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EFFECTS OF BIOLOGICALLY PRODUCED  
SURFACTANTS ON THE MOBILITY AND BIODEGRADATION  
OF PETROLEUM HYDROCARBONS

by

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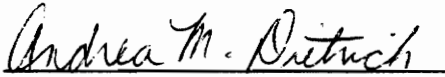
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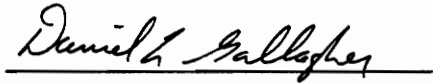
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**Effects of Biologically Produced  
Surfactants on the Mobility and Biodegradation  
of Petroleum Hydrocarbons**

by

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Civil Engineering

(ABSTRACT)

A laboratory investigation was conducted to determine the effects of biologically produced surfactants (biosurfactants) on petroleum hydrocarbons and their potential for the removal of hydrocarbons from groundwater systems. Biosurfactants have been found to be produced by microorganisms during growth on insoluble substrates for the purpose of increasing substrate solubility so as to promote biological degradation. In this study, three types of biosurfactants were produced by microorganisms grown on gasoline and a mixture of glucose with vegetable oil. Solubilization and biodegradation of selected gasoline compounds in the presence of biosurfactants were measured in both static batch and flow through column systems. Batch experiments were conducted in culture tubes, using only liquid phases. A clean sand was used in the column system to monitor physical and chemical interactions yet minimize adsorption effects. A mixed culture of gasoline degrading microorganisms along with isolated cultures grown on selected compounds

were used in the biodegradation studies.

The biosurfactants produced and used in this study acted similarly to synthetic surfactants and increased, to various degrees, the solubility of the monitored gasoline compounds. Biosurfactants produced from growth on glucose and vegetable oil were very effective surfactants, markedly increasing solubility of the gasoline compounds, but inhibiting biological degradation of these same compounds. Biosurfactants produced by microorganisms from growth on gasoline were effective surfactants, but they did not inhibit biodegradation of the gasoline compounds. This indicated that the biosurfactants may be substrate or microorganism specific, produced for growth on a particular insoluble substrate by a specific microorganism. Biosurfactants produced from growth on gasoline or an insoluble hydrocarbon could therefore be used to enhance solubility and subsequent biodegradation of that same hydrocarbon.

The effectiveness of the biosurfactants during application by injection or recirculation for groundwater remediation would be limited by the adsorption and removal of the biosurfactant to the soil. The surfactant demand (by adsorption) of the soil would have to be met before the effects of the biosurfactants would become apparent. Biosurfactants added to groundwater could also create an additional oxygen demand in a system already low in oxygen.

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## TABLE OF CONTENTS

	Page
List of Figures.....	vii
List of Tables.....	x
INTRODUCTION.....	1
LITERATURE REVIEWED.....	3
Petroleum Hydrocarbons.....	3
Hydrocarbons in the Subsurface.....	5
Removal of Subsurface Hydrocarbons.....	8
Use of Surfactants.....	10
Properties of Surfactants.....	11
Biosurfactants.....	14
Biosurfactants, Lipids, and the Cell Membrane.....	15
Biosurfactant Production.....	16
Glycolipids.....	18
Biodegradation of Gasoline.....	20
Hydrocarbon Utilizing Microorganisms.....	22
Bioavailability.....	23
METHODS AND MATERIALS.....	26
Experimental Approach.....	26
Biosurfactant Production.....	27
Analytical Methods.....	30
Solubility Enhancement.....	33
Batch Systems.....	34
Column Systems.....	35

Biodegradation.....	37
Gasoline Degrading Microorganisms.....	37
Batch Systems.....	38
Column Systems.....	41
Hydrogen Peroxide.....	41
RESULTS.....	43
Production of Biosurfactants.....	43
Characterization of Biosurfactants.....	46
Solubility Enhancement with Biosurfactants.....	47
Batch Systems.....	47
Column Systems.....	55
Biodegradation with Biosurfactants.....	58
Batch Systems.....	58
Column Systems.....	66
DISCUSSION.....	74
Biosurfactant Production.....	74
Solubility Enhancement.....	75
Biodegradation.....	81
SUMMARY AND CONCLUSIONS .....	84
LITERATURE CITED.....	87
APPENDIX A : Gas Chromatograms of Biosurfactants.....	91
APPENDIX B : Preliminary Microcosm Study.....	94
APPENDIX C : Preliminary Microcosm Study.....	97

## LIST OF FIGURES

	Page
Figure 1. Reactor extraction procedure.....	31
Figure 2. Schematic of column set.....	36
Figure 3. Batch microcosm dosing procedure.....	40
Figure 4. Surface tension reduction during growth on the two substate groups.....	44
Figure 5. Effect of two, 300 mg/L TOC, biosurfactant solutions on the solubility of four gasoline compounds.....	48
Figure 6. Effects of 300 mg/L biosurfactant solutions on enhanced solubility in the presence of gasoline and mineral oil....	50
Figure 7. Effects of 300 mg/L biosurfactant solutions on the percent increase of solubility enhancement in the presence of gasoline and mineral oil.....	51
Figure 8. Comparison of solubility enhancement from mineral oil and gasoline as caused by three biosurfactant solutions at 600 mg/L TOC.....	53
Figure 9. Comparison of the percent increase in solubility from mineral oil and gasoline as caused by three biosurfactant solutions at 600 mg/L TOC.....	54
Figure 10. Column effluent concentration levels of the four monitored gasoline compounds.....	57
Figure 11. Percent increase in column effluent concentration levels of the four monitored compounds.....	58



Figure 12. Biological degradation of toluene and m-xylene in the presence of two biosurfactant solutions at 300 mg/L TOC.....	60
Figure 13. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of two biosurfactant solutions at 300 mg/L TOC.....	61
Figure 14. Biological degradation of toluene and m-xylene in the presence of three biosurfactant solutions at 300 mg/L TOC.....	64
Figure 15. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of three biosurfactant solutions at 300 mg/L TOC.....	65
Figure 16. Biological degradation of toluene and m-xylene in the presence of three biosurfactant solutions at 600 mg/L TOC.....	67
Figure 17. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of three biosurfactant solutions at 600 mg/L TOC.....	68
Figure 18. Concentration in column at sample point C after a one day retention.....	70
Figure 19. Amount removed during a one day retention in the column as determined by the change in compound concentration between sample points B and C.....	71
Figure 20. Amount removed during a two day retention in column as determined by the change in compound concentration	

between sample points B and D.....	73
Figure A1. Comparison of the chromatograms produced by vegetable oil and the glucose and oil biosurfactant.....	92
Figure A2. Comparison of the chromatograms produced by gasoline and the gas centrifugate and solids biosurfactants.....	93
Figure B1. Biological degradation of toluene and m-xylene in the presence of various biosurfactants.....	95
Figure B2. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of various biosurfactants.....	96
Figure C1. Biological degradation of toluene and m-xylene in the presence of various biological and synthetic surfactants.....	99
Figure C2. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of various biological and synthetic surfactants.....	100

## LIST OF TABLES

	Page
Table 1. Gasoline Composition.....	4
Table 2. Physical/Chemical Properties of Selected Gasoline Constituents.....	6
Table 3. Medium and Substrates.....	29

## INTRODUCTION

Petroleum hydrocarbons which have migrated from spills and leaks into the subsurface present a difficult remediation problem because of the variety of compounds present and their interaction with the subsurface environment. Hydrocarbons are often difficult to remove because they will adsorb to mineral surfaces and organic material and can be retained by capillary action as an immiscible, separate phase, distinct from subsurface water in both the saturated and unsaturated zones. To counter these effects, synthetic surfactants have been proposed and tested for the removal of adsorbed compounds from soils and in the mobilization of immiscible petroleum hydrocarbons retained in the subsurface. Biologically produced surfactants have been shown to have characteristics similar to synthetic surfactants, but they are more readily degraded and may enhance degradation of other insoluble compounds that may be present.

Biosurfactants have been noted to be produced by microorganisms during growth on substrates which are insoluble in water, generally petroleum hydrocarbons. The production of biosurfactants is justified if their presence can increase the microbial growth rate by increasing the utilization rate of the substrate. Biosurfactants can affect the substrate removal rate by increasing the solubility of the substrate, thereby making more of it available for degradation, and by facilitating transport of the substrate across the cell membrane. Because

biosurfactants can affect both substrate solubility and degradation, their presence could be particularly beneficial if used in conjunction with in situ biological degradation of petroleum hydrocarbons during aquifer remediation. During biological degradation of petroleum hydrocarbons in the subsurface, biosurfactants may be produced naturally by the hydrocarbon degrading microorganisms present. Application and use of biosurfactants during aquifer remediation could therefore include injection of biosurfactant solutions or the recirculation of groundwater containing biosurfactants through zones of residual hydrocarbon contamination.

In order to apply or use biosurfactants for groundwater remediation, a better understanding of their characteristics and behavior is needed. For this reason, the specific objectives of this study were:

- to determine whether biologically produced surfactants can be used to solubilize petroleum hydrocarbons
- to determine how a biosurfactant might affect degradation of the solubilized hydrocarbon associated with it
- to assess the potential of biosurfactants for removing residual petroleum hydrocarbons from soil or groundwater systems.

## LITERATURE REVIEW

The processes which occur when spilled hydrocarbons first migrate into the subsurface and are finally removed through biological degradation are complex and slow, often taking many years to complete. Because they can mobilize and solubilize petroleum hydrocarbons, biologically produced surfactants should have the potential to increase the removal rate of hydrocarbons from the subsurface. Whether biosurfactants would increase the removal rate of the hydrocarbons is dependent upon the type of hydrocarbon, the subsurface conditions, and the type and application of the biosurfactants. This literature review discusses petroleum hydrocarbons and their fate in the subsurface, the production of biosurfactants, the affects of biosurfactants on the hydrocarbons, and the ultimate fate of hydrocarbons associated with the biosurfactants.

### PETROLEUM HYDROCARBONS

Because of it's widespread use, gasoline is one of the most common contaminants in the subsurface. Gasoline is a mixture of organic compounds, consisting mainly of aromatics, branched and straight chain alkanes (C<sub>5</sub>-C<sub>10</sub>), and cycloalkanes. Ranges of the amounts of the constituents present in gasoline are shown in Table 1. The various compounds all have different values of aqueous solubility, volatility

Table 1  
Gasoline Composition

Hydrocarbons	Typical Composition (percentage)
Alkanes	52.6
Monocycloalkanes	34.6
Dicycloalkanes	5.2
Alkylbenzenes	
Benzene	up to 5.0
Toluene	up to 20.0
Xylenes	up to 20.0
Indanes and Tetralins	0.9
Naphthalenes	0.3

(after McDuffie, 1982)

and partitioning coefficients, as shown in Table 2. Because of the different characteristics, the gasoline components will solubilize into water, and partition or adsorb to soil in varying amounts. The most soluble portion consists of the monoaromatics; benzene, toluene, ethylbenzene, and the three xylenes, collectively called the BTEX compounds. The compounds in this group all have low partitioning coefficients and are very mobile groundwater contaminants. Compounds with lower solubility and larger partitioning coefficients are generally less mobile and include the polycyclic aromatics and alkanes (Nyer and Skladany, 1989).

Other mixes of petroleum hydrocarbons commonly encountered are fuels composed of larger molecular weight compounds such as kerosene, diesel and heating oils. These fuels consist predominantly of progressively longer alkane chains from C<sub>10</sub>-C<sub>18</sub> with kerosene to C<sub>10</sub>-C<sub>24</sub> with diesel and heating oils. The compounds that make up these fuels generally have lower levels of aqueous solubility and volatility, increased viscosity and larger partitioning coefficients than those found in gasoline. Because of these characteristics, these fuels typically provide less mobile groundwater contaminants as compared to gasoline. For the same reasons, once they are in the subsurface, they can be more difficult to remove.

#### HYDROCARBONS IN THE SUBSURFACE

Gasoline or other hydrocarbons which have leaked into the



Table 2

## Physical/Chemical Properties of Selected Gasoline Constituents

Compound	Molecular Weight	Vapor Pressure (25 OC, mm Hg)	Aqueous Solubility (25 OC, mg/L)	Henry's Law Constant (Atm/mM)	Partition Coefficient (log Kow)
<u>Aromatics</u>					
Benzene	78	95	1780	7.0	2.11
Toluene	92	29	535	6.7	2.69
O-Xylene	106		175		
M-Xylene	106		200		
P-Xylene	106		200		3.15
Ethylbenzene	106	7	152	6.6	3.15
Propylbenzene	120		60		
Naphthalene	128		32	10.0	3.36
Biphenyl	154		7.5		4.09
Trimethylbenzene	120				3.7
<u>Alkanes</u>					
Butane	58		79		
Pentane	72		2		
Hexane	86				
Cyclohexane	84		130		
Heptane	100		50		
Octane	114		16		
Iso-octane	114				

(after McDuffie, 1982)

subsurface would go into the unsaturated zone (if present), with the resulting flow pattern depending on the soil characteristics, moisture content, and the volume of spilled hydrocarbons. Assuming unconsolidated sediments or generally isotropic porous media, hydrocarbons can be drawn into and retained in the unsaturated zone by capillary action (Lehr, 1988). If the volume of the hydrocarbons is large enough, the free phase will flow cohesively downwards under the influence of gravity.

Free or second phase petroleum hydrocarbon refers to any amount of hydrocarbon present as a distinct liquid phase, separate from the water phase. Second phase hydrocarbons can describe individual drops present in both saturated and unsaturated porous media, though the term is often used to refer to a layer of hydrocarbons residing above the water table. This free, or second phase flow, can cease if the volume of the spilled hydrocarbons balances with the amount needed to obtain a residual saturation in the soil as caused by capillary effects (Schille, 1981). Capillary effects are increased in fine grained sediments, but decrease with increasing moisture content (Bouwer, 1978).

Brown et al. (1978) found that the residual gasoline saturation in dry soils varied from 32 and 122 g/kg gasoline in coarse and fine sand, respectively. Sands at field capacity moisture contents had lower residual saturation values, with retentions of 27 and 44 g/kg gasoline in medium and fine sand. If the residual saturation capacity is exceeded, the hydrocarbons will continue to flow downward, and will migrate towards the capillary fringe above the water table. With the

increasing moisture content towards the water table, residual saturation levels decrease, and the hydrocarbon flows quickly and mixes in with the water in the capillary fringe. As the free phase hydrocarbons continue downwards, the capillary fringe above the water table is depressed, and the hydrocarbon begins to form a lens riding above the water table (Testa and Paczkowski, 1989). This basic flow leaves hydrocarbons in four phases; in soil vapors, as a second free phase, as adsorbed to soils and organics, and dissolved in groundwater (Wilson and Brown, 1989).

While the hydrocarbon mixes such as gasoline or diesel fuel would all end up to some degree in each of the four phases noted, the different fractions of the hydrocarbons would end up partitioning to different degrees between these four phases. Gasoline, being the most volatile and soluble, would be found at proportionally greater levels in the vapor and dissolved phases. The larger molecular weight fuels, such as diesel fuel, would be affected more by capillary action and may be retained more in the unsaturated zone, or flow laterally at a slower rate. These fuels would also partition and adsorb to soil and organics at proportionally greater levels than gasoline compounds.

#### REMOVAL OF SUBSURFACE HYDROCARBONS

Typical remediation activities for the removal of hydrocarbons include hydraulic removal by pumping, soil excavation, vapor extraction under reduced pressure, and biological removal through degradation

(Nyer, 1985). All of these methods have their limitations. Pumping out the free phase hydrocarbon usually removes less than 50% of the original volume (Wilson and Conrad, 1984). During two phase flow (water and hydrocarbon), a second fluid cannot be completely removed and replaced by water in an aquifer (Bouwer, 1978). Excavated contaminated soil must still be treated or disposed of, and with increasing contaminant depth this option becomes increasingly impractical. Vapor extraction methods are limited by the depth and extent of the contamination since unlike hydraulic removal, vapor extraction techniques cannot utilize gravity in creating pressure differences. Vapor extraction will also only remove the most volatile compounds (Nyer, 1985). In the case of gasoline, benzene, toluene, ethylbenzene and the xylenes are typically targeted for removal, leaving a residual of larger molecular weight, less volatile compounds. With the heavier fuels, vapor extraction methods remove compounds slowly or not at all.

Biological degradation of petroleum has been proposed as one method for the ultimate removal of these groundwater contaminants (Wilson and Brown, 1989). Biodegradation can be limited by the rate of degradation. Factors limiting the degradation rate include the oxygen transfer rate to the aquifer, the limited solubility of the compounds, toxic effects of pure gasoline, and the limited bioavailability of adsorbed compounds. A typical method for adding oxygen is in the form hydrogen peroxide, but due to its instability it is often not considered economical to use (Hinchee and Downey, 1987). Degradation is typically fastest for the most soluble portion of the gasoline,

typically the BTEX group, while the less soluble and adsorbed compounds degrade slowly.

