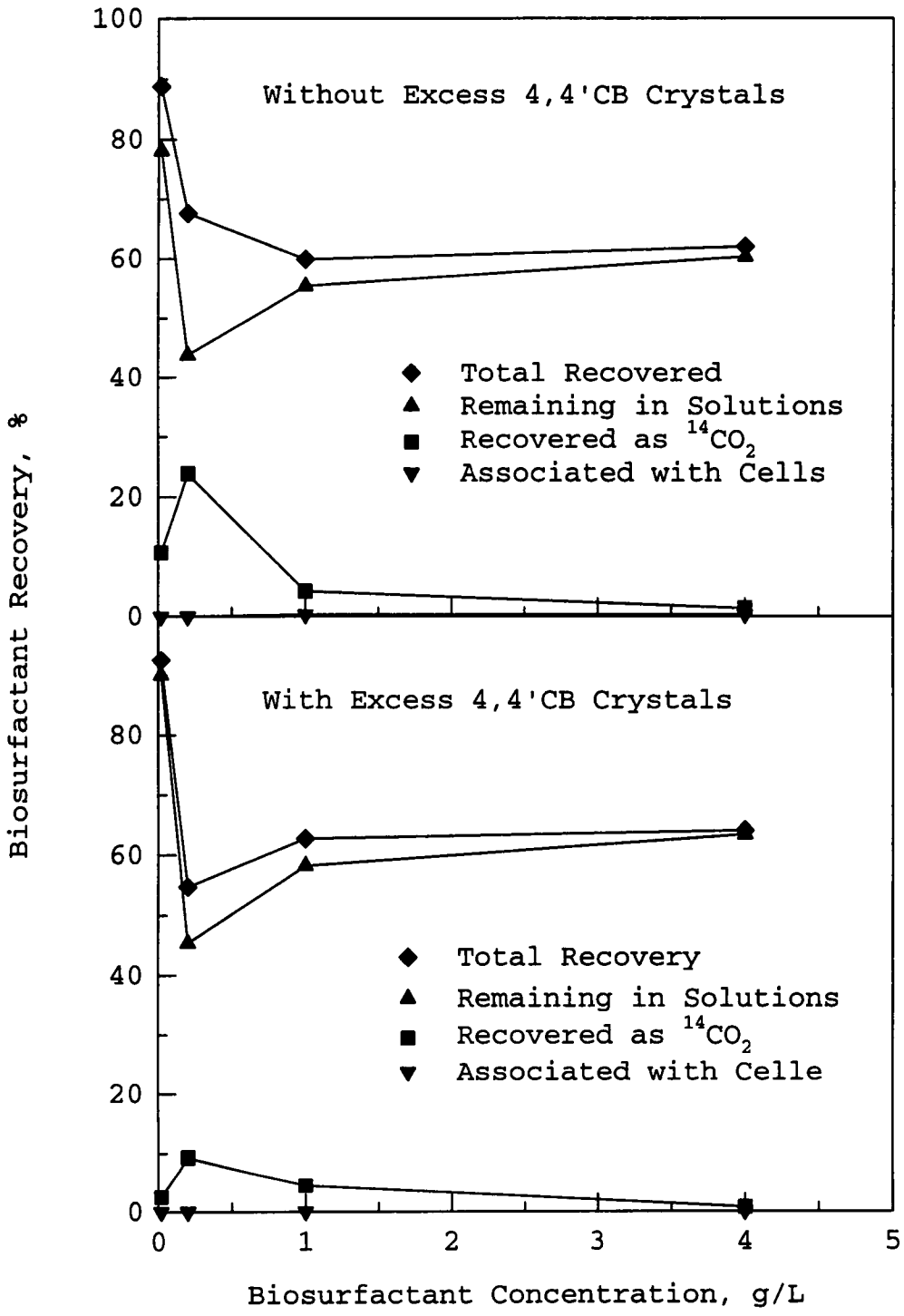


Figure 4.22 Mass Balance of ^{14}C-labelled Biosurfactant at the End of Incubation.

4.4 SORPTION, DESORPTION AND MINERALIZATION OF 4,4'CB FROM CONTAMINATED SOILS

4.4.1 Sorption of 4,4'CB to Soils

Two natural soils with initial total carbon fractions (f_{tc}) of 0.5 and 1.5 were pretreated (section 3.2) before PCB sorption experiments. Soil characteristics before and after treatments are listed in Table 4.5. Approximately 36 and 51% of the initial total carbon, respectively, was lost due to soil pretreatment.

After pretreatment, soils with f_{tc}s of 0.32 and 0.74 were synthetically contaminated with ^{14}C-4,4'CB to a level of 227168 and 237532 DPM/g soil (1.65 and 1.73 µg/g soil), respectively (Figure 4.23). Sorption was linear up to 6 batch treatments then began to level off as binding sites became saturated with PCB. The sorption capacity of the high f_{tc} soil was slightly greater than that of the low f_{tc} soil possibly due to the difference in carbon content. The partitioning coefficient between soil and water can be defined as

Table 4.5 Soil Changes as a Result of Pretreatment.

		Low f_{tc} Soil	High f_{tc} Soil
f_{tc}	Initial	0.50	1.5
	After pretreatment	0.32	0.74
	Loss as initial, %	36.0	50.7
	After contamination*	0.27	0.60
	Loss as initial, %	46.0	60.0
Mass (g)	Initial	1.02	1.01
	After pretreatment	0.97	0.93
	Loss as initial, %	4.9	7.9
Water in soil (g)	Initial	0.62	0.73
	After pretreatment	0.62	0.65
	Loss as initial, %	0.0	11.0

* exclude the contaminated 4,4'CB in soils.