#### USE OF SURFACTANTS

Some of the problems with the different types of remediation listed previously can be explained by certain characteristics of the hydrocarbon. During hydraulic removal of free phase hydrocarbons, the recovery of the immiscible fluid is low, since a second phase present with water in porous media acts to reduce porosity. To remove the second phase most effectively, individual drops must be able to coalesce and flow together, or be emulsified with water and then removed. During biological degradation of gasoline, degradation occurs on the soluble portion, therefore compounds of low solubility degrade slowly. The solubility of the compounds can severely limit degradation rates of the heavier, low solubility fuels such as diesel fuel. The use of surface active agents, or surfactants, has been proposed as a way to counteract some of these effects (Ellis, 1985). Surfactants can lower interfacial tensions and change the wetting characteristics of the hydrocarbons (Rosen, 1978), thus enhancing flow of the immiscible fluid through the aquifer. Surfactants can also increase the solubility of the compounds, thus increasing removal rates when pumping and possibly make compounds more available for degradation. The use of surfactants has been investigated for the removal from soil of PCBs (Mc Dermont et al., 1989), anthracene (Vigon and Rubin, 1989), and DDT and trichlorobenzene (Kille

and Chiou, 1989). Their investigations determined that in column or batch tests, the organics could be removed at an increased rate, and to lower residual levels. In addition, it was determined that the lower the solubility of the compound, the greater the effect the surfactant had in increasing solubility. The petroleum industry has also investigated the use of surfactant solutions in enhanced oil recovery (Hill, 1973). Enhanced oil recovery methods have also utilized biologically produced surfactants from bacteria grown in the reservoir rock containing the oil. This method utilized gas and organic acid production as well the biologically produced surfactants to increase the yield of the reservoir (Finnerty and Singer, 1983).

A problem common to these studies was the degree of adsorption of the surfactant to the porous media. The adsorption is minimized in the presence of sand, but can become significant with increasing clay content and surface area. McDermont et al. (1987) compared Triton, a non-ionic surfactant, with Surco 233, an anionic surfactant for the removal of PCBs from clay soils. While both increased the solubility of the PCB, the Triton was basically ineffective due to the large amount of adsorption to the soil, with 15-20% by weight adsorbed as compared to the Surco 233 with less than 1% adsorbed.

#### PROPERTIES OF SURFACTANTS

Surfactants can achieve these high levels of petroleum product solubility and mobilization because of their ability to lower surface and interfacial tensions, and to form soluble colloidal groups of

surfactant molecules called micelles. A surfactant is defined (Rosen, 1978) as any substance that when present at low concentrations in a system has the ability to adsorb onto the surfaces or interfaces of the system and alter the free energy of those surfaces or interfaces. Surfactants have a characteristic molecular structure consisting of a lyophobic group, which has little attraction for the solvent, and a lyophilic group which has a strong attraction for the solvent. Synthetic surfactants are typically grouped based on the charge associated with the lyophilic group, cationic, anionic, and non-ionic. The surfactant will orient itself so that lyophobic group is at the interface, and the lyophilic group is in the solvent. This surfactant orientation, combined with the dissolved surfactant molecules, results in an increase in the free energy of the system (Rosen, 1978). This increase means that less work is needed to bring a surfactant molecule to the surface than a solvent molecule, as is required for a change in surface area. Changing the surface area by an amount  $dA$  involves an amount of work  $dW$ ,

$$dW = \gamma dA \quad \text{where } \gamma$$

is the surface tension. In order for a drop of liquid to migrate through a porous media, the surface area of the liquid must expand to migrate through the narrow openings near the points of grain contact. Any reduction in surface tension lowers the amount of work needed to do this.

Surfactants affect surface tension as described by the Gibbs surface tension equation (Atkins, 1986),

$$d\gamma = -\Gamma dU$$

where  $\Gamma$  is the surface excess per unit area, or the amount of surfactant present at the interface above the bulk solution concentration, and  $U$  is the chemical potential of the surfactant. At low surfactant concentration,

$$dU = RTd\ln C$$

where  $C$  is the surfactant concentration. Then

$$d\gamma = -(RT/C)\Gamma dC$$

and at constant temperature,

$$(\delta\gamma/\delta C) = -RT\Gamma/C.$$

If a surfactant accumulates at the interface, its surface excess is positive, so  $(\delta\gamma/\delta C)$  is negative and the surface tension decreases (Atkins, 1986). Any reduction in surface tension can therefore be defined to indicate the presence of one or more surfactants.

Once surfactants have accumulated at the interfaces, there will be no further reduction in surface tension, and the surfactant will begin to form colloidal groups, or micelles, soluble in the bulk solution. The basic structure of the micelle has the hydrophobic end of the surfactant at the center of a sphere of molecules with the hydrophilic end of the molecule at the outside towards the aqueous solvent. This structure gives the surfactant its detergent properties, since hydrophobic molecules can partition into the micelle towards the center. The increase in solubility of hydrophobic compounds reported previously is the result of this partitioning. The concentration of the surfactant at which micelles will form is referred to as the critical micelle concentration (CMC). This concentration will vary for different



surfactants and for different systems.

Because of the ability of surfactants to lower surface and interfacial tensions as well as form micelles, they can be used to both mobilize and solubilize petroleum hydrocarbons for removal and degradation.

## BIOSURFACTANTS

A variety of microorganisms produce metabolites which have surface active properties, and are considered to be biologically produced surfactants, or bio-surfactants. Bio-surfactants present a broad range of surfactant types and properties. They have shown a potential for the cleanup of oil spills on land and water (Jobson et al., 1972), and are usually easily bio-degraded (Cooper and Zajic, 1980). It is also generally believed that organisms growing on an insoluble substrate will benefit from the presence of a compound that can emulsify that substrate.

The biosurfactants produced are typically lipids with the properties resulting from the polar and apolar groups on a single molecule. The apolar or hydrophobic group is usually the hydrocarbon chain of a fatty acid. The polar groups may include ester and alcohol functional groups of neutral lipids, the carboxylate group of fatty acids, phosphate containing portions of phospho-lipids, and the sugars of glycolipids (Cooper and Zajic, 1980).

## BIOSURFACTANTS, LIPIDS, AND THE CELL MEMBRANE

Two of the most common groups among the biosurfactants are the glycolipids and the phospholipids (Syldatk and Wagner, 1987). These two types of compounds also comprise a major portion of the cell membrane in microorganisms. The simplest of the many different structures these lipids have is similar to a triglyceride, but with one of the fatty acids removed and replaced with a phosphate or sugar group. This replacement results in a hydrophilic group (sugar or phosphate) and two hydrophobic groups (two fatty acid chains) on a single molecule. This molecular structure causes the alignment in the cell membrane to be a double layer of lipids, with hydrophilic end groups facing out from the membrane to both sides, while the hydrophobic groups align at the center of the membrane (Brock and Madigan, 1988). This type of membrane allows regulation of the transport of material in and out of the cell. Polar compounds are restrained from entering the cell by the inner hydrophobic portion of the lipid double layer, while less polar compounds such as alcohols, fatty acids and benzene may enter the cell by becoming dissolved in the membrane (Brock and Madigan, 1988).

While the exact mechanism is not clear, biosurfactants have been credited with facilitating cellular uptake and utilization of generally insoluble substrates (Kosaric, et al., 1987). When associated with the cell, they promote transport across the membrane, while as extracellular compounds, they emulsify the substrate. Boulton and Ratledge (1987) felt that growth on mildly polar dissolved hydrocarbons was not facilitated

by the presence of a surfactant, noting that hydrocarbons with shorter chain lengths ( $<C_{10}$ ) are not likely to be affected. Syldatk and Wagner (1987) describe the action of the biosurfactant in two modes. When cell bound, the biosurfactant is orientated in the cell wall such that the hydrophobic end is towards the medium, reversing the hydrophilic effects at that location, thereby facilitating transport. When excreted out of the cell, biosurfactants will form microemulsions which can then be transported across the membrane. Extracellular lipids produced as biosurfactants must have some degree of aqueous solubility for this reason.

#### BIOSURFACTANT PRODUCTION

Biosurfactants will be produced for substrate transfer requirements as previously stated, and under a variety of conditions when the biosurfactant is not used for substrate transfer. These biosurfactants include various lipids as with the growth associated compounds, and fatty acids produced either as metabolites during growth or produced from simpler substrates in the TCA cycle. In the case of *P. aeruginosa*, the type of biosurfactant produced during growth on sugar or insoluble hydrocarbons does not vary with the substrate, it is the same type of biosurfactant, a glycolipid. Other microorganisms can also produce the same biosurfactant when grown on a variety of soluble and insoluble substrates (Duvnjak and Kosaric, 1985), but the yield may vary. Boulton and Ratledge (1987) have shown that the type of

biosurfactant produced can be varied with the substrate supplied. The microorganism will incorporate the substrate into the biosurfactant as part of the chemical structure if possible (Syldatk and Wagner, 1987). In the case of *P. aeruginosa*, which produces the rhamnolipid, the sugar group and the lipid group can be synthesized or directly utilized, depending on the type of substrate.

When the biosurfactant is not used to facilitate substrate transport, it is typically cell bound and can be released during various stages of growth. This often occurs after maximum biomass is reached or under some nutrient limiting conditions (Cooper and Goldenberg, 1987, Ramsey et al., 1988). The lipids produced and retained in the cell or excreted as biosurfactants are similar to the lipids present in the cell membrane. The basic structure of two hydrocarbon chains from fatty acids with a hydrophilic group is common to both biosurfactants and cellular lipids (White et al., 1978). Noting that biosurfactants may be excreted under some stressful condition (nutrient limitations) and not always for growth, it would appear that general growth and subsequent cell lysis would release some amount of lipids and surfactant compounds from the cell. Cooper and Goldenberg (1987) observed that cell lysis was important for the production of bio-surfactants from *Bacillus cereus*, as maximum surfactant production was achieved after the maximum biomass was produced. Other research (Duvnjak and Kosaric, 1985, Cooper et. al., 1979) found similar results indicating that many surfactants are cell bound and can be released under a variety of conditions.

Lipids derived from the cell membrane have been shown to have

extremely low CMC values, and can start to form micelles at concentrations as low as  $10^{-6}$  moles/L (White et al., 1978). This means they can form micelles before a significant reduction in surface tension is noted. They have a particularly strong affinity for other lipids with a similar structure, due to the two fatty acid groups present. This causes the lipids to align themselves in a double layer micellular sheet, which can spontaneously reform a hollow, spherical cell membrane structure with solvent both inside and out (White et al., 1978). Cooper and Goldenberg (1987) also proposed that what are often reported as surface active biopolymers are actually aggregates of surface active lipids.

It appears that general microbial growth will produce some amount of biosurfactants. When these compounds are produced in amounts large enough to lower surface tension, they are termed biosurfactants. Small amounts of similar compounds can also be produced, not noted as biosurfactants, but still possess strong surfactant characteristics. Production of notable amounts of biosurfactants is typically regulated by substrate or nutrient manipulations.

#### GLYCOLIPIDS

Based on the substrate supplied, one of the possible types of biosurfactants produced in this research are glycolipids with the sugar rhamnose or the sugar sophorose attached to a hydroxycarboxylic acid. In addition, it is likely that a large number of other bio-surfactants

are produced, such as a variety of carboxylic acids and assorted polar lipids.

Rhamnose lipids were isolated (Hauser and Karnousky, 1958, Edwards and Hayashi, 1965) and identified as being produced by several strains of *Pseudomonas aeruginosa*. The rhamnose lipid was produced during growth on a variety of substrates, typically hydrocarbons or glucose. When grown on hydrocarbons, however, *P. aeruginosa* produced up to an order of magnitude more rhamnolipid as compared to growth on glucose (Itol et al., 1971). Hisatsuka et al. (1971) isolated the rhamnolipid, added it to *P. aeruginosa* growing on hydrocarbons (n-alkanes), and observed a significant increase in the rate of growth. Addition of the rhamnolipid to *P. aeruginosa* growing on glucose did not increase the rate of growth. Itol and Suzuki (1972) demonstrated that the rhamnolipid was essential for growth of *P. aeruginosa* on hydrocarbons.

Species of the yeast *Torulopsis* produce glycolipids that have some similarity to the bacteria rhamnolipids (Gorn et al., 1961, Tulloch et al., 1967). These glycolipids contain a disaccharide, sophorose, attached to a hydroxycarboxylic acid. With the sophorose lipids it was found that the structure and yield could be influenced by the addition of secondary substrates (Tulloch et al., 1962, Jones and Howe, 1968). Inoue and Itol (1982) demonstrated the surfactant properties of sophorose lipids produced by *Torulopsis bombicola* when grown on alkanes. Cooper and Paddock grew *T. bombicola* on glucose and vegetable oil and produced a large yield of mixed glycolipids with excellent surfactant properties.

The two examples of the glycolipids produced by the bacteria species *Pseudomonas* and yeast *Torulopsis* are typical of biologically produced surfactants. They are presented here as examples of the bio-surfactants produced by two microorganisms grown on substrates similar to those used in this research.

These results indicate that with general microbial growth, bio-surfactants may be produced at various stages of growth. The bio-surfactants may be produced for growth and substrate uptake, or as the result of cell lysis. The bio-surfactants may have widely varying molecular structures due to the nature of the microbe and substrate, but can still have the similar properties which define them as surfactants.

#### BIODEGRADATION OF GASOLINE

The in situ biodegradation of gasoline has been proposed as the only method that will restore a contaminated aquifer to its original condition (Nyer, 1985). Zobell (1946) studied the microbial utilization of petroleum hydrocarbons and noted that such microorganisms are widespread in nature. Microbial degradation of the hydrocarbons present in gasoline is complex, due to the variety of the components, the diversity of the microbial population, and the environmental conditions.

In the degradation of whole gasoline, the n-alkanes (straight chained) are typically degraded first with a monoterminial attack. Usually a primary alcohol is formed followed by an aldehyde and a monocarboxylic acid (Miller and Johnson, 1966, Ratledge, 1978). Further

degradation of the carboxylic acids proceeds by beta-oxidation with subsequent formation of two-carbon unit shorter fatty acids and acetyl coenzyme A, with eventual liberation of carbon dioxide. Fatty acids, some of which are toxic, have been found to accumulate during hydrocarbon degradation (King and Perry, 1975, Atlas and Bartha, 1973).

Branched alkanes, such as pristane, have different degradation pathways. Pristane undergoes omega oxidation to form dicarboxylic acids (Pirnik, 1977, Schaeffer et al. 1979). Cycloalkanes are particularly resistant to microbial degradation, but degradation of both substituted and unsubstituted cycloalkanes has been reported (Atlas, 1981). Degradation of substituted cycloalkanes appears to occur more readily than unsubstituted forms, particularly if there is an n-alkane substituent which would be degraded first (Atlas citing Soli, 1973). Several unsubstituted cycloalkanes have been reported to be degraded by co-metabolism of the n-alkanes, with the formation of a ketone or alcohol, with subsequent degradation by ring cleavage (Perry, 1979).

The degradation of aromatic compounds by bacteria generally involves the formation of a diol followed by cleavage and formation of a diacid such as cis,cis-muconic acid (Fewson, 1981). Extensive methyl substitution can inhibit initial oxidation (Atlas citing Atlas, 1981), and condensed ring aromatics are relatively more resistant to degradation (Cripps and Watkinson, 1978). The degradation of mixtures of petroleum hydrocarbons is complex, and degradation rates for the individual compounds can vary in the presence of others due to co-metabolism, and the presence of intermediate



metabolites (Atlas, 1981). Jamison et al. (1976) found that degradation rates for hydrocarbons from a high octane gasoline were not in agreement with rates as determined from pure cultures. Acetate, an intermediate product in hydrocarbon degradation, was found to inhibit utilization of hexadecane, and pristane degradation has been reported to be inhibited by the presence of hexadecane (Atlas, 1981). Biodegradation of oil in tundra soils was accompanied by the accumulation of various polar lipids in the soil column, but these compounds were not further identified (Sexstone and Atlas, 1978). The production of various lipids described as surfactants is well documented however. The intermediate and final products of hydrocarbon degradation in the environment are numerous and difficult to determine. The microbial products may help hydrocarbon degradation as in the case of bio-surfactants, or hinder degradation as with recalcitrant or toxic products.

#### HYDROCARBON UTILIZING MICROORGANISMS

A diverse group of bacteria and fungi have demonstrated the ability to degrade petroleum hydrocarbons. Some of the most important genera (based on frequency of isolation) of hydrocarbon degraders in aquatic environments are; *Pseudomonas*, *Archromobacter*, *Arthrobacter*, *Micrococcus*, and *Nocardia* (Bartha and Atlas, 1977). A large number of *Pseudomonas* species have been isolated which can utilize hydrocarbons and their degradation has been well studied (Atlas, 1981). Hydrocarbon degradation in the environment would depend on the type of microbial population present and its acclimation to the hydrocarbons. In situ

bioremediation techniques typically utilize the existing population to degrade subsurface hydrocarbons by the addition of nutrients and oxygen.

#### BIOAVAILABILITY

The biosurfactants produced by the specific microorganisms discussed previously were usually noted to be an aid to the growth on insoluble substrates. This was tested, as in the case of *P. arueginosa*, by the isolation, identification and addition of the biosurfactant to active cultures and observing changes in growth. With the addition of the biosurfactant, rates increased during growth on insoluble substrates (oils or alkanes), and remained unchanged during growth on an infinitely soluble substrate (glucose). Whether the biosurfactant was specific to the substrate or the microorganism that produced it was not investigated. Due to the usually non-specific nature of surfactants in solubility enhancement, it is unlikely that the biosurfactant would solubilize only certain compounds. It seems more likely, due to the structure of the cell membrane, that the biosurfactant would be specific to the microorganism that produced it, thereby enhancing growth only for that microorganism.

In the degradation of whole gasoline by a mixed culture, a large variety of biosurfactants may be produced. Since some portions of gasoline are relatively soluble in water, many microorganisms would not need to produce any biosurfactant for substrate uptake to occur. Only microorganisms utilizing the longer chain alkanes might need to produce

such compounds. Other surface active compounds such as cellular lipids or fatty acids may be produced concurrently with growth from the mixed cultures, but may not be used to mobilize and transfer a substrate. In this case, the biosurfactants may act more like a synthetic surfactant, or simply as dissolved or colloidal organic matter. It is well established that hydrophobic compounds will adsorb or bind up with particulate or dissolved organic matter (Weber et al., 1983, Karickhoff et al., 1979). In addition the degree of adsorbance usually can be characterized by the octanol/water partitioning coefficient (Abdul et al., 1987).

The biological fate of hydrophobic compounds which have adsorbed or partitioned into other organic matter is not well understood. Farmer (1989) found that during the degradation of toluene in soil microcosms, the adsorbed toluene was not available for degradation. The desorption of toluene from the soil was found to be the rate limiting process. Moore (1989) used synthetic surfactants to try to speed up the rate of desorption of toluene in soil microcosms. The surfactants adsorbed strongly to the soil, creating another organic phase for the toluene to partition into, thereby decreasing the degradation rate. Moore (1989) also noted that the surfactant in solution did not bind the toluene enough to interfere with the degradation. Robinson (1990) determined that 2,4,6-trichlorophenol partitioned into dissolved organic matter, typically humic material, which retarded degradation. Desorption of the trichlorophenol from the dissolved organics was determined to be the rate limiting process in the final degradation. If the biosurfactants

behave as dissolved organic matter, then the partitioning and subsequent degradation of the compounds may behave similarly.

## METHODS AND MATERIALS

### EXPERIMENTAL APPROACH

To test the potential of biosurfactants for removing a separate phase of gasoline from a groundwater aquifer, both static batch and flow-through column systems were used. The two setups were designed to simulate a second phase of gasoline present on top of the water table. Both systems were used to observe the physical, chemical and biological effects of the biosurfactants on gasoline solubility and mobilization. Because of the toxicity of pure gasoline to microorganisms, the biological effects had to be observed after removing the separate phase of gasoline. This was conducted in both systems, either by distance separation with two separate columns in the flow system, or physical removal and separation in the batch system.

Two types of biosurfactants were generated for use in this study. One group was produced by microorganisms when grown on a non-petroleum based substrate, glucose and vegetable oil, which has been established as a substrate for the production of biosurfactants (Cooper and Paddock, 1984.) The other type was produced from microbial growth on gasoline. Based on previous research described in the literature, two types of biosurfactants were used because some biosurfactants may be substrate specific, and only capable of enhancing solubility and growth for particular compounds, while other biosurfactants may be more non-

specific. Biosurfactants which are substrate specific would be produced from growth on gasoline, and could enhance both solubility and degradation of gasoline. Biosurfactants which are not substrate specific might not enhance degradation, but should enhance solubility and would be inexpensive to produce from a variety of cheap substrates.

Whole gasoline was used in the majority of the experiments, but only four compounds were monitored. These compounds were toluene, m-xylene, 1,2,4-trimethylbenzene and naphthalene. These compounds were selected because of the high concentration of aromatics that appear in groundwater contaminated with gasoline, and for the different solubility and partitioning values present among the group.

Microorganisms capable of degrading the four monitored gasoline compounds were used in the biodegradation experiments. None of the microorganisms were isolated and indentified. Sterile controls in the batch and column tests were obtained using sodium azide to inhibit biological activity.

#### BIOSURFACTANT PRODUCTION

Biosurfactants were produced by microorganisms grown in two liter batch reactors. The biosurfactants were produced by microorganisms isolated from activated sludge in the case of the glucose and vegetable oil substrate, or obtained from a mixed culture of gasoline degrading bacteria supplied by Sybron Chemicals Inc. of Salem, Virginia. The two

groups of microorganisms used were cultured on the two substrates. During growth it was observed that surface tension of the reactor liquids were lowered. When this occurred, these two groups of substrates and microorganisms were then considered to be producing biosurfactants.

To produce the first biosurfactant, glucose, vegetable oil and yeast extract were added initially to two liters of buffered, minimal-salts medium with proportions in accordance with Cooper and Paddock (1984). The mix was aerated and seeded with microorganisms capable of utilizing the substrate to produce biosurfactants which lowered the surface tension of the solution to less than 35 dynes/cm. The substrate and nutrients used are shown in Table 3.

To produce the other biosurfactant, gasoline utilizing microorganisms were grown in the minimal-salts medium solution in a two liter batch reactor. Exxon unleaded, 89 octane gasoline was added every 2-4 days, with more frequent additions with the occurrence of more biomass. Biomass was determined by filtering a known volume of reactor liquid thru a tared fiber filter, with the dry weight of retained material used to determine the solids or biomass per volume. Surface tension was monitored daily, and was lowered to a stable level between 40-50 dynes/cm.

When surface tension reduction reached a stable level, the biosurfactants were extracted. The extraction procedure was modified from Ramsey et. al. (1988) and Cooper and Goldenberg (1987). In the glucose/oil reactor, the whole mix broth was centrifuged at 12,000 rpms,

Table 3.  
Medium and Substrates

Minimal-Salts Medium	
Buffer Solution	Salts Solution
3.8 g $\text{KH}_2\text{PO}_4$ (.02794 M)	4.0 g $\text{MgSO}_4$ (.33250 M)
12.5 g $\text{K}_2\text{HPO}_4$ (.07184 M)	0.2 g $\text{NaCl}$ (.03422 M)
1.0 g $(\text{NH}_4)_2\text{PO}_4$ (.00763 M)	0.2 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (.00720 M)
	0.2 g $\text{MnSO}_4 \cdot 14\text{H}_2\text{O}$ (.00539 M)
	0.2 g $\text{CaCl}$ (.02651 M)
Add 1mL salts solution to the above dissolved in distilled water to make one liter.	Add the above to distilled water to make 100 mLs.
Substrates	
Glucose and Vegetable Oil Produced Biosurfactant	Gasoline Produced Biosurfactant
75 g Glucose	Add unleaded gasoline at the equivalent of 500 ppm daily.
75 g Vegetable Oil	
3.5 g Yeast Extract	
Add the above to 1.5 L of minimal-salts solution, seed with microorganisms and aerate.	Seed the above with microorganisms and aerate.



emulsified oil was skimmed off the top, and the liquid decanted off the pelletized cells. The liquid was then acidified to pH 3.0, mixed with a 3:1 chloroform:butanol solution, shaken in a separatory funnel, and the chloroform:butanol layer containing the biosurfactant removed. This extraction process was then repeated on the same liquid with a 3:1 mix of chloroform and methanol. The extracts were mixed and the solvents were evaporated off to obtain a concentrated residual. Biosurfactants were extracted from the gasoline fed reactor by simply acidifying the whole broth mixed liquor to pH 3.0, and contacting/extracting the liquid with the solvent mixes as noted previously, followed by evaporation to obtain a concentrated residual. At other stages, as noted in the research, the cells and the broth were separated and extracted separately in the same manner. A schematic of the biosurfactant extraction procedure is shown on Figure 1.

Both sets of biosurfactants were reconstituted in distilled water. The glucose and oil produced biosurfactant was difficult to completely solubilize. Excess biosurfactant floating on the surface was removed by repeated separations with a separatory funnel. The solutions were measured for total organic carbon (TOC), and diluted to matching TOC values for later work.

#### ANALYTICAL METHODS

Aqueous samples were collected from both the batch systems and the column systems and extracted with methylene chloride. A one

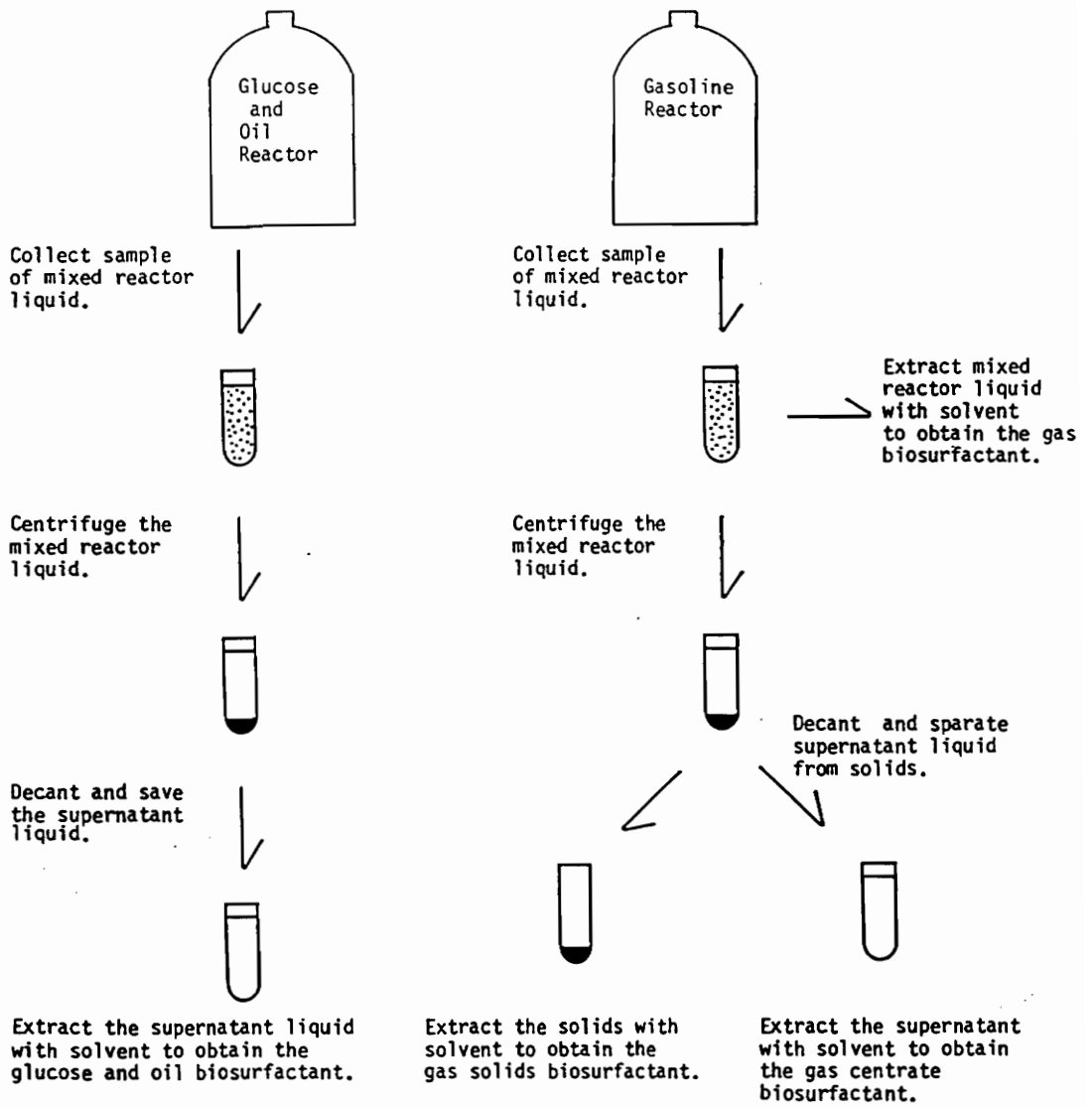


Figure 1. Biosurfactant extraction procedure.

milliliter aqueous sample was contacted and shaken with 3 mLs of methylene chloride with 60 mg/L of 1,4-dichlorobenzene present as an internal standard. During studies on degradation conducted early in the research, the amount of methylene chloride used was 0.5 mLs for each one mL of sample. Preliminary tests with the monitored compounds present showed good recovery. The recovery of the extraction was determined by noting the levels of the monitored compounds from samples extracted from sterile controls. If extracted samples from sterile controls had constant levels of monitored aromatic compounds, then the extraction recovery was considered to be constant. Since this research looked at changes in the aromatic compounds present, a constant recovery, even if less than 100 %, was considered to be good.

With time, however, some of the recoveries decreased, most likely due to the presence of the biosurfactants, as recoveries remained constant in distilled water controls without a biosurfactant. The biosurfactants were apparently forming a small emulsified layer at the interface between the water and solvent. A 3 mL methylene chloride extraction was later determined to effectively break up any microemulsions formed and extract the majority of the compounds. This was determined by repeated extractions of the same liquid sample, and by direct injection of the aqueous sample into the GC to search for aromatics. The percent recovery was generally 90-100 % when the methylene chloride to water ratio was 3:1. Data obtained from microcosm studies shown in the main text were all by extraction with the 3:1 methylene chloride to water sample ratio.

After extraction of the sample with methylene chloride, the contents of the sample were determined using a Hewlett Packard model 5890 gas chromatograph with a flame ionization detector. For gasoline analysis, the oven temperature was programmed at 35°C for 4 minutes, then increasing at 8°C/min for 21 minutes to 200°C. The column was a J&W DB-Wax, 15 meter long, .25mm ID, with a .50  $\mu\text{m}$  film thickness. For comparison of the substrates used and the biosurfactants produced, a J&W DB-1 capillary column was used. The oven temperature was held at 35°C for 4 minutes and then raised to 325°C at 7°C/minute and held for 15 minutes. Injector and detector temperatures were held at 300°C and 325°C respectively. Samples were typically analyzed with split injection, and the injected sample size was typically 1  $\mu\text{L}$ . The purge valve was shut for up to one minute for splitless injection during trace analysis of the sample as needed.

Analysis of TOC and CO<sub>2</sub> present in the samples was performed by a Dorhman TOC analyzer. TOC was determined by furnace combustion with the subsequent measurement of CO<sub>2</sub> produced. Since a portion of the TOC present were volatiles, purging samples to remove CO<sub>2</sub> also removed volatile compounds. Therefore, depending on the analysis, some TOC values included both CO<sub>2</sub> and volatiles, as well as other TOC contributors. Analysis of CO<sub>2</sub> by the same Dorhman equipment was by direct injection into the oxidation reactor. The persulfate solution was replaced with acidic (pH 2.0) distilled water however, the U.V. lamp turned off, and CO<sub>2</sub> was measured directly by the detector.

Surface tension measurements were made using a Fisher Scientific

surface tensiometer, which operates under the de Nouy method. The tensiometer was calibrated and standardized against distilled water, which has a surface tension of 74 dynes/cm.

## SOLUBILITY ENHANCEMENT

### Batch Systems

Enhanced solubility of gasoline into water as caused by biosurfactants was tested by contacting biosurfactant solutions with a second phase layer of gasoline. Thirty mLs of either distilled water or a biosurfactant solution were contacted with 3 mLs of gasoline in 40 mL amber jars. The jars were screw capped with teflon lined septum, and were inverted and centrifuged at 1000 rpms to force gasoline away from the septa. The inverted jars were then placed on the shaker table for 24 hours, and an aqueous sample was removed out the bottom through the septa. The sample was extracted with methylene chloride and analysed.

Standard deviations for the solubility tests were determined on experiments with triplicate samples. The standard deviations were determined on a percent removal basis so as to combine data to form a larger data base for a more accurate value. Standard deviations were determined for each of the four monitored compounds based on the percent increase in solubility in the presence of the two organic phases, gasoline and mineral oil. This gave eight different standard deviation values. When plotting the percent increase, these values are used and

shown directly as an interval on the plot. When showing the actual solubility levels, the associated coefficient of variation is determined, and used to find a standard deviation for the set of solubility values shown.

### Column Systems

Three pairs of glass columns were constructed to observe leaching and mobilization of the gasoline. The columns were glass liquid chromatography columns. The larger of the paired columns was 25 mm in diameter and 450 mm in length. The larger column was used to observe the leaching and solubility enhancement of the gasoline. The smaller column was used to observe degradation and was fitted with side sampling ports. It's dimensions and operation are discussed in the column system of the biodegradation section. A schematic of the column set-up is shown in Figure 2. The columns were filled simultaneously with distilled water and a clean, white, acid washed sand. The columns were tapped and agitated to compact the sand while being filled. This sand was used to minimize adsorption affects, and was seived between #40 and #100 size screens for uniformity. The sand had a porosity of 43 % as determined by volume displacement, which gave the larger column a pore volume of 80 mLs. The sand had a low adsorption capacity for toluene, .0231 mg. toluene/gram sand, as determined by the adsorbance until breakthrough during a flow through column test. Adsorbance for the other compounds was not tested, but it would be expected to occur at similar or higher levels.