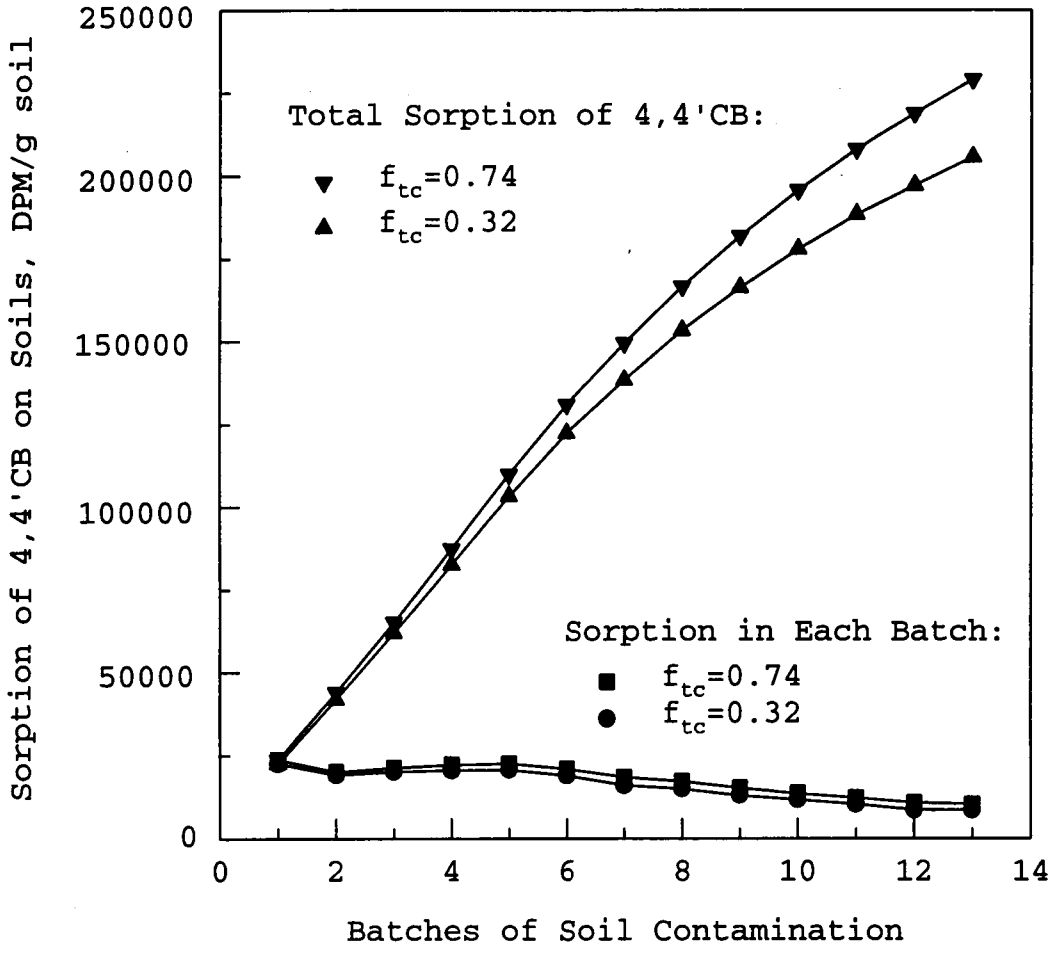


Figure 4.23 Sorption of 4,4'CB to Soils Containing Different Total Carbon Contents (f_{tc}).

$$K_p = C_s/C_w \qquad (4.6)$$

Where

C_s: concentration of 4,4'CB sorbed onto soil, mg/g_{soil};

C_w: concentration of 4,4'CB remaining in water, mg/ml.

The larger the K_p, the more likely a hydrophobic organic contaminant will partition to the soil phase. K_p values for 4,4'CB binding to the test soils can be determined using first batch contamination data. After first batch treatment, C_s values were 5668 and 5967 DPM/g_{soil}, and C_w values were 138 and 142 DPM/ml for the low and high f_{tc} soils, respectively. The K_p was calculated as 41.1 and 43.9 ml/g_{soil} for the soils with f_{tc} 0.32 and 0.74, respectively.

4.4.2 Desorption of 4,4'CB

Contaminated soils were washed three times using biosurfactant concentrations ranging from 0-4.0 g/L (Figure 4.24). The total amount of 4,4'CB desorbed from each soil increased with increasing biosurfactant concentration and number of wash treatments. Washing efficiency was higher in the low f_{tc} soil than the high f_{tc} soil. At a biosurfactant concentration of 4.0 g/L, 57.2% of the sorbed 4,4'CB was

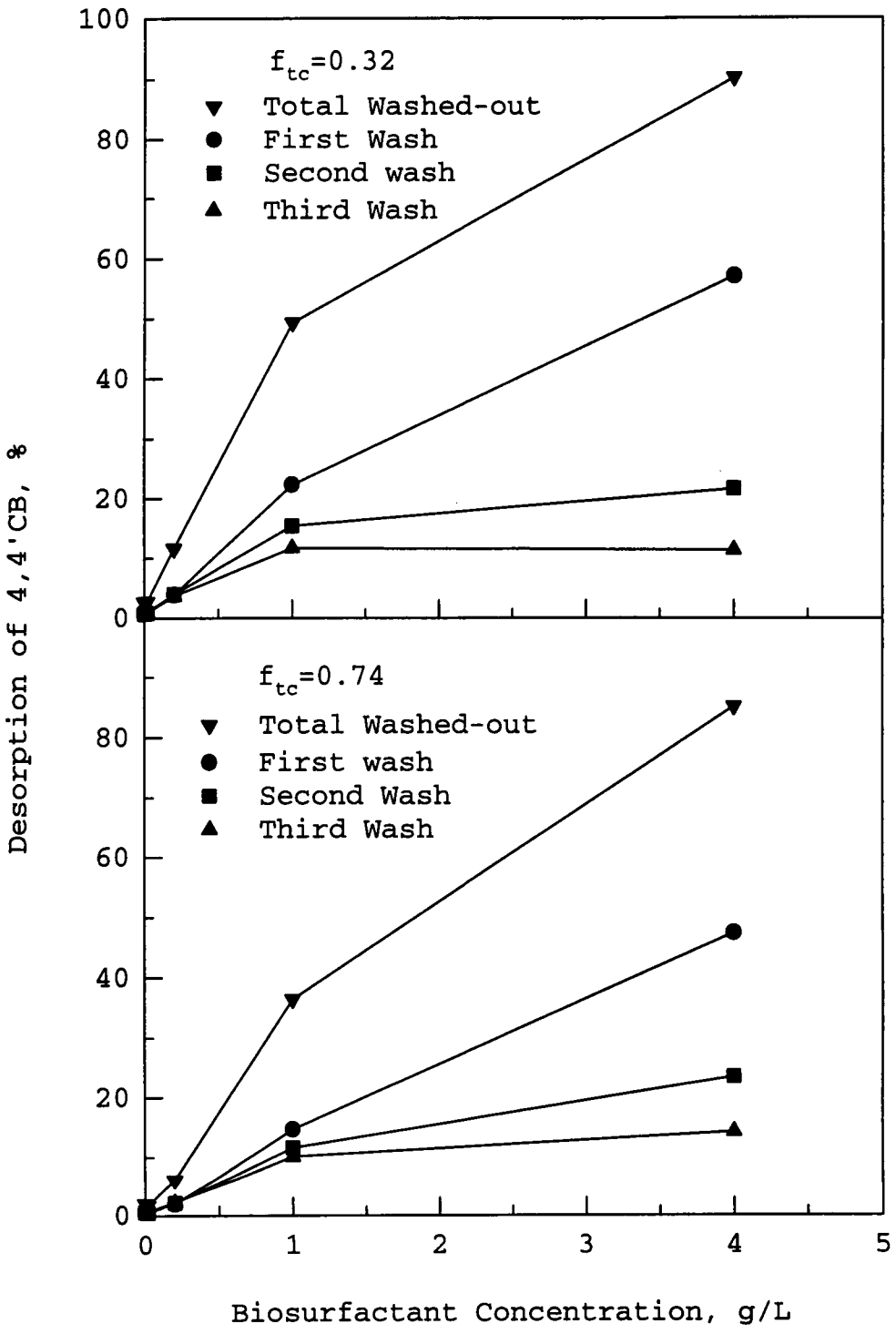


Figure 4.24 Desorption of 4,4'CB from Different f_{tc} Soils Using Biosurfactant Solutions.

removed from the low f_{tc} soil after one wash. In the high f_{tc} soil, 47.5% of the sorbed 4,4'CB was desorbed at this biosurfactant concentration. After three batch washes, the total amount of 4,4'CB removed from the low and high f_{tc} soils was 90.3 and 85.3%, respectively. In comparison, only 3.2% and 2.7% of the sorbed 4,4'CB was removed from the same soils using the control solution (biosurfactant concentration = 0 g/L) after three batch washes. The extent of 4,4'CB removal from low and high f_{tc} soils was enhanced by 87.1 and 82.6%. Under the procedures tested, biosurfactant significantly improved 4,4'CB desorption from the test soils.

4.4.3 Sorption of Biosurfactant

The loss of biosurfactant due to sorption on soil during treatment will result in a decrease of available solution phase biosurfactant for desorption of PCBs. Further, the binding of biosurfactant to soil will change the soil surface characteristics. Biosurfactant losses to soil during first batch washes are listed in Table 4.6. 29% and 32.4% of the 4.0 g/L biosurfactant solution sorbed to the low and high f_{tc} soils during the soil washing.

Table 4.6 Sorption of Biosurfactant to Soils.