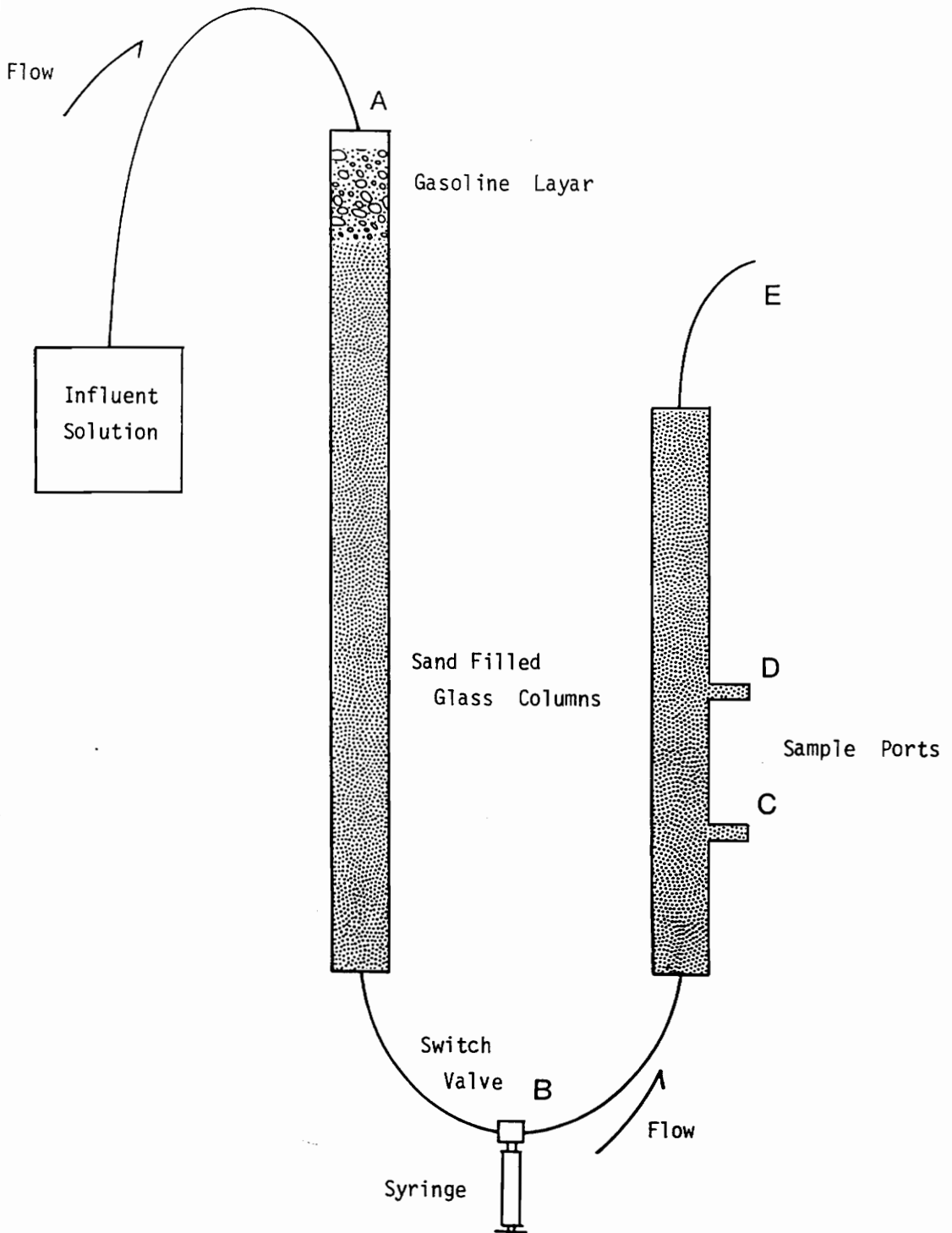


Figure 2. Schematic of column set.

At the top portion of the larger column a coarser grained sand was used, into which was placed 25 mLs of gasoline. The different solutions were added at the top, they percolated through the gasoline and sand and out the base of the column. Liquid was pumped through the column using syringes in 9ml and 10mL plugs, for a total flow of 19 mls each day. The syringe was located in between the two columns at the base of each. It was filled with effluent from the column containing gasoline, the valve was then turned and the effluent from the gasoline column was passed into the second column. The flow rate for each plug was 10 mLs per minute.

The liquids flowing into the columns were either biosurfactant solutions or controls. These solutions were purged with nitrogen gas to remove oxygen and stop degradation. Samples were collected at the bottom of the column, at sample point B, and extracted and analysed to determine effluent water solubility levels of gasoline for the column system.

## BIODEGRADATION

### Gasoline Degrading Microorganisms

A mixed culture of gasoline degrading microorganisms was supplied by Sybron Chemicals Inc., of Salem, Virginia. The culture was maintained in 200 mL flasks on a shaker table. Gasoline was added at a concentration of 500 mg/L with 30% of the volume wasted and replaced with fresh nutrient buffer solution every 2-4 days. Other cultures were



started from this mix, and acclimated separately for growth on each of the four monitored compounds. One compound was added to each of the four cultures in the same fashion as noted previously for gasoline. When seeding microcosms with gasoline degrading microorganisms, a portion of each culture was mixed and added to the microcosms.

### Batch Systems

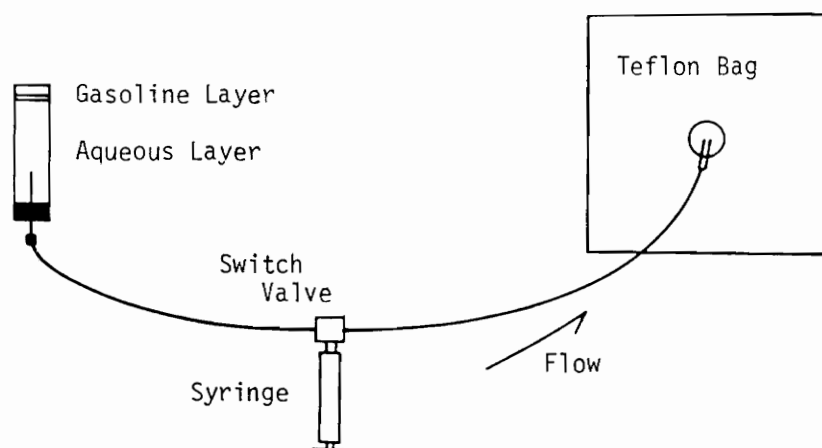
Biodegradation of solubilized gasoline in the surfactant containing solutions was measured in batch 9.4 mL, screw capped microcosms. Biodegradation of the gasoline compounds was observed either with solubilized whole gasoline present, as noted on the figures showing degradation, or with only the four monitored compounds present. When observing biodegradation with solubilized gasoline present, the biosurfactant solutions with solubilized gasoline were those contacted with gasoline in the manner described previously for the testing of solubility enhancement with biosurfactants. Each separate solution was obtained from the surfactant containing solution which contacted an overlying gasoline layer. A schematic of the process is shown in Figure 3. Solutions were diluted as needed to match the concentrations of gasoline present in the other mixes. The amount each solution was diluted was based on the amount of gasoline that was solubilized. The more effective the biosurfactant was at increasing solubility, the more the solution was diluted so that the initial concentrations in the microcosms were similar. Nutrient buffer was added, and each mix was seeded with the same dose of acclimated microorganisms and placed in the

microcosms.

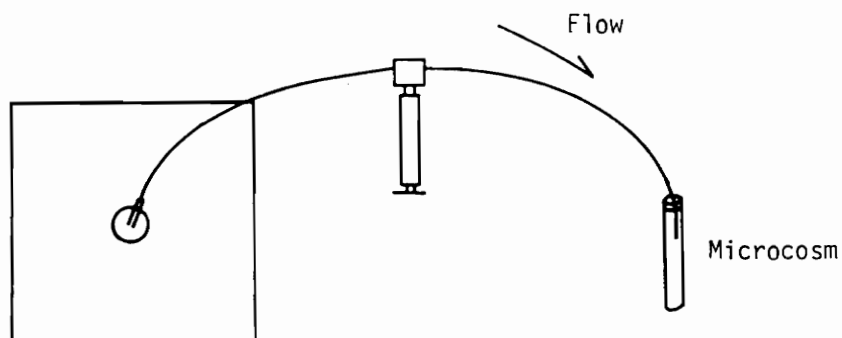
In degradation studies with only the four monitored compounds present, the four compounds were mixed in the teflon bag with nutrients, biosurfactant solutions and acclimated microorganisms, and then dosed into the individual microcosms. The microcosms were then monitored through sample collection and analysis in the same manner as the microcosms with solubilized gasoline present.

All microcosms were mixed daily using a vortex mixer, and hydrogen peroxide was added to supply oxygen on an as needed basis depending on the amount of observed degradation or the CO<sub>2</sub> produced. Each microcosm was used once to produce a data point. By using each microcosm only one time, volatile losses were minimized, and a large sample could be obtained from a single microcosm. Each data point was obtained from a separate microcosm. Therefore the data can appear somewhat scattered, as degradation rates may vary slightly between different microcosms.

Controls on the microcosms were obtained by the addition of sodium azide to inhibit biological growth. The biologically active microcosms and the control microcosms were treated identically except for the addition of the sodium azide to the controls at the beginning of each experiment. Both active and control microcosms were mixed from the same solution containing nutrients, aromatic compounds, and acclimated microorganisms, and both had the same amount of hydrogen peroxide added during the course of the experiment. Decreases in the concentration of aromatics present in the controls should be due to non-biological



The aqueous solution was drawn from the aqueous layer underlying the gasoline layer, mixed with nutrients in the teflon bag, and seeded with gasoline utilizing microorganisms.



The mixed solution was then pumped with the syringe and added to the individual microcosms.

Figure 3. Batch microcosm dosing procedure.

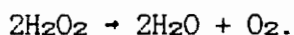
removal mechanisms such as volatile losses, or chemical reactions with hydrogen peroxide or sodium azide.

### Column Systems

A flow-through column with side sampling ports was also used to observe the biodegradation of gasoline. The column in which degradation occurred in was 25mm in diameter, and 270mm in length, and had a pore volume of 57 mls. The column was filled with sand and water in the same manner as described previously for the first column. The columns had two side sampling ports, positioned to sample the midpoint of a plug of liquid sized at one third of the pore volume. Therefore a 19 mL plug of liquid was transferred each day into the column, giving each plug a three day residence time in the column. The injected liquid was the effluent from the from the first column, which were used in pairs, as shown in Figure 2 previously. Each plug of liquid injected was dosed with hydrogen peroxide to provide 9 mg/L dissolved oxygen. If peroxide were added above this level, rapid degradation of peroxide would result in excess oxygen and subsequent bubble formation would reduce porosity.

### HYDROGEN PEROXIDE

Hydrogen peroxide was added to both column and batch studies as a source of oxygen. The peroxide provides oxygen according to the equation,



The reaction proceeds rapidly in the presence of biological activity due to the enzyme catalase, which is produced by microorganisms to degrade toxic oxygen species during aerobic respiration. This reaction is generally catalyzed by the presence of iron, and inhibited by phosphate. A simple rate test showed that in the nutrient buffer solutions with biological activity, the peroxide decays to stoichiometric values within 25 minutes.

## RESULTS

### PRODUCTION OF BIOSURFACTANTS

A preliminary study was conducted to measure the production of biosurfactants using different substrates. Several substrates or substrate combinations were tested, including glucose, glucose and vegetable oil, naphthalene, kerosene and gasoline. Evaluation of the production of biosurfactants was made by measuring surface tension in the reactor liquid.

It was found that the glucose and oil combination produced the greatest reduction in surface tension, lowering it to 35 dynes/cm. Commercial synthetic surfactants typically reduce surface tension to between 30 and 35 dynes/cm. For the petroleum hydrocarbons, there was no significant difference in the reduction of surface tension between the different substrates. All reduced surface tension to between 45 and 55 dynes/cm. Since the mobilization of whole gasoline was to be investigated, and gasoline was to be monitored for degradation, further experiments focussed on its use for biosurfactant production.

With the addition of the gasoline and glucose and oil substrates, surface tension was immediately lowered to between 65-70 dynes/cm, down from the 74 dynes/cm of water. This initial quick drop is caused by the addition of the substrates. After this point, as shown graphically on Figure 4, surface tensions decreased at a slower, generally linear rate

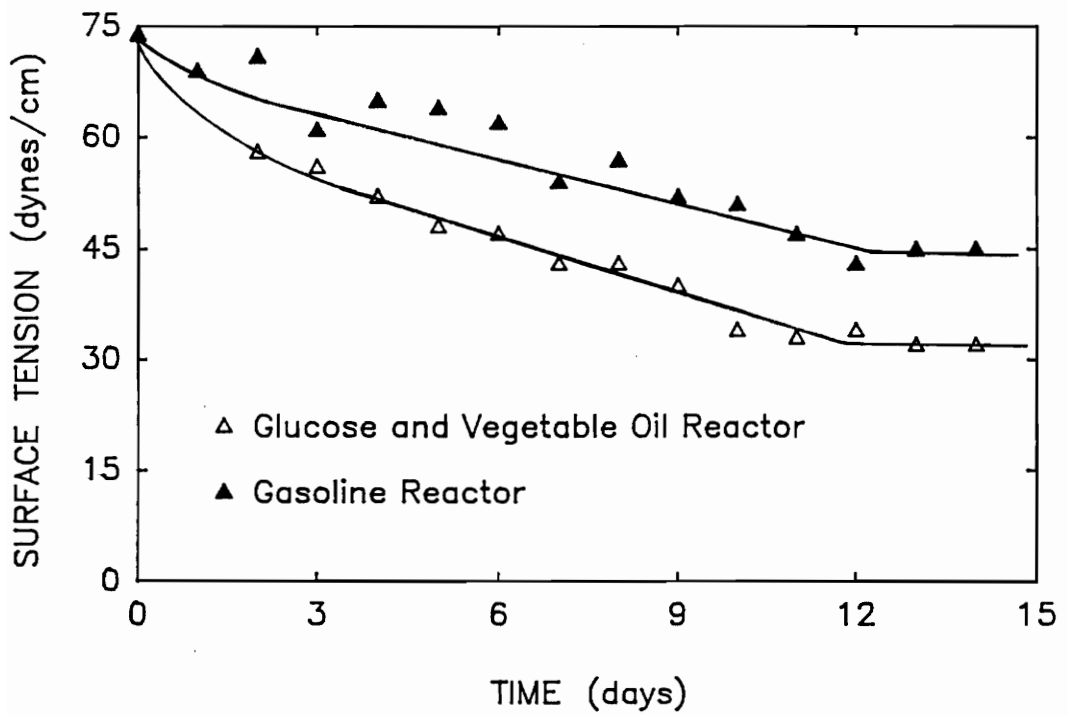


Figure 4. Surface tension reduction during growth on the two substrate groups.

as microbial growth and biosurfactant production occurred. The complete utilization and removal of these substrates at the end of two weeks would mean that the biosurfactants produced are the only compounds present that would lower surface tension in the reactors. Therefore surface tension is plotted on Figure 4 starting from 74 dynes/cm., the surface tension of water, and decreasing to the final levels with no adjustments made for the initial drop since it is not due to biosurfactant production.

Microorganisms producing biosurfactants were grown in two liter batch reactors with mixing and aeration provided by diffused air. Surface tensions were reduced to stable levels within a two week growth period. Biosurfactants from the gasoline utilizing microorganisms were also produced during the latter stages of the research in a 20 liter batch reactor with a five week growth period. The larger reactor and longer detention time were used to raise the concentration of the biosurfactant. Plots of the reduction of surface tension with time are shown in Figure 4 for the two week growth period. Because of the slow rate of surface tension reduction in both reactors, it is uncertain whether the microorganisms produced the biosurfactants to assist in growth and substrate utilization, or it occurred as a byproduct of growth, for example, as a result of cell lysis. The steady or linear rate of surface tension reduction suggests that the biosurfactant production was related to growth. Biosurfactants produced during the later stages of growth are often attributed to cell lysis or other causes besides growth (Cooper and Goldenburg, 1987). Since the



characterization of growth and biosurfactant production was not the focus of this investigation, the production mechanism was not studied.

Once biosurfactant production was completed, subsequent extraction yielded a more refined biosurfactant material. Since surface tension is not always equally reduced by different biosurfactants such as cellular lipids or fatty acids acting as surfactants, biosurfactant mixes were equated based on TOC.

#### CHARACTERIZATION OF BIOSURFACTANTS

Biosurfactants were defined by their ability to lower surface tension. Because of the large variety of compounds which may be present in the biosurfactant mixes produced from the bioactivity, characterization and isolation was determined to be outside of the scope of this investigation. Some type of identification was needed however, to show that the substrates added as a food source for the microorganisms were not acting as surfactants or providing another phase for partitioning of organics. Since glucose is water soluble it would not act as a second phase for partitioning. Excess gasoline or vegetable oil could act as a surfactant if it remained undegraded. Their presence or absence was determined from peak retention times from gas chromatography data. A non-polar column was used, and was able to separate gasoline compounds as well as triglycerides. Chromatograms of the substrates and the biosurfactants are presented in Appendix A. Based on the peak identification, the substrates do not appear to have

been carried over into the biosurfactant mixes.

## SOLUBILITY ENHANCEMENT WITH BIOSURFACTANTS

### Batch Systems

Batch experiments were used to study the effects of biosurfactants on hydrocarbon solubility and biodegradation. By contacting an overlying gasoline phase with water containing various biosurfactant mixes, solubility enhancement could be evaluated.

Distilled water and various biosurfactant mixes were contacted with gasoline in a 10:1 ratio of water to gasoline. The different biosurfactant mixes all increased solubility of the hydrocarbons, but in varying amounts. Different types of biosurfactant mixes were tested early in the research, but were all produced by microorganisms grown on the glucose and oil or gasoline. Figure 5 shows the increase in solubility for the four gasoline compounds, as affected by two biosurfactants. The two biosurfactants are the gas biosurfactant, produced in the gasoline fed reactor, and the gluc/oil biosurfactant, produced by microorganisms growing on glucose and vegetable oil. These two surfactants were equated based on TOC values of 300 mg/l. The biosurfactant produced from glucose and oil generally caused the greatest increase in solubility. Trimethylbenzene showed the greatest percent increase in solubility, but in general, compounds with the lowest aqueous solubility had the largest percent increase in solubility

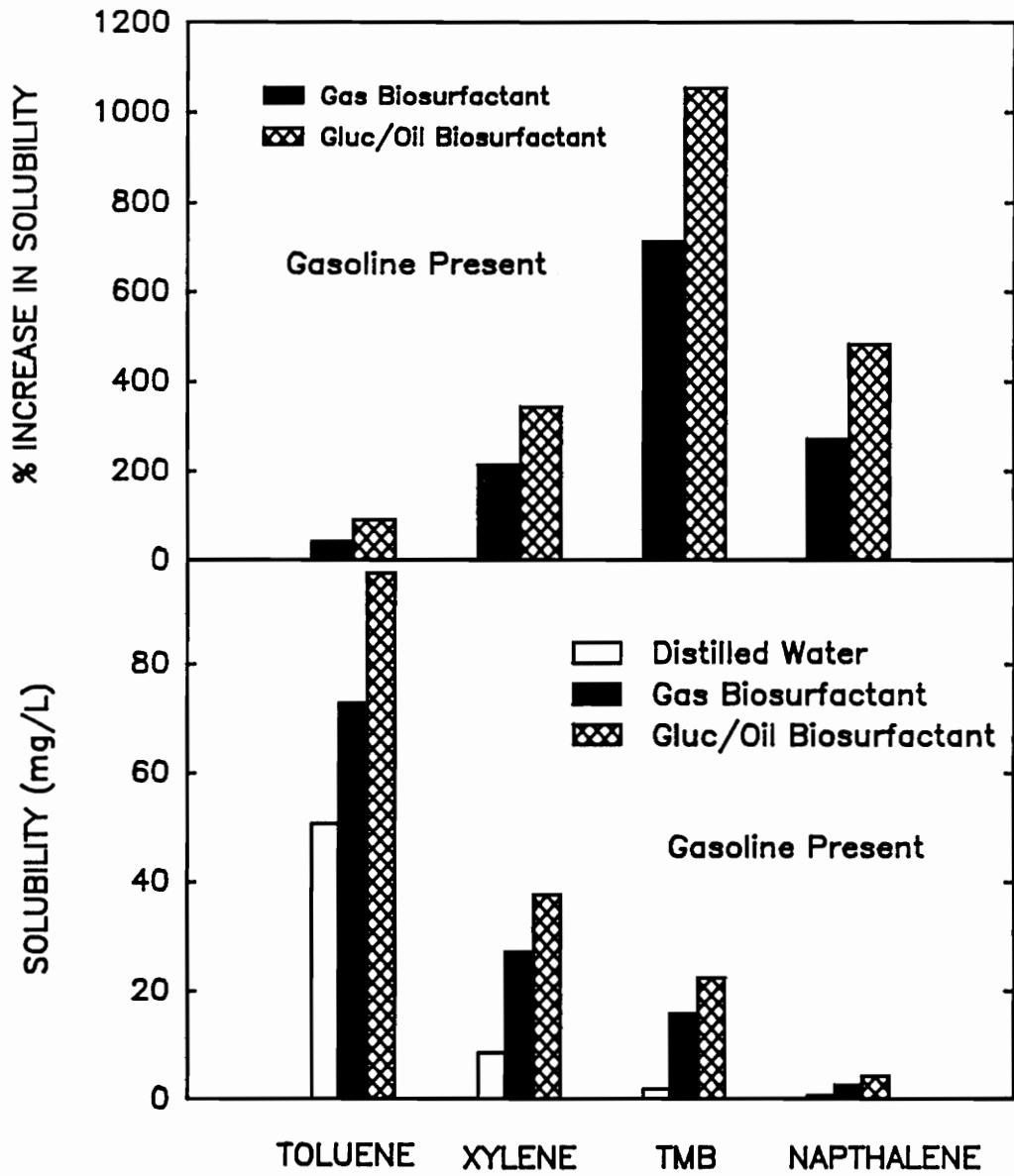


Figure 5. Effect of two, 300 mg/L TOC biosurfactant solutions on the solubility of four gasoline compounds.

when contacted with the biosurfactants. A more specific trend may be that as the number of methyl substituent groups increased, the biosurfactants increased the solubility proportionally.

The different biosurfactant mixes were also tested against different organic phases to observe increases in solubility. Both gasoline and mineral oil spiked with the four monitored compounds were tested individually against the different biosurfactants mixes. In these tests, the gasoline biosurfactant extracted from the reactor culture was replaced by two other biosurfactants. One surfactant was extracted from the liquid portion which remained following centrifugation of the bioreactor solution and the other was from the solids or biomass material removed during centrifugation.

In one test, gasoline was layered on top of distilled water and the different biosurfactant solutions, and shaken for 24 hours as described previously and shown in Figure 3. In another test, mineral oil spiked with the four monitored compounds was layered on top of distilled water and the biosurfactant solutions and was also shaken for 24 hours. After the samples were shaken, an aqueous sample was collected and the concentrations of the four monitored compounds were measured. As shown on Figure 6, when contacted with the hydrocarbon spiked mineral oil, the gasoline solids biosurfactant increased solubility more than the glucose and oil biosurfactant. As shown on Figure 7, when contacted with gasoline, the glucose and oil biosurfactant increased solubility more than the other biosurfactant. This interaction may be caused by the differences in polarity between

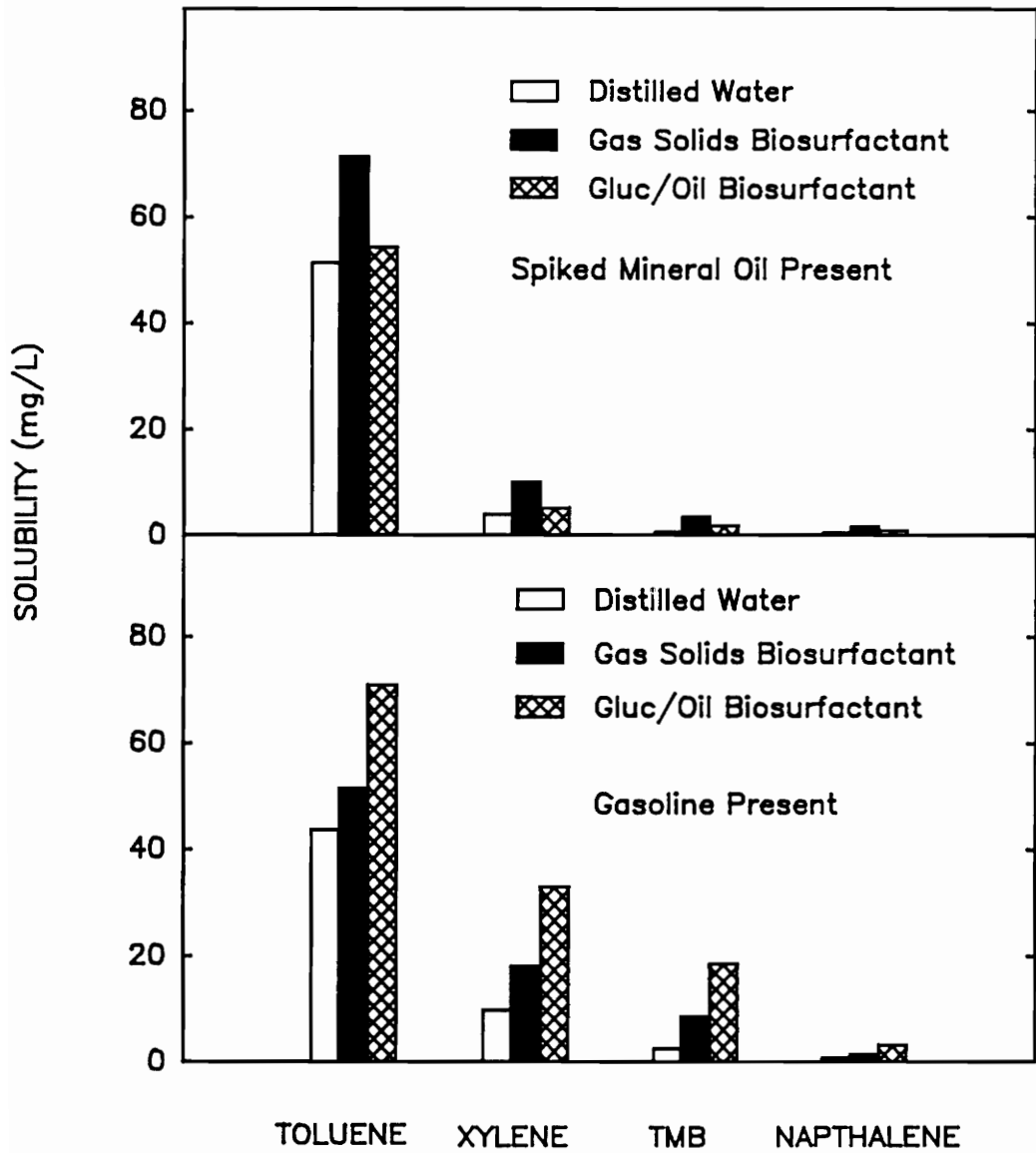


Figure 6. Effects of 300 mg/L biosurfactant solutions on enhanced solubility in the presence of gasoline and mineral oil.

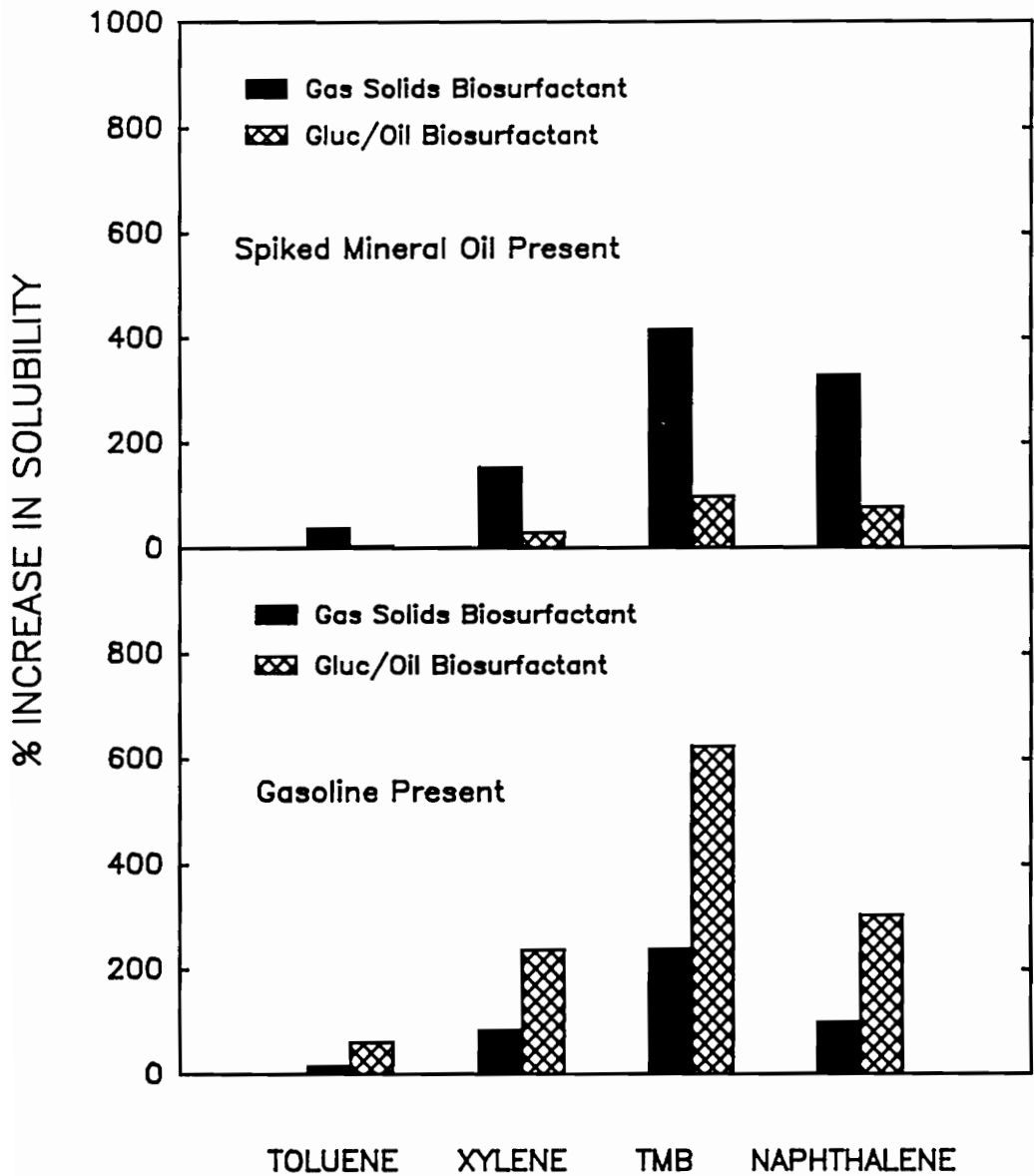


Figure 7. Effects of 300 mg/L biosurfactant solutions on the percent increase of solubility enhancement in the presence of gasoline and mineral oil.

the biosurfactants and the organic solvent phase. Such interactions are usually characterized by the hydrophile-lipophile balance (HLB) value assigned to different surfactants. Surfactants with a hydrophilic end group of relatively low polarity may simply partition into the organic phase, and be rendered ineffective. The reason for the difference in effectiveness between the biosurfactants is not known.

The gas centrate biosurfactant was not tested for solubility enhancement in the previous experiment because of the limited amount produced from the reactor containing the gasoline utilizing microorganisms. An adequate amount of this material at a TOC level of 300 mg/L could not be obtained. Preliminary tests with the gas centrate biosurfactant at TOC levels of 150 mg/L showed that this biosurfactant was relatively ineffective at solubilizing the monitored compounds. To better observe the effects of this biosurfactant, a larger amount or higher concentration was desired. To obtain more biosurfactant from the reactor supernatant, the reactor size was increased and the time allowed for growth of the mixed culture was increased. This allowed for more biosurfactant to accumulate in the reactor.

Tests using three types of biosurfactants at higher TOC levels (600 mg/L) showed trends in solubility enhancement similar to those found in previous experiments. This latter experiment was completed using gasoline produced biosurfactants grown in a 20 liter reactor with a five week growth period. Data from this experiment, presented in Figures 8 and 9, show that the biosurfactants were all similar with regard to their ability to increase the solubility of the gasoline

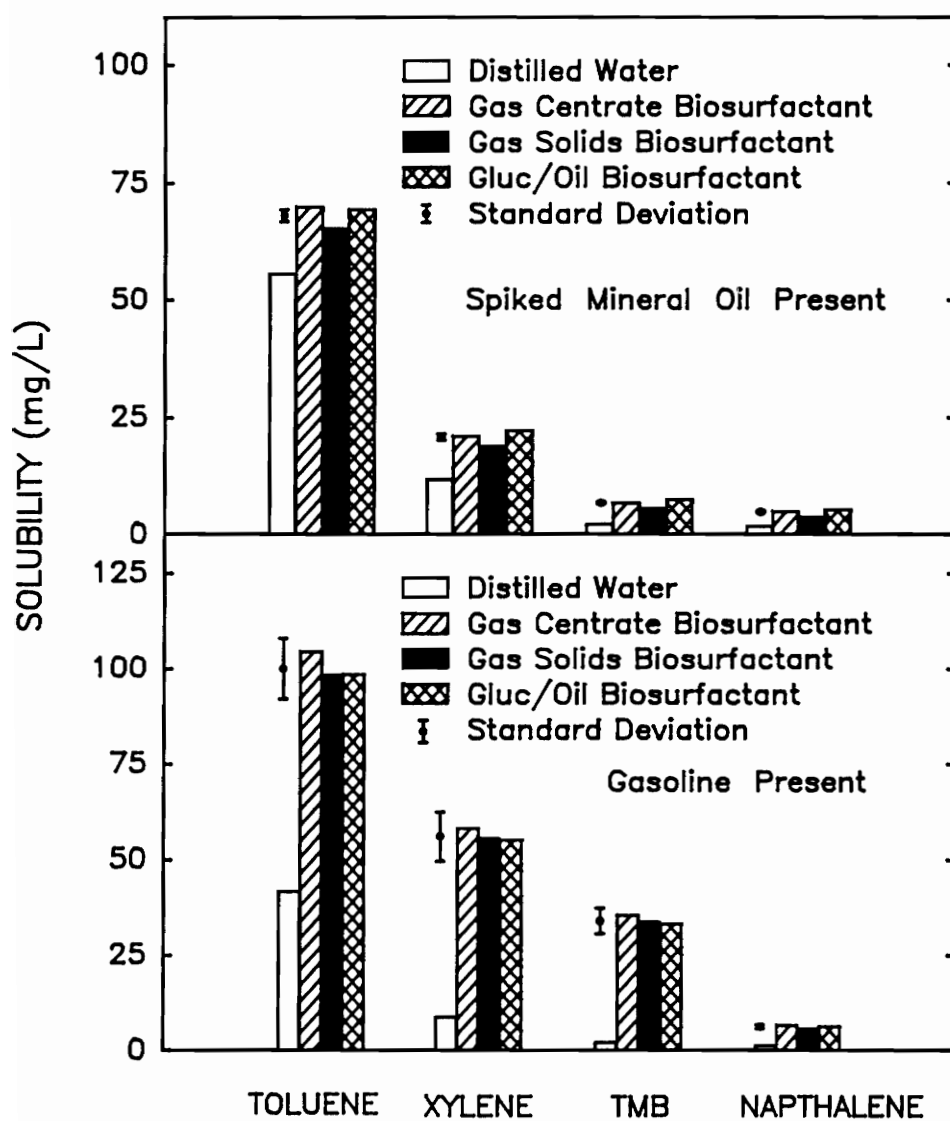


Figure 8. Comparison of solubility enhancement from mineral oil and gasoline as caused by three biosurfactant solutions at 600 mg/L TOC.