Soil f_{tc}	Biosurfactant initial conc. (mg/L)			
	20	200	1000	4000
0.32	Biosurfactant conc. after equilibrium (mg/L)			
	6.2	133	809	2840
	Biosurfactant sorbed in soil (mg/g_{soil})			
	0.044	0.216	0.613	3.72
0.74	Biosurfactant conc. after equilibrium (mg/L)			
	4.9	102	761	2690
	Biosurfactant sorbed in soil (mg/g_{soil})			
	0.052	0.340	0.833	4.55

To characterize the sorption of biosurfactant onto the soils, the data were fitted to Langmuir and Freudlich isotherms (Figure 4.25). The Freudlich isotherm appeared to better describe binding of biosurfactant to the soils in the study. Because the Langmuir isotherm assumes that sorption occurs as a single homogeneous layer and the Freudlich isotherm assumes that binding on the surface is heterogeneous, it appears that biosurfactant sorption to the test soils is not restricted to a mono-layer. Researches (Liu et al., 1992) confirmed that sorption of nonionic surfactants to soils with f_{oc} of 1-1.5 was well described by a Freundlich isotherm when the concentration of surfactant was below the CMC.

4.4.4 Mineralization of 4,4'CB in Soil Wash Solutions.

Mineralization of 4,4'CB in soil wash solutions (first treatment) by *Alcaligenes eutrophus* (A5) showed that, once again, total mineralization increased with increasing biosurfactant concentration (Figure 4.26 and 4.27). After a 549 hour incubation, the percent 4,4'CB mineralized was 36.9, 15.1, 16.8, 15.5, and 21.2% in the low f_{tc} (0.32) soil wash solution; and 38,1, 35.8, 30.5, 20.5, and 24.2% in the high f_{tc} (0.74) soil wash solution for biosurfactant concentration of 0, 0.02, 0.2, 1.0, and 4 g/L. The percent

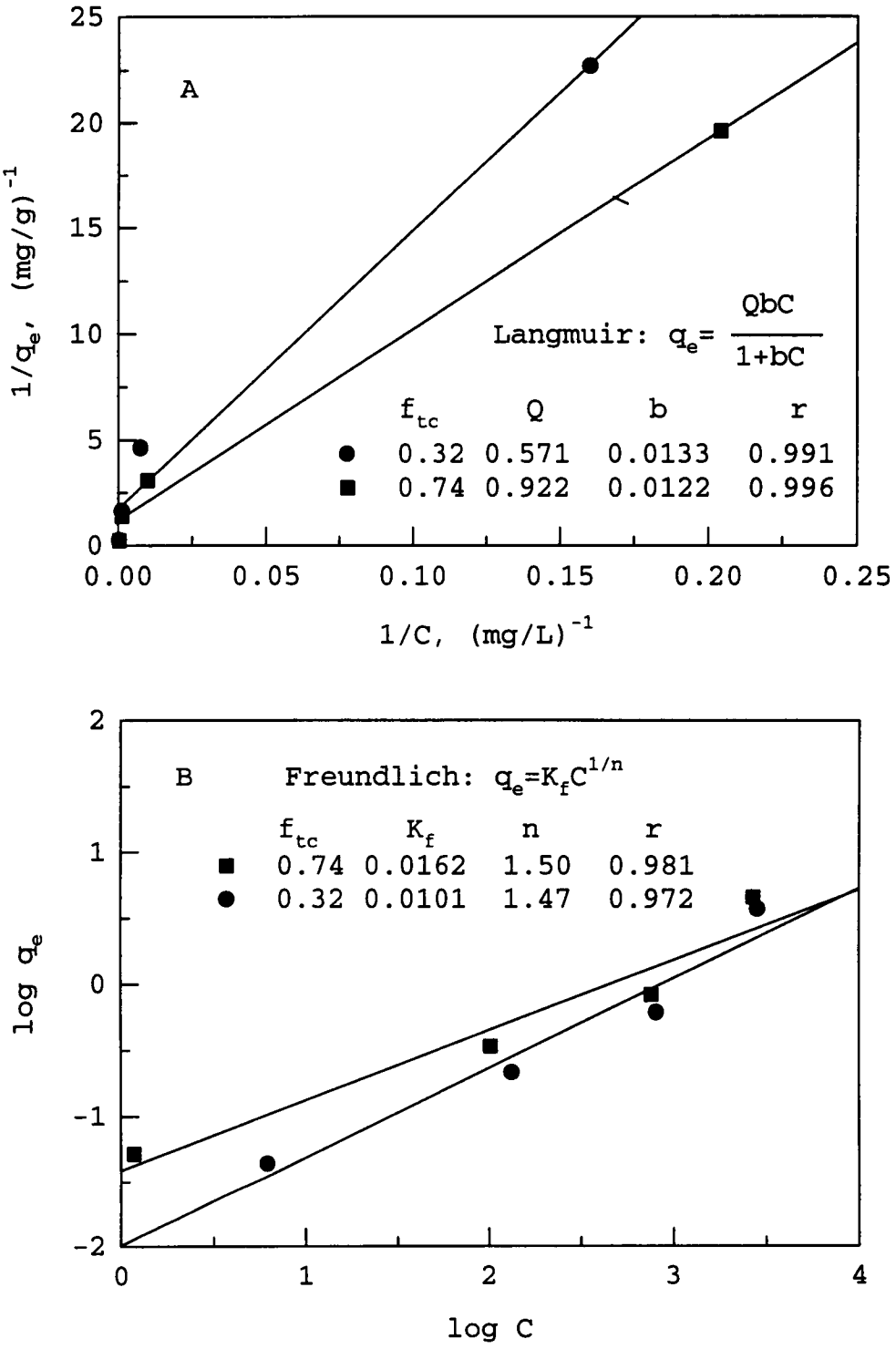


Figure 4.25 Isotherms for the Sorption of Biosurfactant on Soils with Different f_{tc}. A: Langmuir Isotherm; B: Freundlich Isotherm.

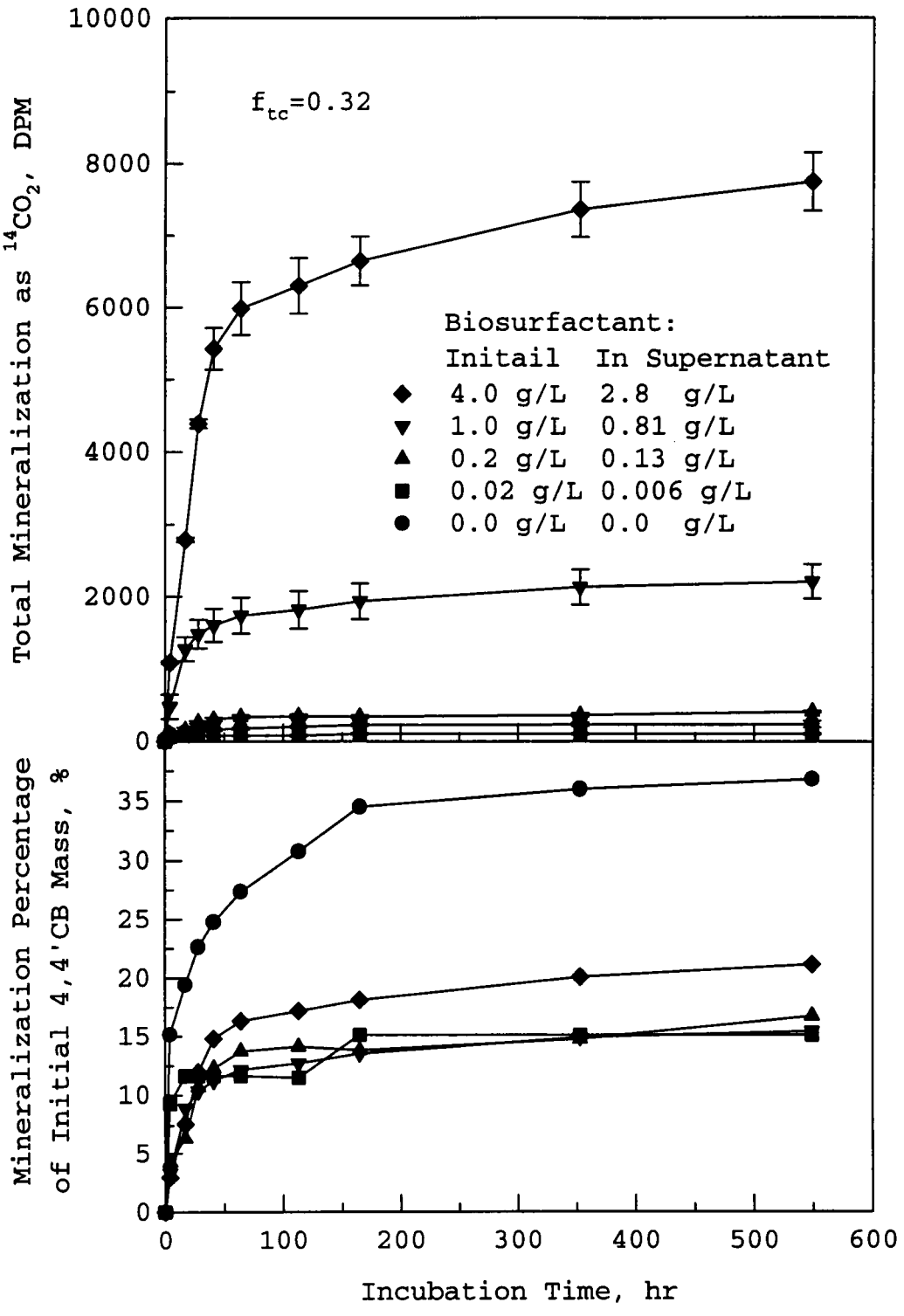


Figure 4.26 Mineralization of 4,4'CB Extracted from Soil (f_{tc}=0.32) Using Biosurfactant Wash Fluids.

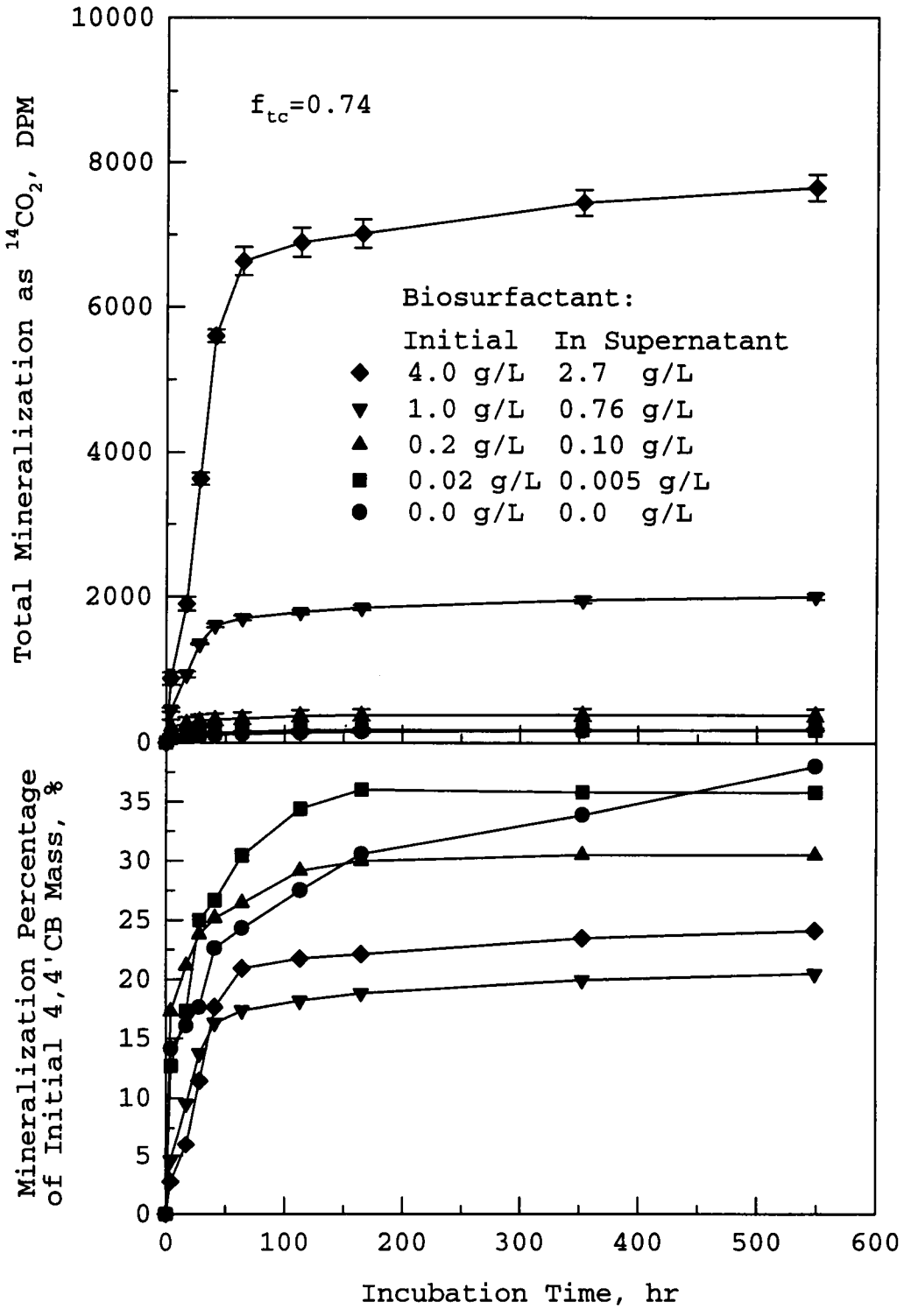


Figure 4.27 Mineralization of 4,4'CB Extracted from Soil (f_{tc}=0.74) Using Biosurfactant Wash Fluids.

of 4,4'CB mineralized in the wash fluid was greater in solutions without biosurfactant than that for solutions containing biosurfactant. It is possible that complexing of biosurfactant or the 4,4'CB with dissolved soil organic matter interfered with 4,4'CB mineralization. It was observed that the 4,4'CB mineralization percentages were higher in the high f_{tc} soil wash solution than in the low f_{tc} soil wash solution at the same biosurfactant concentration. Nutrients washed off from the high f_{tc} soil may help to improve mineralization.

Mineralization rates of 4,4'CB in the soil wash solutions are presented in Figures 4.28 and 4.29. The higher the biosurfactant level in the solution, the higher the mineralization rate.

As was the case for solution phase 4,4'CB mineralization, soil wash mineralization appears to proceed at two distinct rate, fast (r_1) and slow (r_2). The two rates can be defined by a critical time point (t_c). The two rates can be approximated using linear function; the slopes representing the average mineralization rates. Critical time points and average mineralization rates are listed in Table 4.7. Mineralization rates versus biosurfactant concentration and substrate (4,4'CB) concentration are