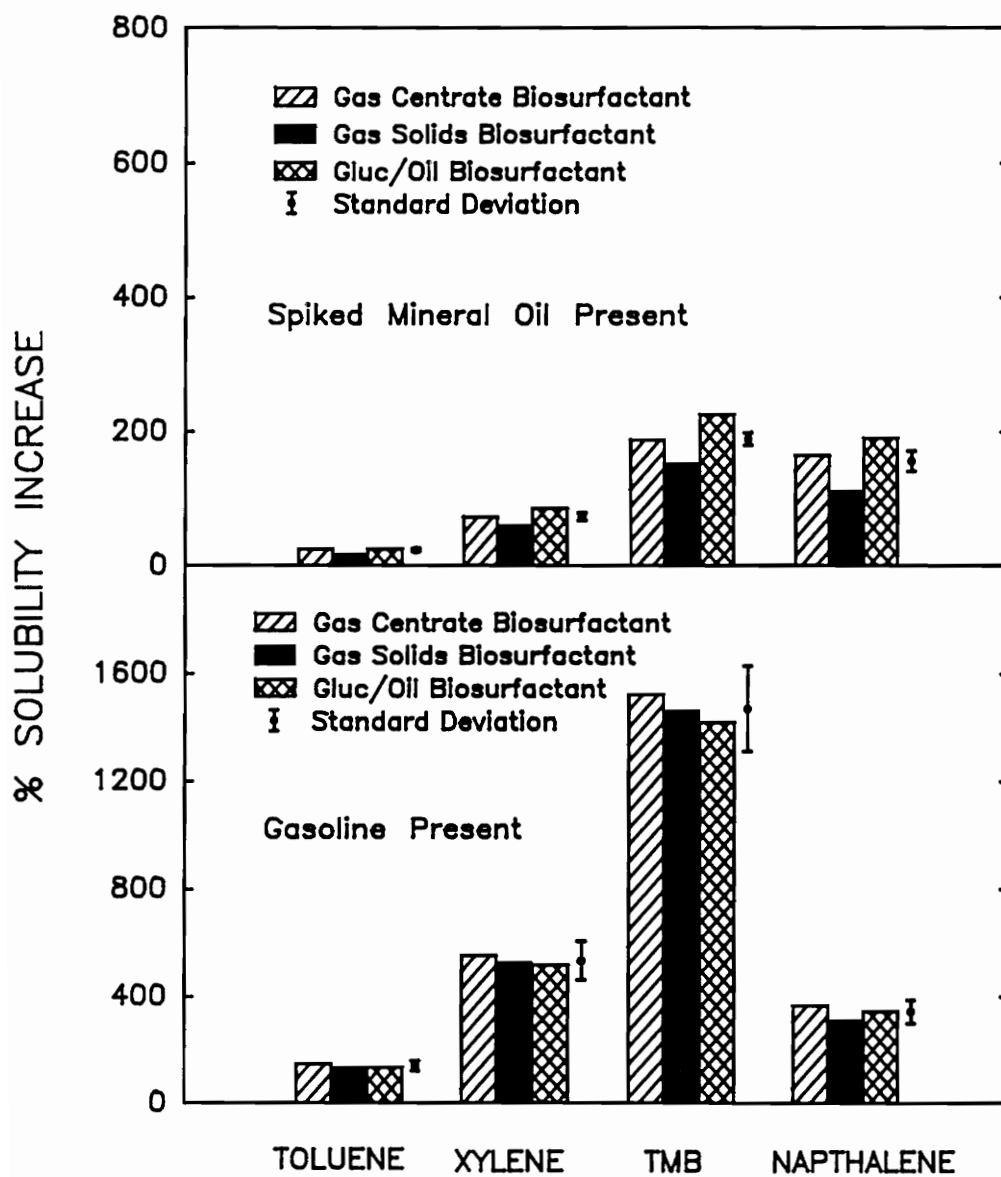


Figure 9. Comparison of the percent increase in solubility from mineral oil and gasoline as caused by three biosurfactant solutions at 600 mg/L TOC.

compounds. At the higher TOC levels, the gasoline-produced biosurfactants apparently increased in strength proportional to the biosurfactant grown on glucose and oil. The gasoline produced centrate biosurfactant was slightly stronger than the other two biosurfactants, and the gasoline produced solids biosurfactant was stronger than the one used in the previous experiment shown in Figures 6 and 7. The reason for this difference is unclear, but it may be caused by different age and growth patterns of the cells present in the reactor which produced the biosurfactants. The gasoline produced biosurfactants used to generate the data in Figure 8 and 9 were produced from a reactor with gasoline utilizing microorganisms that had an older cell age (up to five weeks) than the previous reactors (up to two weeks).

#### Column System

Sand filled columns were used to observe the potential effects of biosurfactants on the removal of second phase hydrocarbons in an aquifer system. The use of the columns added information concerning the degree of adsorption of the biosurfactant to sand, and the potential for the mobilization of hydrocarbons in the subsurface.

Three different solutions were passed through a layer of gasoline at the top of glass columns filled with water saturated sand. This was done in order to observe the effects of the biosurfactant on the hydrocarbon. The three solutions were; a nutrient solution without a biosurfactant, a nutrient solution without a biosurfactant but with

sodium azide to inhibit biological activity (sterile control), and a nutrient solution with the glucose and oil biosurfactant.

Gasoline constituents were monitored at the bottom of the larger column at point B, as shown previously in Figure 2. Soluble gasoline components first appeared in the effluent just after one column pore volume (80 mLs) was passed. This confirmed reasonably good plug flow conditions as did the chloride tracer study noted previously. All solutions had similar concentrations of solubilized gasoline over the first 250 mLs (3.13 bed volumes). After 250 mLs of biosurfactant solution had passed through the column, levels of gasoline compounds present in the effluent biosurfactant solution began to increase, as shown on Figure 10. The failure of the biosurfactant solution to increase soluble gasoline levels up to that point is thought to be due to adsorption of the biosurfactant to the sand. Regardless of the cause, the biosurfactant appeared to be initially retained by the sand. A retardation factor (RF) for this system can be defined as  $dW/dC = RF$ , where  $dW$  is the distance water has traveled in time  $t$ , and  $dC$  is the distance the biosurfactant has traveled in time  $t$ . Using the ratio of the volumes of solution passed (250 mLs) to the pore volume (80 mLs) would produce a  $RF = 3.13$ . This value assumes that the increase in solubility marks the beginning of the presence of the biosurfactant in the effluent. Correspondingly, it may be a conservative RF estimate since the solubility continued to increase after this point.

The plots of the percent increase in solubility are shown on Figure 11. The increase in solubility for the compounds was greatest for

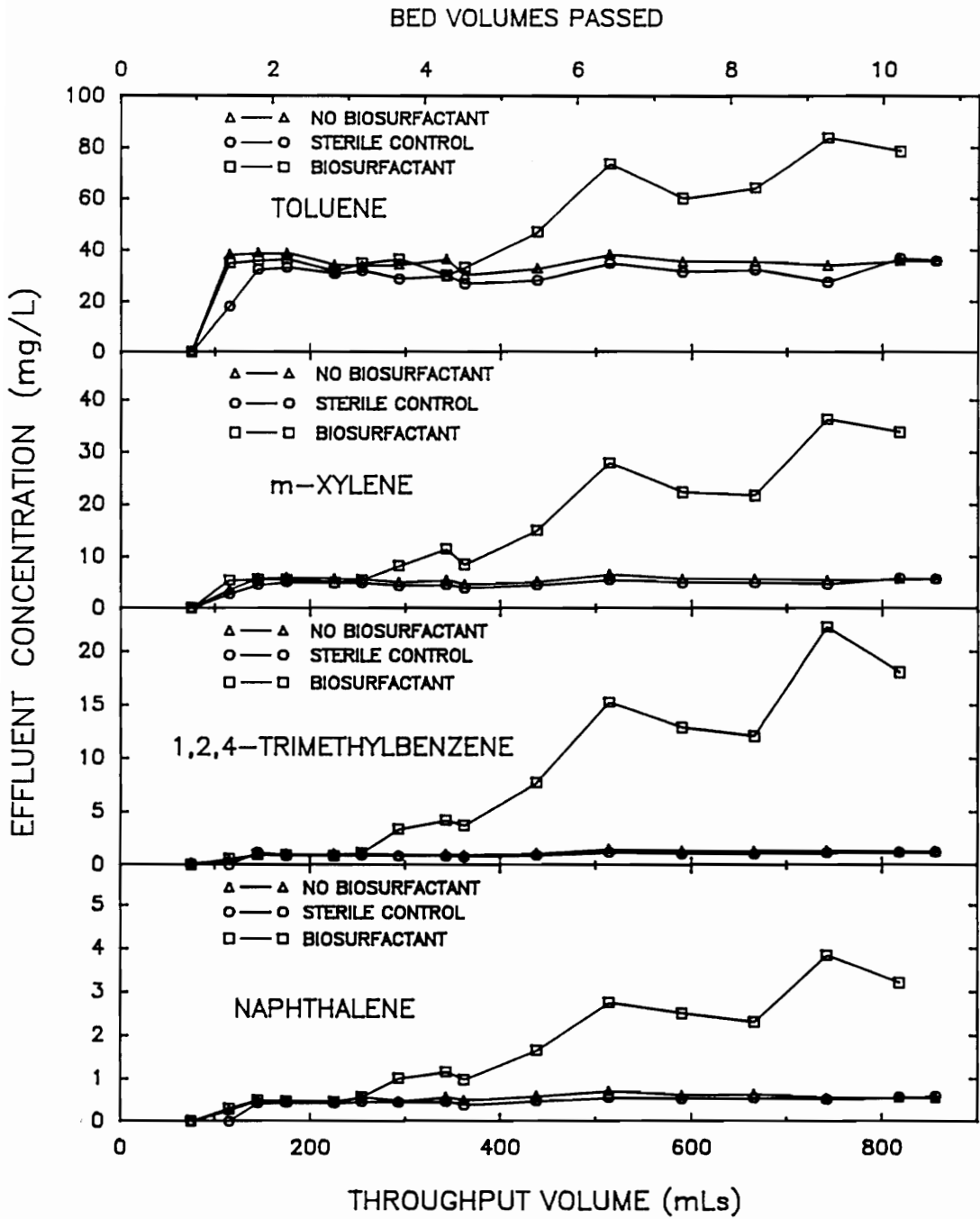


Figure 10. Column effluent concentration levels of the four monitored gasoline compounds.

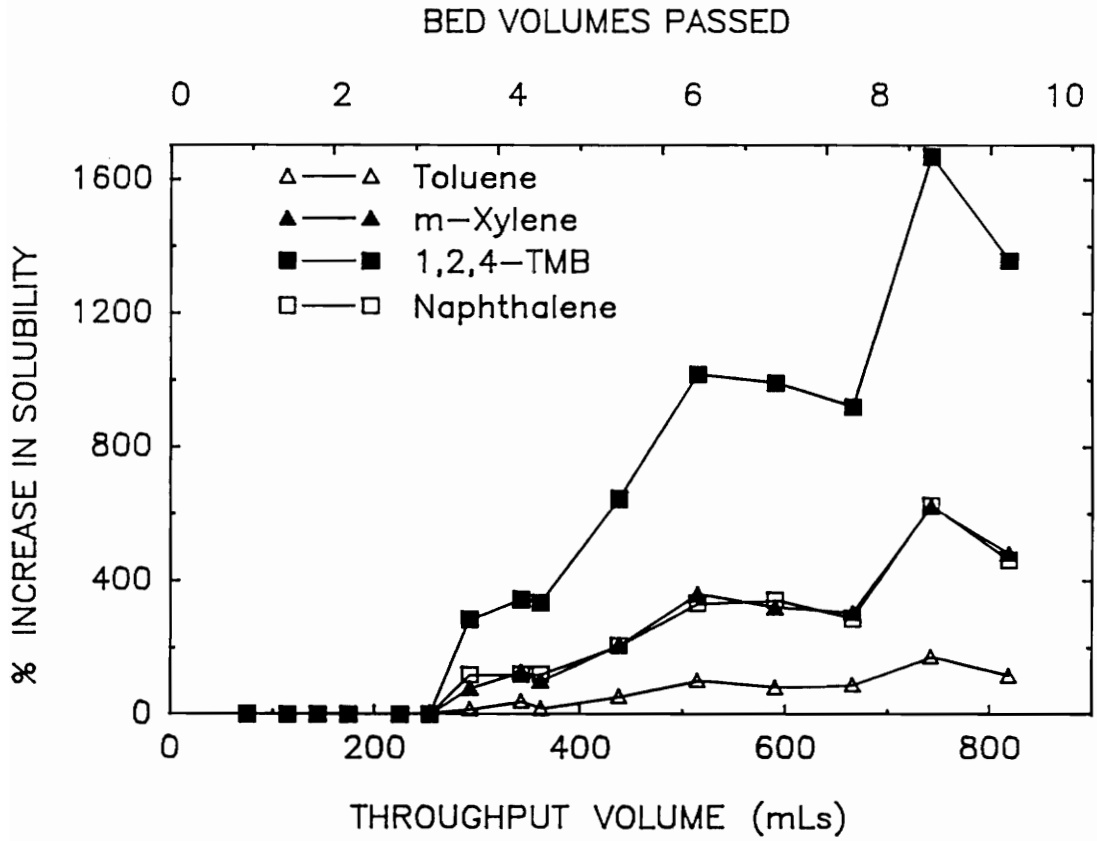


Figure 11. Percent increase in column effluent concentration levels of the four monitored compounds.

trimethylbenzene, as it was in the batch tests. The control columns had soluble gasoline compounds present at levels similar to those in the batch tests.

## BIODEGRADATION WITH BIOSURFACTANTS

### Batch Systems

Biodegradation of the gasoline compounds in the presence of biosurfactants was studied using 9 mL culture tubes, or microcosms. Preliminary microcosm study described in Appendix B and Appendix C indicated that the biosurfactant produced by the gasoline utilizing microorganisms did not interfere with the degradation of the compounds. Biosurfactants produced by microorganisms from growth on glucose and oil, while greatly increasing solubility, appeared to retard degradation of the compounds. These preliminary studies were used to design more refined biodegradation studies utilizing only two types of biologically produced surfactants, one from gasoline degradation, the other from degradation of glucose and oil.

Figures 12 and 13 show the changes in the compound levels in the microcosms after the biosurfactant solutions had been contacted with gasoline, then separated and seeded with gasoline utilizing microorganisms. Toluene was partially degraded in the presence of the gasoline biosurfactant, and was easily degraded in the presence of the glucose and oil biosurfactant. Other less soluble compounds were not

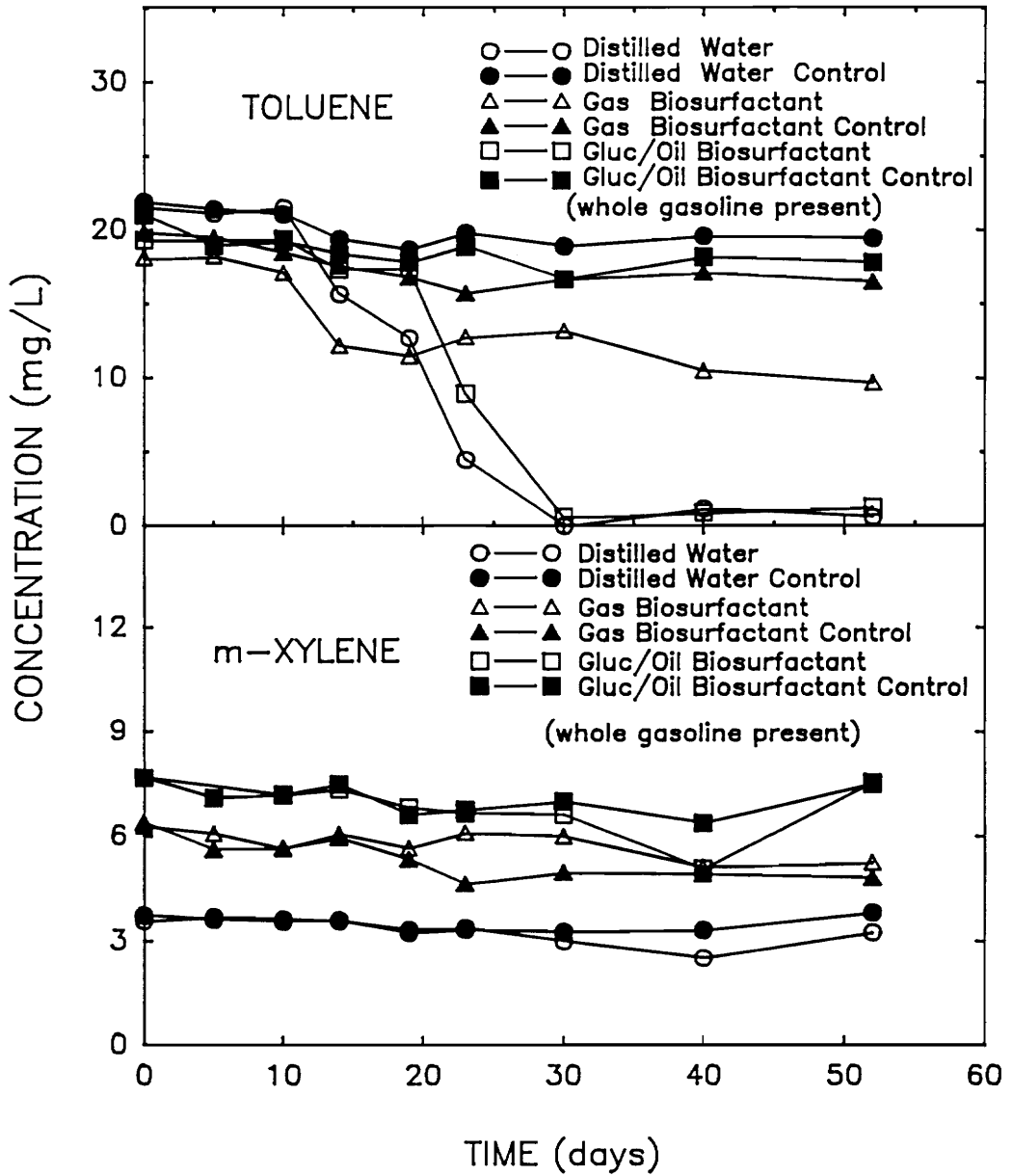


Figure 12. Biological degradation of toluene and m-xylene in the presence of two biosurfactant solutions at 300 mg/L TOC.

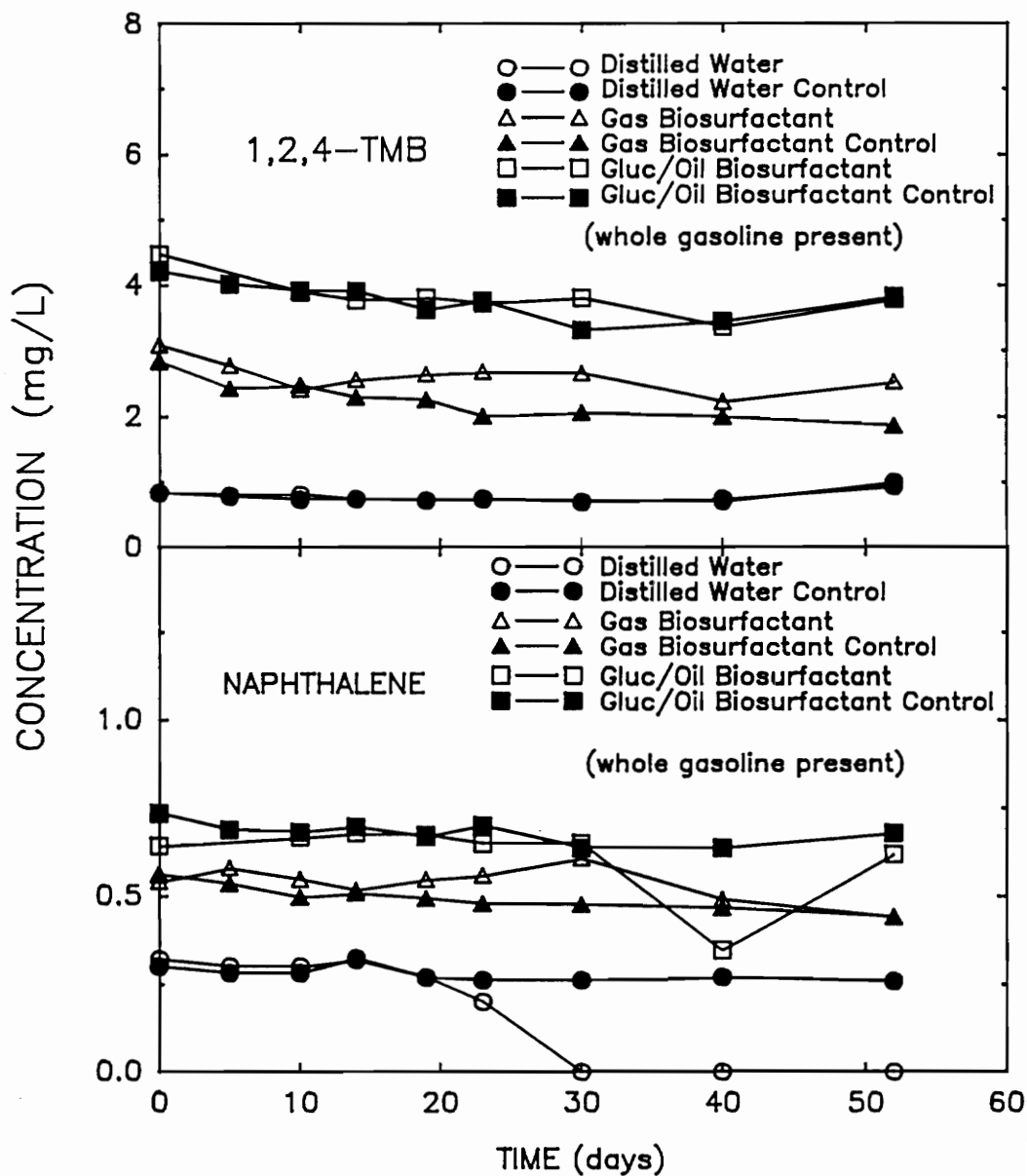


Figure 13. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of two biosurfactant solutions at 300 mg/L TOC.



degraded in the presence of either biosurfactant. In this microcosm experiment, the gasoline produced biosurfactant inhibited degradation. This differs from previous experiments where the gasoline-produced biosurfactant did not greatly affect degradation.

There are several reasons why the degradation may have been affected in this experiment. Inhibition might be caused by certain organic materials and extracted with the biosurfactant. Alternatively, the toluene may have been more strongly bound to the biosurfactant and therefore less available for degradation. Since this microcosm study was set up with whole solubilized gasoline present there may have been biological inhibition due to additional gasoline constituents present beyond the four compounds monitored. Monitoring of CO<sub>2</sub> during the later stages of this microcosm study showed increased levels of CO<sub>2</sub> production in the glucose and oil biosurfactant microcosms. This might indicate that the biosurfactant was being degraded, releasing toluene which then could be subsequently degraded. It could also indicate that with the increase in solubility of the four monitored compounds, other unmeasured and easily degradable compounds were present and were being degraded simultaneously. Subsequent batch tests therefore used only the four specific compounds which were being monitored. The concentrations of CO<sub>2</sub> and TOC in the microcosms were also measured.

Based on observations from the experiment noted previously, changes were made in the procedure of the next microcosm study to clarify the data collected. Instead of contacting the the solutions with gasoline, only the four compounds, toluene, m-xylene, 1,2,4-

trimethylbenzene, and naphthalene were added to the biosurfactant solutions. This eliminated the presence of other organics in the microcosms which could be degraded preferentially to the compounds being monitored. In addition, two types of gasoline produced biosurfactants were used, one from the centrate (referred to as the gas centrate biosurfactant) and one extracted from the solid material collected after centrifugation of the reactor contents. The plots of concentration versus time for the four compounds are shown on Figures 14 and 15. The gas centrate biosurfactant did not appear to inhibit degradation of the compounds. The gas solids biosurfactant appeared to partially inhibit the degradation of the compounds, allowing approximately 50-75% degradation of the compounds present. The glucose and oil biosurfactant appeared to inhibit degradation of all compounds, with toluene remaining at its initial concentration after 26 days. At the biosurfactant concentrations used in this experiment, the gas centrate biosurfactant was relatively ineffective at increasing solubility, but it did not inhibit biological degradation. The biosurfactant produced from glucose and oil increased solubility the most, but also inhibited biological degradation the most.

The naphthalene present in the gas solids biosurfactant control mix was initially added at too low a concentration to be detected by GC analysis. Naphthalene was measured initially in the active gas solids biosurfactant microcosms but was not detected in the remaining microcosms sampled after this point. Results concerning the effects of the gas solids biosurfactant material on naphthalene degradation are

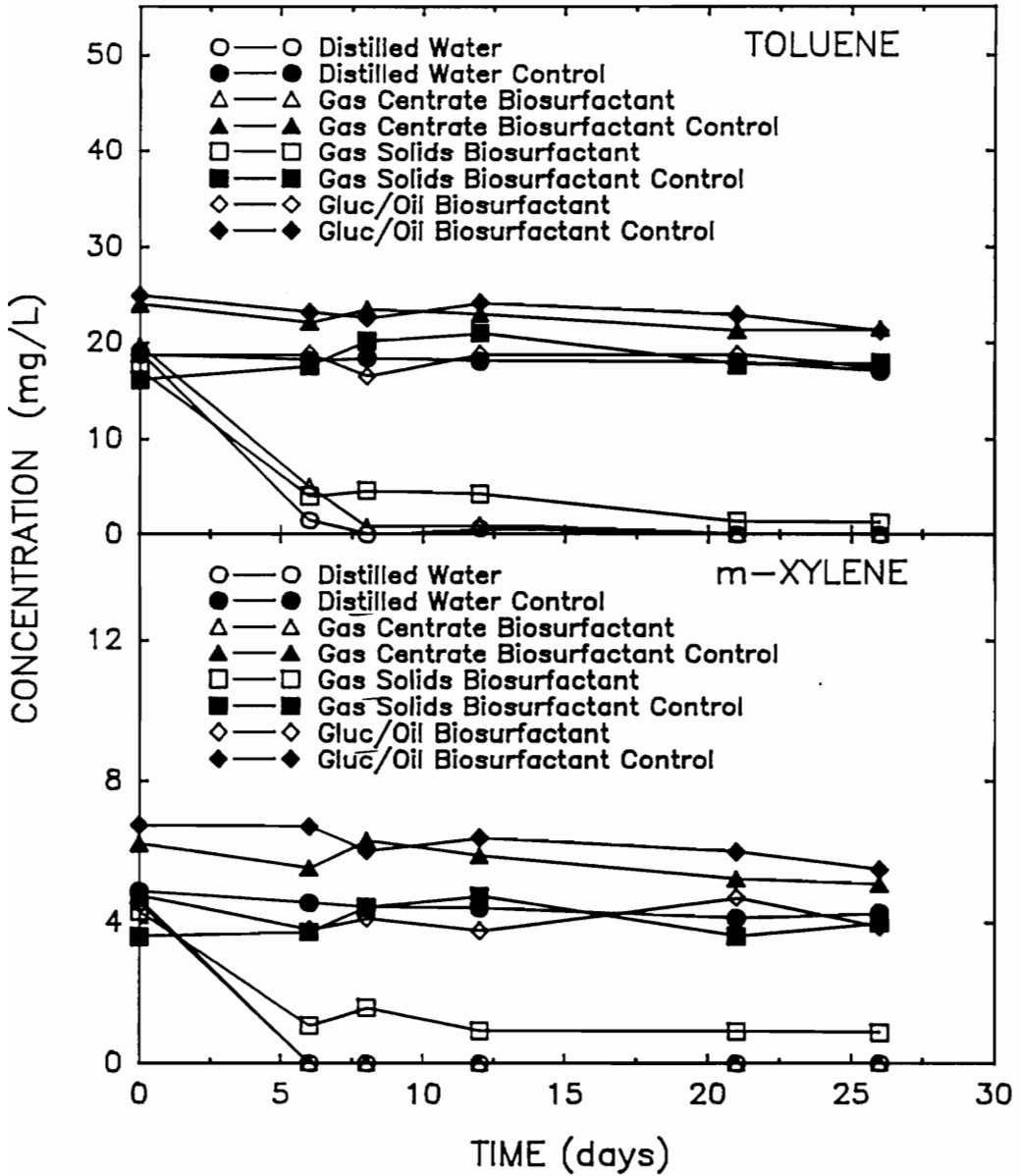


Figure 14. Biological degradation of toluene and m-xylene in the presence of three biosurfactant solutions at 300 mg/L TOC.

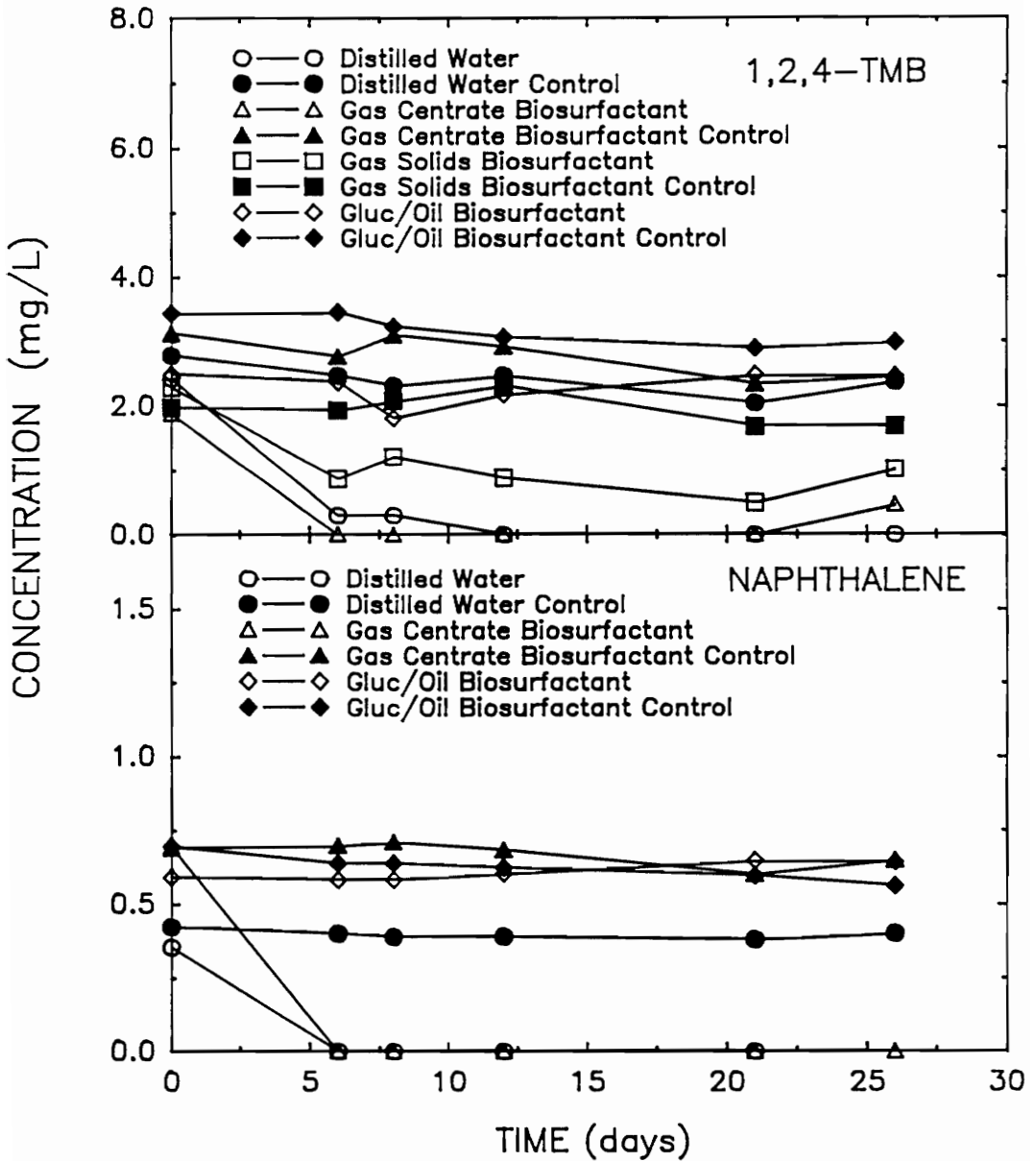


Figure 15. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of three biosurfactant solutions at 300 mg/L TOC.

therefore inconclusive.

A subsequent experiment conducted in the same manner with a different batch of the same biosurfactants gave slightly different results regarding the effects of the biosurfactants on degradation. The plots of concentration against time for the four compounds in the presence of the biosurfactants are shown in Figures 16 and 17. The gas centrate biosurfactant did not affect degradation, the gas solids biosurfactant reduced degradation, and the glucose and oil biosurfactant completely stopped degradation of toluene, naphthalene and trimethylbenzene, but allowed partial degradation of xylene.

The combined effects of solubility enhancement and inhibition of degradation are worth noting at this point. At the higher TOC levels and presumably higher biosurfactant levels used in this experiment, the gas centrate biosurfactant increased solubility substantially, but did not inhibit biological degradation. The other biosurfactants, while increasing solubility, did inhibit biological degradation.