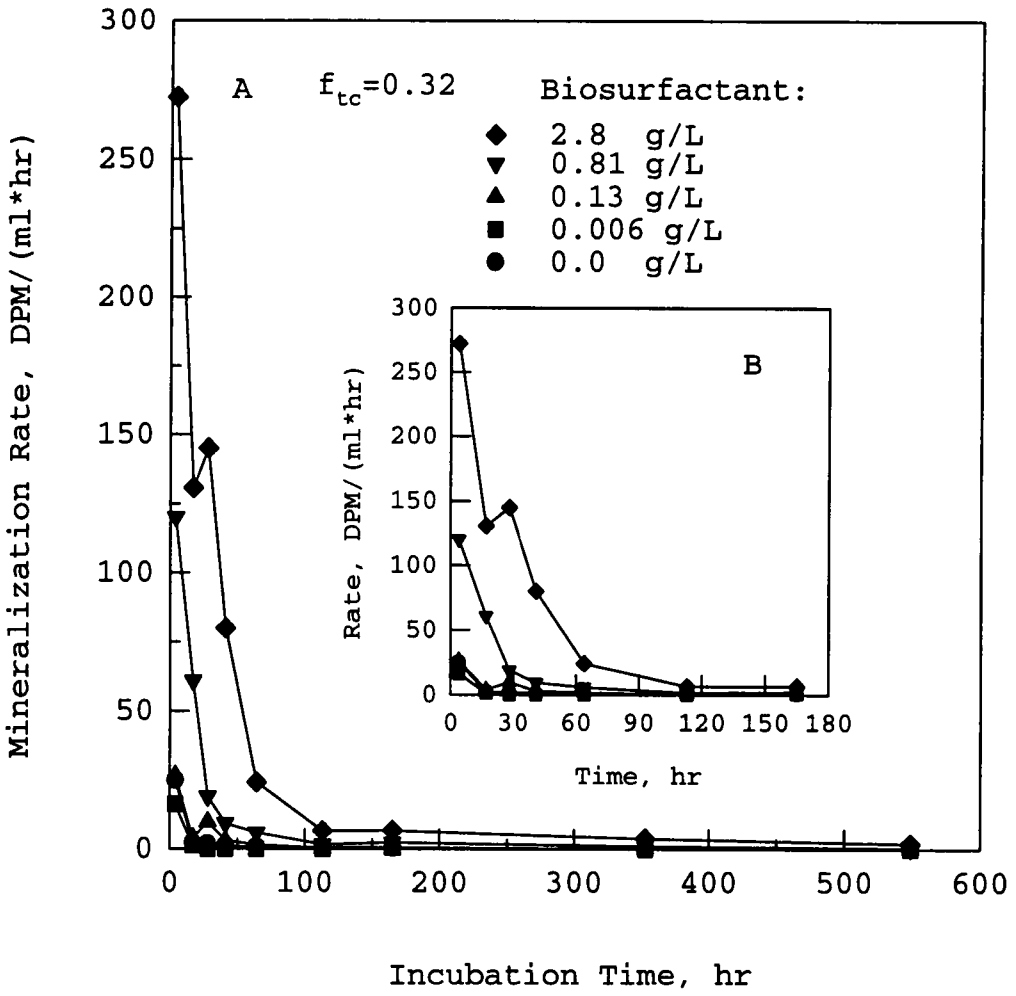


Figure 4.28 Mineralization Rate of 4,4'CB Extracted from Soil (f_{tc}=0.32) Using Biosurfactant Wash Fluids (B is an Amplified Part of A).

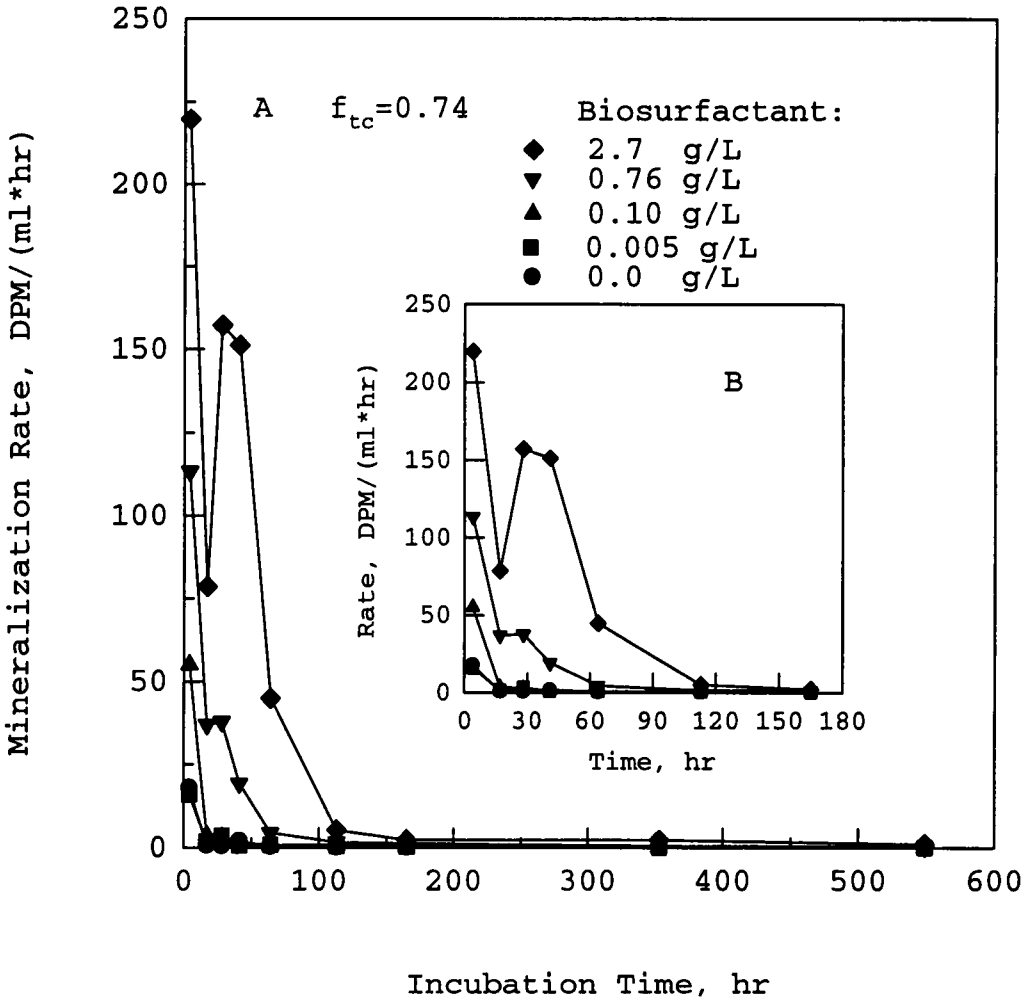


Figure 4.29 Mineralization Rate of 4,4'CB Extracted from Soil (f_{tc}=0.74) Using Biosurfactant Wash Fluids (B is an Amplified Part of A).

Table 4.7 Critical Time (t_c) and Average Mineralization Rate of 4,4'CB in Soil Wash Fluids.

Biosurfactant Concentration (g/L)		0.0	0.006	0.13	0.81	2.8
4,4'CB in Solution (DPM/L)		657	687	2468	14289	36621
f_{tc}=0.32	t_c (hr)	17	28	41	41	64
	r_1 (DPM/(ml*hr))	7.5	2.9	7.4	39	146
	(mg/(L*hr))	0.055	0.021	0.054	0.283	1.06
	r_2 (DPM/(ml*hr))	0.21	0.05	0.22	1.2	3.49
	(mg/(L*hr))	0.002	0.000	0.002	0.009	0.025
Biosurfactant Concentration (g/L)		0.0	0.005	0.10	0.76	2.7
4,4'CB in solution (DPM/L)		505	505	1274	9812	31678
f_{tc}=0.74	t_c (hr)	17	28	41	41	64
	r_1 (DPM/(ml*hr))	4.7	4.5	7.8	39	104
	(mg/(L*hr))	0.034	0.033	0.057	0.284	0.756
	r_2 (DPM/ml*hr)	0.21	0.11	0.14	0.81	2.11
	(mg/(L*hr))	0.002	0.001	0.001	0.006	0.015

shown in Figures 4.30 and 4.31, respectively. The initial average mineralization rate increased with increasing biosurfactant concentration and substrate concentration for both soil wash samples, indicating again that biosurfactant played an important role in the mineralization enhancement of PCB in soil wash solutions. There were no significant differences in rates for a given biosurfactant and 4,4'CB concentration between the high and low f_{tc} soils wash samples, possibly due to the fact that f_{tc}s were close for both soils after washing.

A 4,4'CB mass balance was performed at the end of the mineralization experiment (Figure 4.32). Total recovery of ^{14}C activity ranged from 64% to 89% for both soils. Low recoveries may be due to binding on the surface of the vials and/or volatilization into the head space of the vials.

Comparing 4,4'CB mineralization in the soil wash solutions with solution phase mineralization (without excess 4,4'CB crystals) indicates that the rate and extent of 4,4'CB mineralization were similar under both sets of test conditions. The presence of soil organic matter in the wash solutions did not alter 4,4'CB mineralization enhancement by biosurfactant.

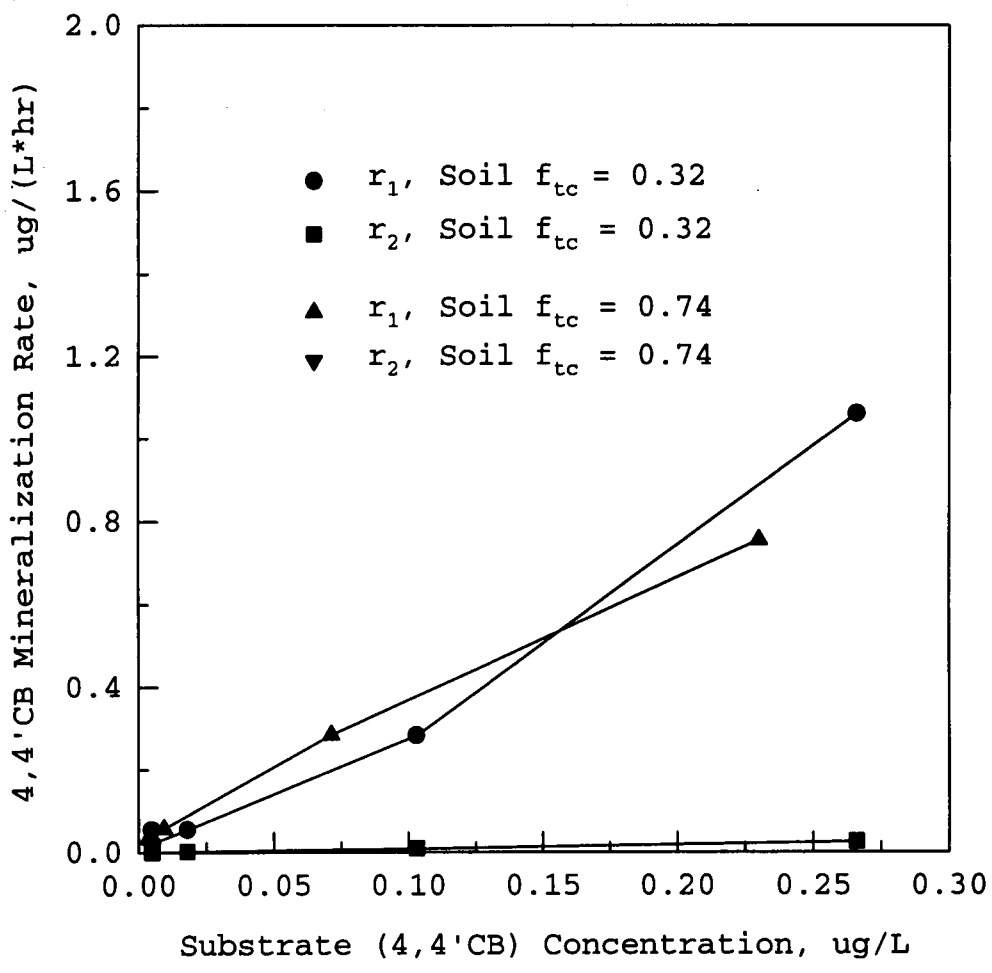


Figure 4.30 Effect of Substrate (4,4'CB) Concentration on the Mineralization Rate of 4,4'CB in Soil Wash Fluids.

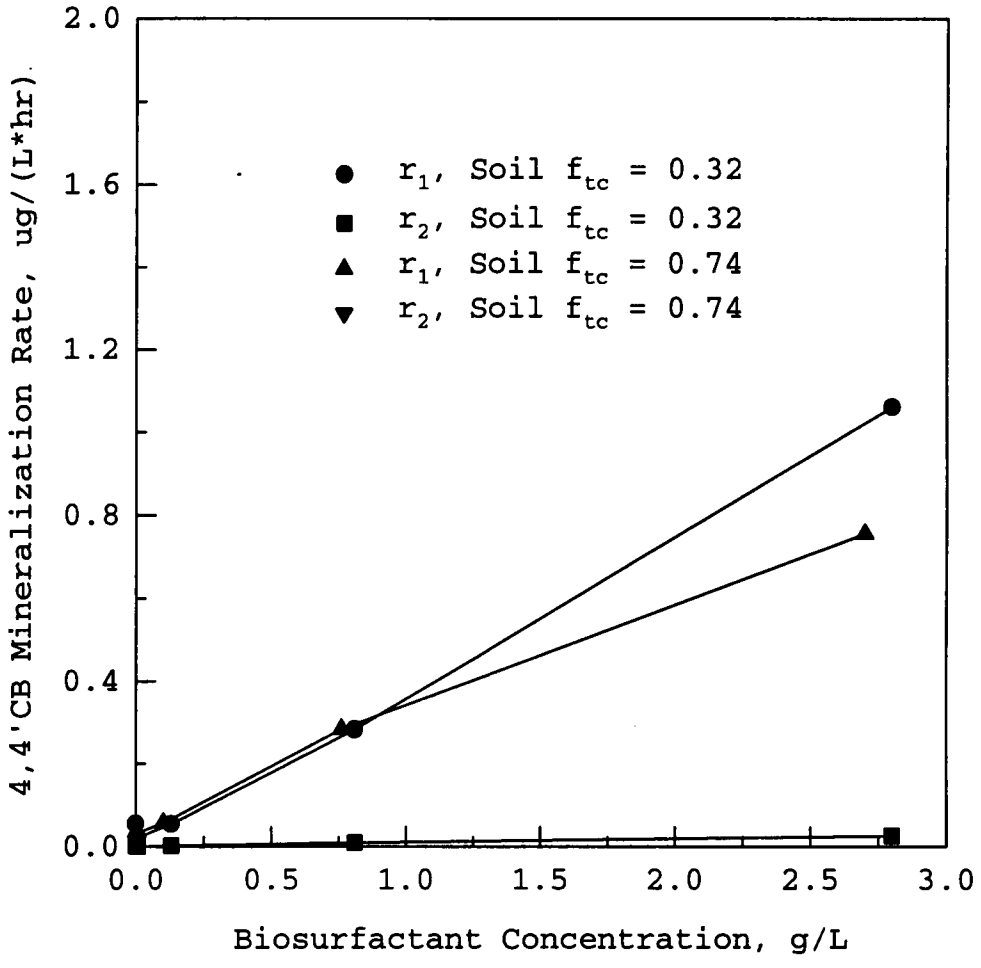


Figure 4.31 Effect of Biosurfactant Concentration on the Mineralization Rate of 4,4'CB in Soil Wash Fluids.

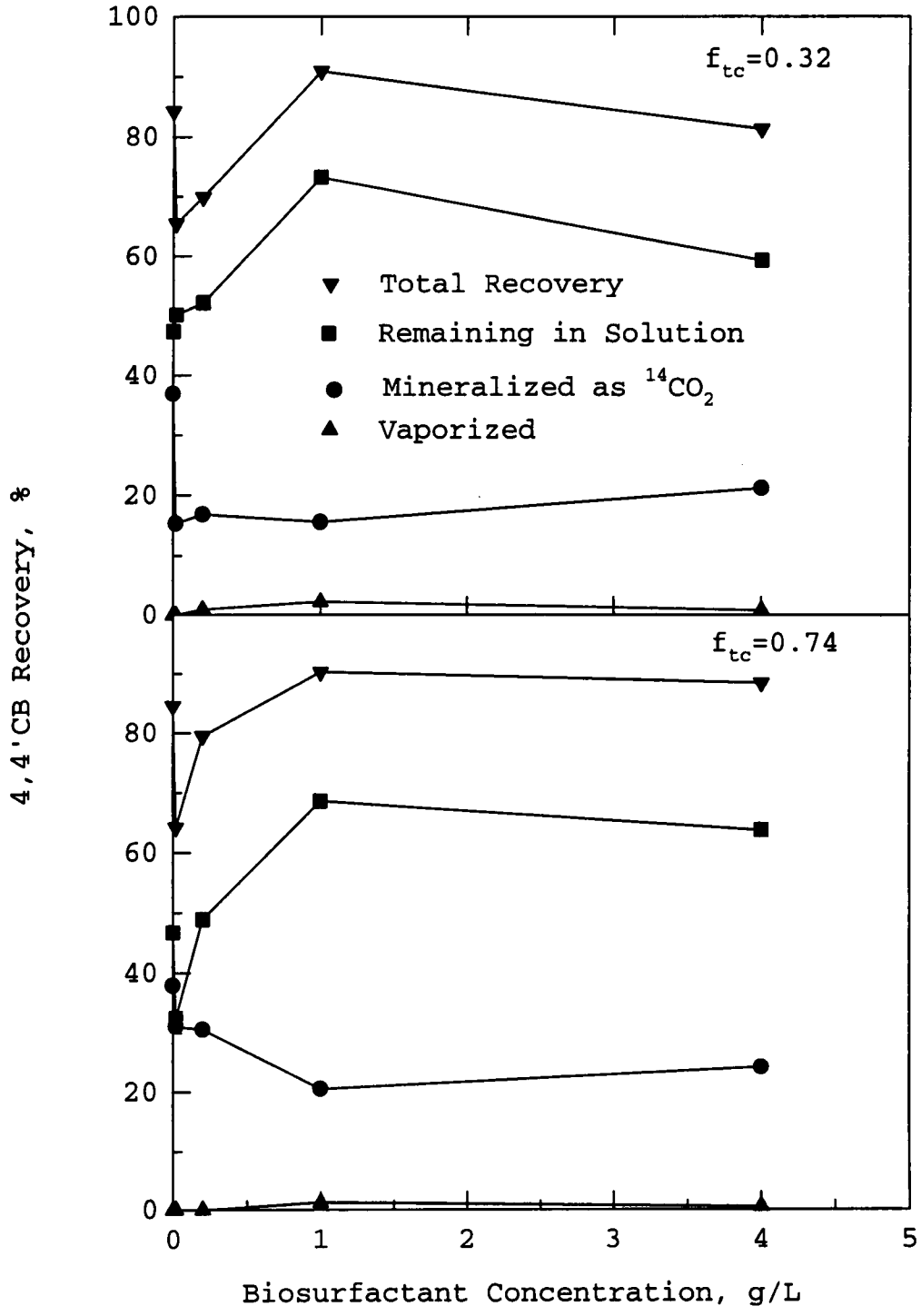


Figure 4.32 Mass Balance of 4,4'CB in Soil Wash Fluids at the End of Incubation

CHAPTER 5 SUMMARY AND CONCLUSIONS

The role of the biosurfactant (Rhamnolipid R1) in the solubilization, mobilization, and mineralization of 4,4'CB was investigated in this study. Based on results obtained in this research, the following conclusions can be derived:

1. Rhmnolipid R1, produced by *Pseudomonas aerugenosa*, was an anionic biosurfactant containing a glycol moiety as the hydrophilic portion and a fatty acid moiety as the hydrophobic portion. In a 0.03 M K_2HPO_4 solution with pH adjusted to 7.0, it has a CMC of 54 mg/L. The surface tension of a solution containing biosurfactant decreased from 75 to 42 dynes/cm at the CMC;

2. Biosurfactant R1 dramatically increased the apparent aqueous solubility of 4CB and 4,4'CB. At a biosurfactant concentration of 4 g/L (74 times the CMC), the aqueous solubility of 4CB and 4,4'CB were 59.13 and 4.21 mg/L, respectively. This was a solubility enhancement of 56 and 105 fold over the intrinsic solubilities of these compounds. For the case where 4,4'CB was the sole solute, solubility was measured as 5.33 mg/L for a biosurfactant

concentration of 4.0 g/L. 4,4'CB solubility increased by 27% when present as the sole solute in solution compared to its solubility in the presence of the co-solute, 4CB. 4CB inhibited solubilization of 4,4'CB through competition for partitioning sites within the biosurfactant micelles.

3. Mineralization of 4,4'CB by *Alcaligenes eutrophus* was enhanced in the presence of biosurfactant. Both the mineralization rate and total amount of 4,4'CB mineralized were elevated through biosurfactant addition. For a biosurfactant concentration of 4.0 g/L, overall average mineralization rates were 1.07 and 4.43 times that measured in controls (no biosurfactant) for solutions with and without excess 4,4'CB crystals. Mineralization enhancements were more pronounced when biosurfactant concentrations above the CMC were utilized. It is clear that micelles, which only form at biosurfactant concentrations above the CMC, play an important role in PCB degradation enhancement;

4. The total mass of 4,4'CB mineralized in biosurfactant solutions (without excess 4,4'CB crystals) exceeded the total initial mass of 4,4'CB dissolved in a biosurfactant free solution, which confirmed that micellized 4,4'CB was biodegradable;

5. The time course of 4,4'CB mineralization in biosurfactant solutions without excess 4,4'CB crystals can be divided into two periods as identified by the critical time point (t_c). For treatment times below t_c, mineralization rates were much higher than treatment times above this point. Different mineralization rates can be identified using linear regression of the raw data to yield values (slopes of the lines) which represent the average mineralization rate over a given treatment time. As the biosurfactant concentrations increase from 0 to 4.0 g/L, mineralization rates increase from 0.164 to 7.43 mg/(L*hr) for treatment times below t_c. This represents a 45 fold increase in the mineralization rate measured for the 4.0 g/L biosurfactant solution over the biosurfactant free solution.

6. Co-mineralization of biosurfactant was at a minimum during periods of high 4,4'CB mineralization. For solutions without excess 4,4'CB crystals, less than 10% of the initial biosurfactant was mineralized at a treatment time of 500 hr. Most of the 4,4'CB mineralization occurred during this time period. For solutions containing excess 4,4'CB crystals, less than 3% of the biosurfactant was co-mineralized after 500 hr and less than 10% after a 980 hr incubation period. The presence of excess 4,4'CB crystals

increased the mass of bioavailable 4,4'CB, thereby resulting in a decrease in mineralization of biosurfactant;

7. A yellow colored material, evidence of incomplete biooxidation of 4,4'CB, accumulated in all biologically active solutions containing 4,4'CB. Color development was more intense in higher 4,4'CB solutions (higher biosurfactant concentration). The disappearance of the colored material corresponded to enhance $^{14}CO_2$ recovery indicating this biodegradation by-product was mineralized but at a limited rate. The fact that the color intensity and duration of stability of the yellow colored material increased with increasing biosurfactant concentration provides indirect support of enhanced 4,4'CB degradation to this intermediate by the biosurfactant;

8. Biosurfactant R1 was an effective agent in mobilizing 4,4'CB from contaminated soils. A 4.0 g/L biosurfactant solution desorbed 57.2 and 47.5% of the soil-bound 4,4'CB in the first wash from soils with total carbon contents of 0.32 and 0.74%. After three washes, 90.3 and 85.3% of the 4,4'CB in the low and high f_{tc} soils was removed.

9. Mineralization of 4,4'CB in the soil wash fluid was also enhanced by the presence of biosurfactant. At a biosurfactant concentration of 4.0 g/L, average mineralization rates (in the treatment period below t_c) were enhanced by factors of 19.5 and 22.1 over the control (0 g/L biosurfactant concentration) for the soils with f_{tc}s of 0.32 and 0.74.

In general, results of this research provide clear evidence that biosurfactant soil washing followed by pure culture biological treatment of the wash fluid is a promising technology for PCB soil remediation.

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APPENDIX

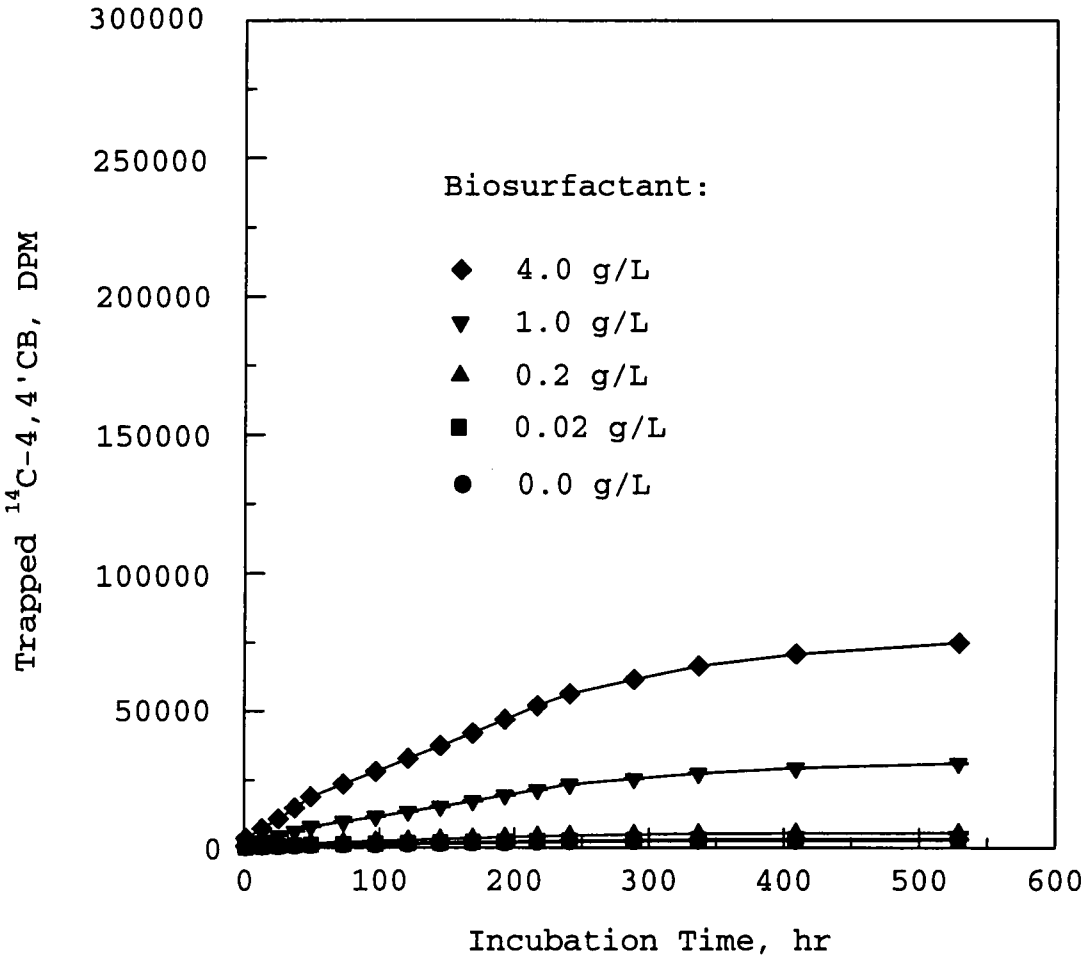

Figure A.1 Total ^{14}C- 4,4'CB Collected from Abiotic Biosurfactant Solutions (Controls) with Heat-killed *A. eutrophus* and without 4,4'CB Crystals.

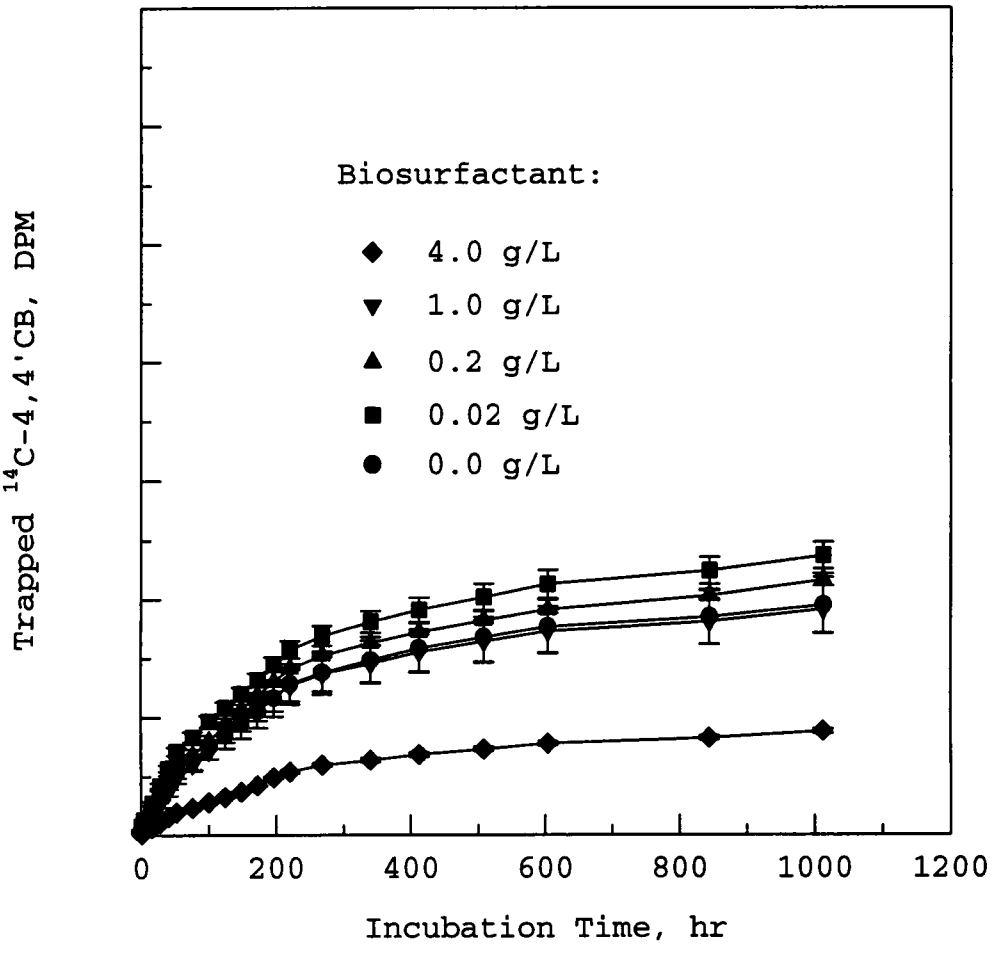

Figure A.2 Total ^{14}C-4,4'CB Collected from Abiotic Biosurfactant Solutions (Controls) with Heat-killed *A. eutrophus* and with 4,4'CB Crystals.

VITA

Zhou Shi was born in Changsha, the People's Republic of China on May 26, 1961. He attended the affiliated elementary school of Hunan Medical University (Changsha) and graduated from The First High School of Changsha in June, 1978. The following September he entered Hunan University and in July, 1982 received the degree of Bachelor of Science in Water and Wastewater Engineering. He reentered the Hunan University in September, 1985 and received a Master of Science in Urban Engineering in October, 1988. In August, 1991, he entered The University of Tennessee, Knoxville and in May, 1995 received a Master of Science degree in Environmental Engineering.

He is currently a graduate student seeking a Doctor of Philosophy degree in Civil Engineering at The University of Tennessee, Knoxville.