#### Biodegradation in Column Studies

Biodegradation of the gasoline compounds in a flow through column system was difficult to measure. The cost of the columns constrained the initial study to three paired column sets. The three solutions tested and noted previously for mobilization of hydrocarbons were also passed through sand filled glass columns which were seeded initially with gasoline utilizing microorganisms. Degradation was determined by

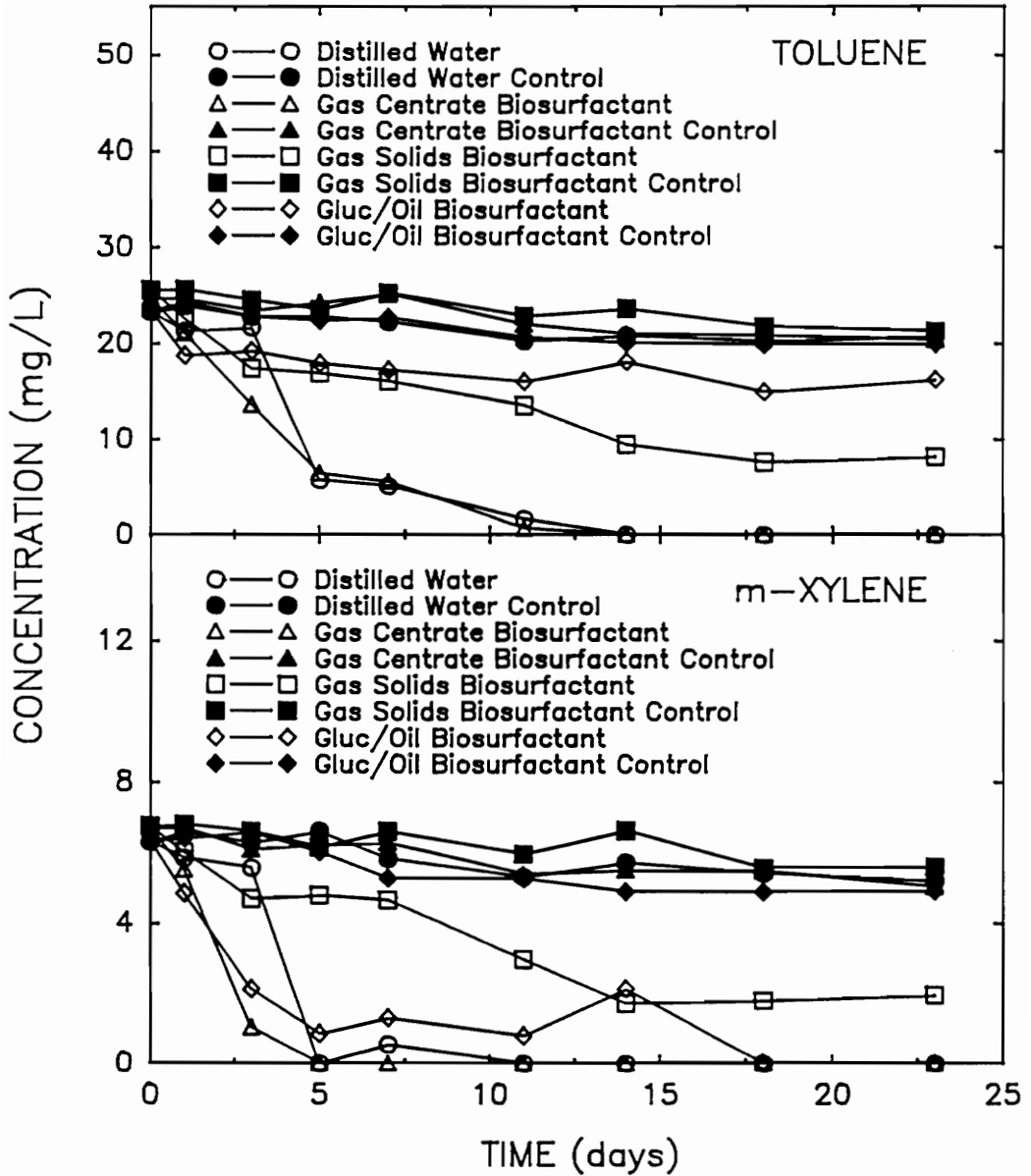


Figure 16. Biological degradation of toluene and m-xylene in the presence of three biosurfactant solutions at 600 mg/L TOC.

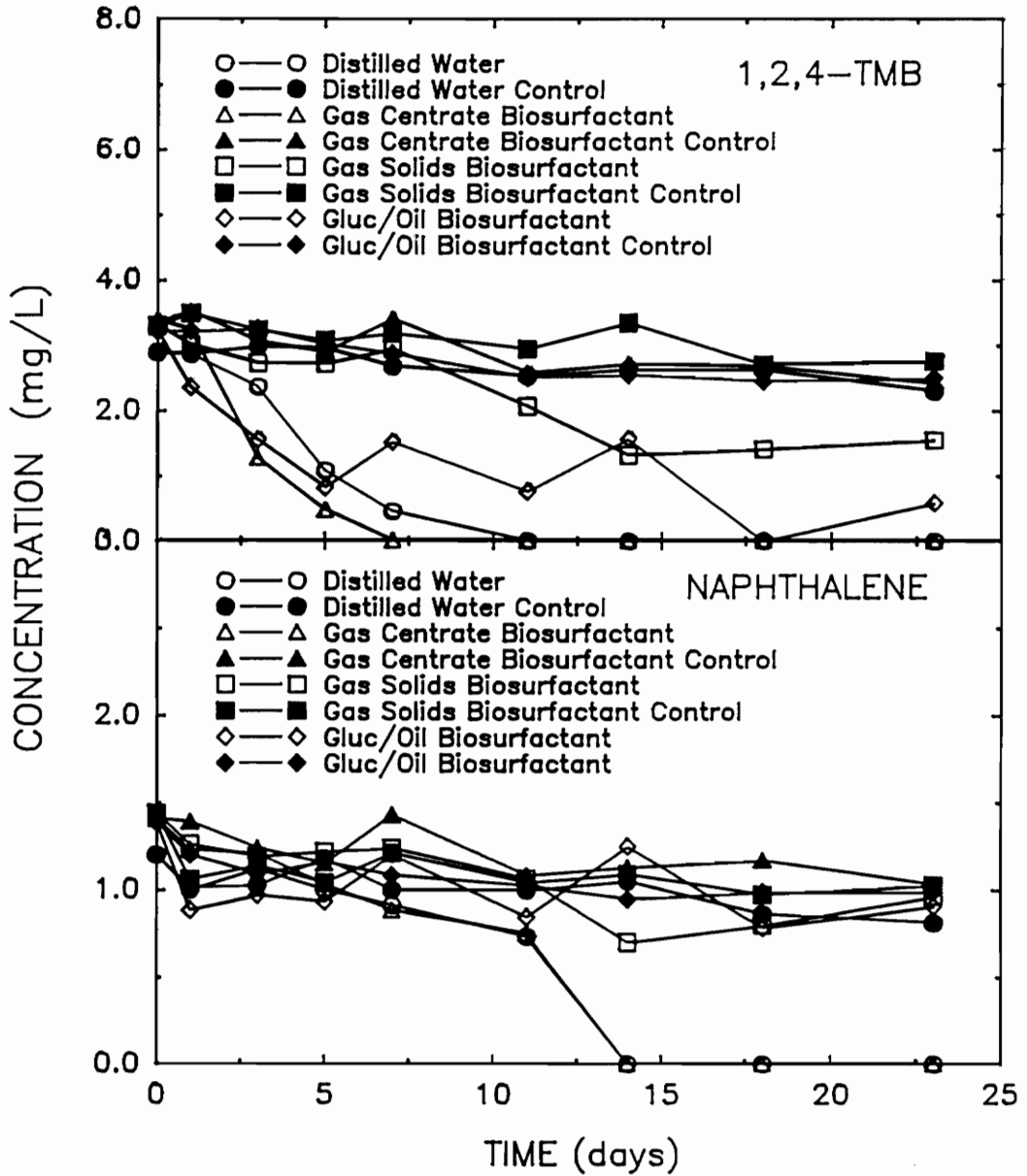


Figure 17. Biological degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of three biosurfactant solutions at 600 mg/L TOC.

comparing the levels of removal in biologically active and inactive (control) sand columns.

No control column was operated for the biosurfactant solution so degradation was difficult to observe. The levels of the compounds present after a one day retention in the column are shown on Figure 18. The one day retention is determined as the time a volume of liquid spends in the column before it is sampled. If the levels of the compounds present in the solution without a biosurfactant are compared with those in its sterile control counterpart, it is noted that toluene is the only compound which decreases in concentration relative to the control, and this occurs only after seven bed volumes have passed through the column.

The adsorption affects are more evident from the plots of the amount of compound removed through adsorption or degradation after a one day retention as a function of throughput volume, as shown on Figure 19. The amount of toluene removed in the sterile control and biosurfactant columns is decreasing as the amount removed in the active column without the biosurfactant is increasing due to degradation. The difference between the amount removed in the sterile control column and the biologically active column without a biosurfactant is approximately equal to the amount of toluene degraded. This was shown in the plot of concentration after a one day retention. The influent toluene levels to the columns varied slightly between the sterile control solution and the solution with no biosurfactant present, therefore the difference between the two plots is an approximation of the degradation.



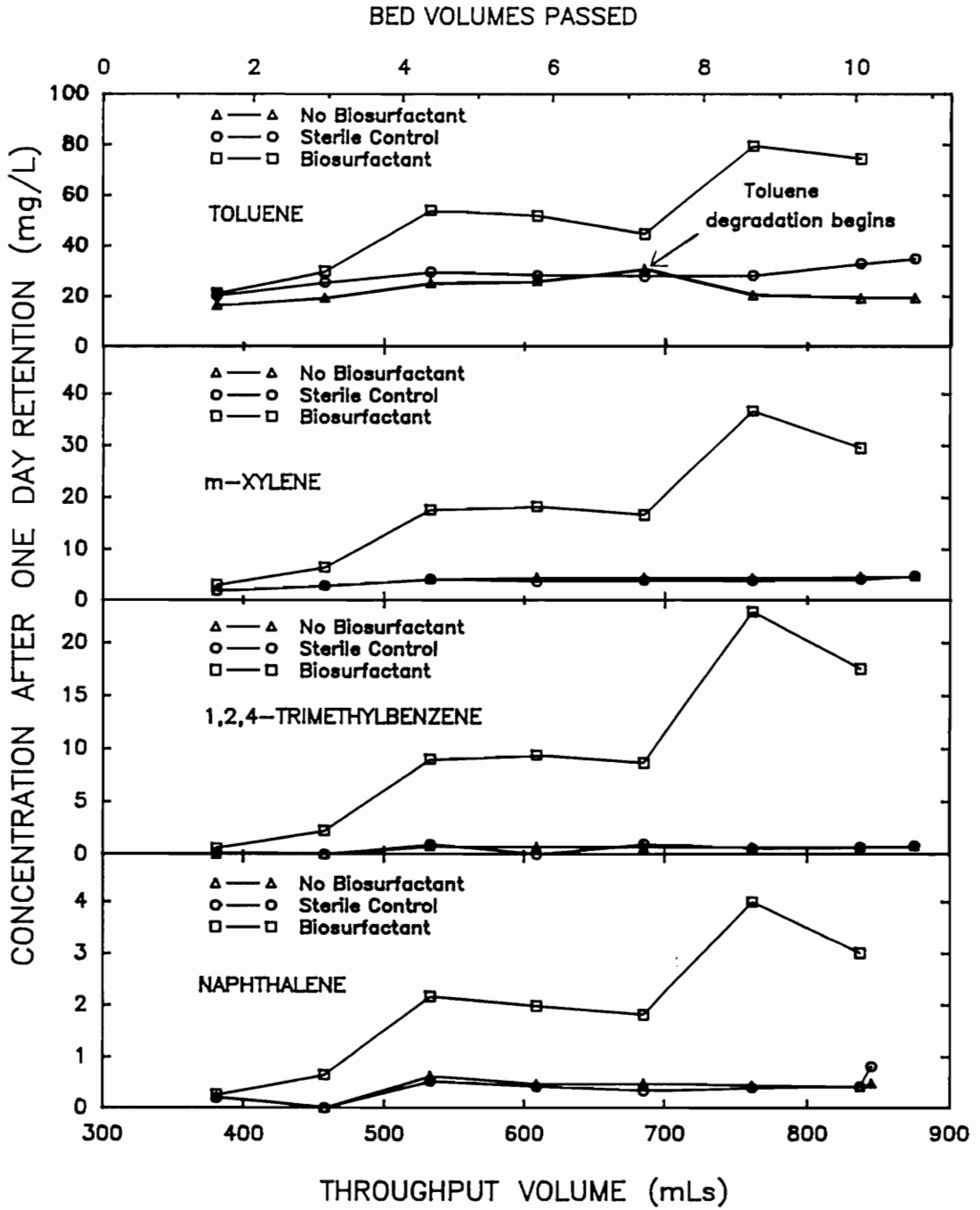


Figure 18. Concentration in column at sample point C after a one day retention.

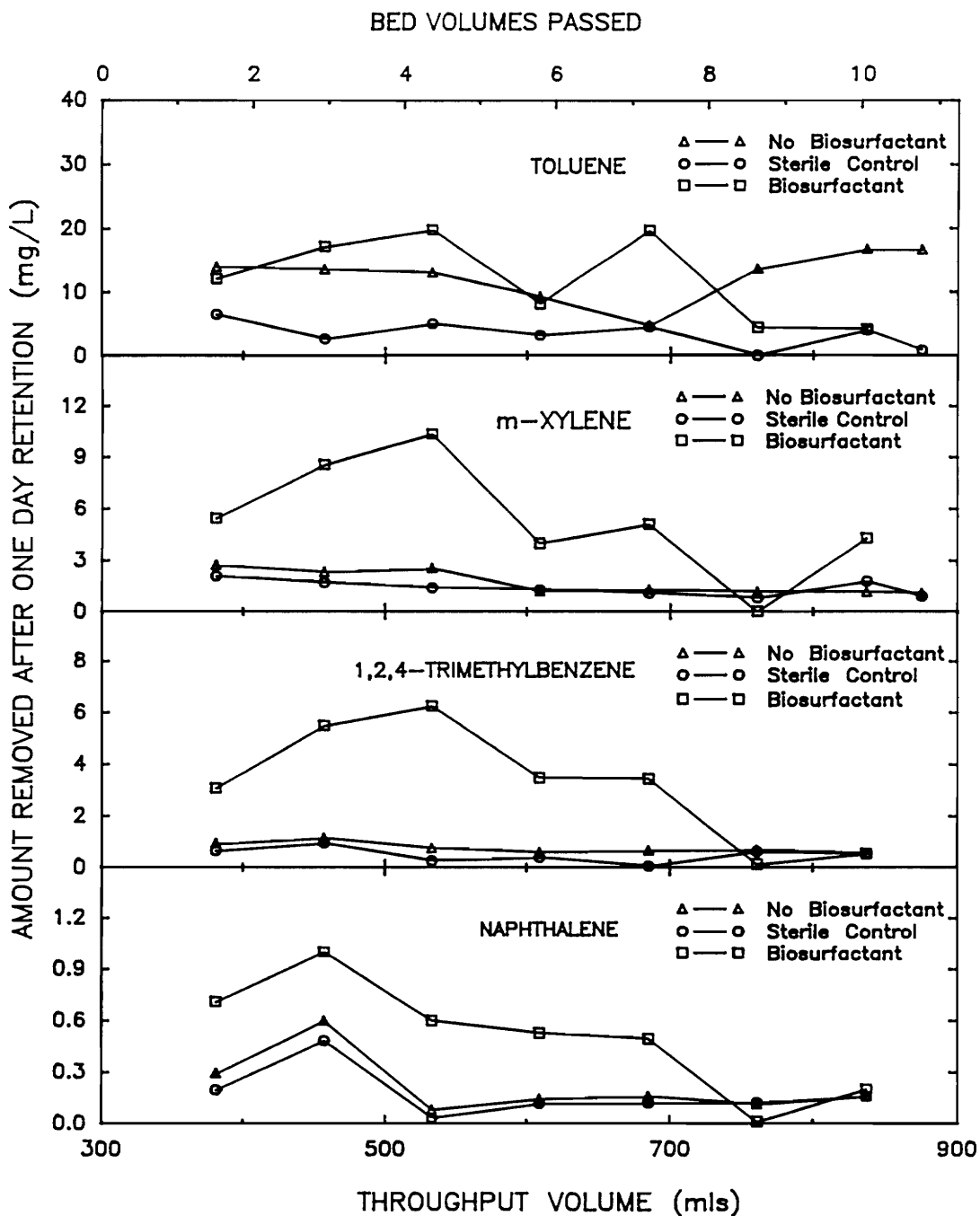


Figure 19. Amount removed during a one day retention in the column as determined by the change in compound concentration between sample points B and C.

Figure 20 shows the amount of compound removed after a two day retention in the column. Adsorption is apparently still taking place at the two day sample port, so removal is still high for the biosurfactant and low for the sterile control. Removal for the active column without a biosurfactant is between these two levels, as both degradation and adsorption are still taking place.

Degradation of monitored compounds other than toluene could not be detected in any of the columns. As in the batch tests, this may be due to other gasoline substrates being present which were degraded preferentially. In addition, oxygen levels in the effluent from the active columns were in the 2-3 mg/L range, which can be a limiting factor in fixed film systems. Oxygen limitations could not be overcome since the influent to the biologically active column was at an oxygen saturation of 9 mg/L with added H<sub>2</sub>O<sub>2</sub>. This is a problem in both the lab and as applied in field conditions.

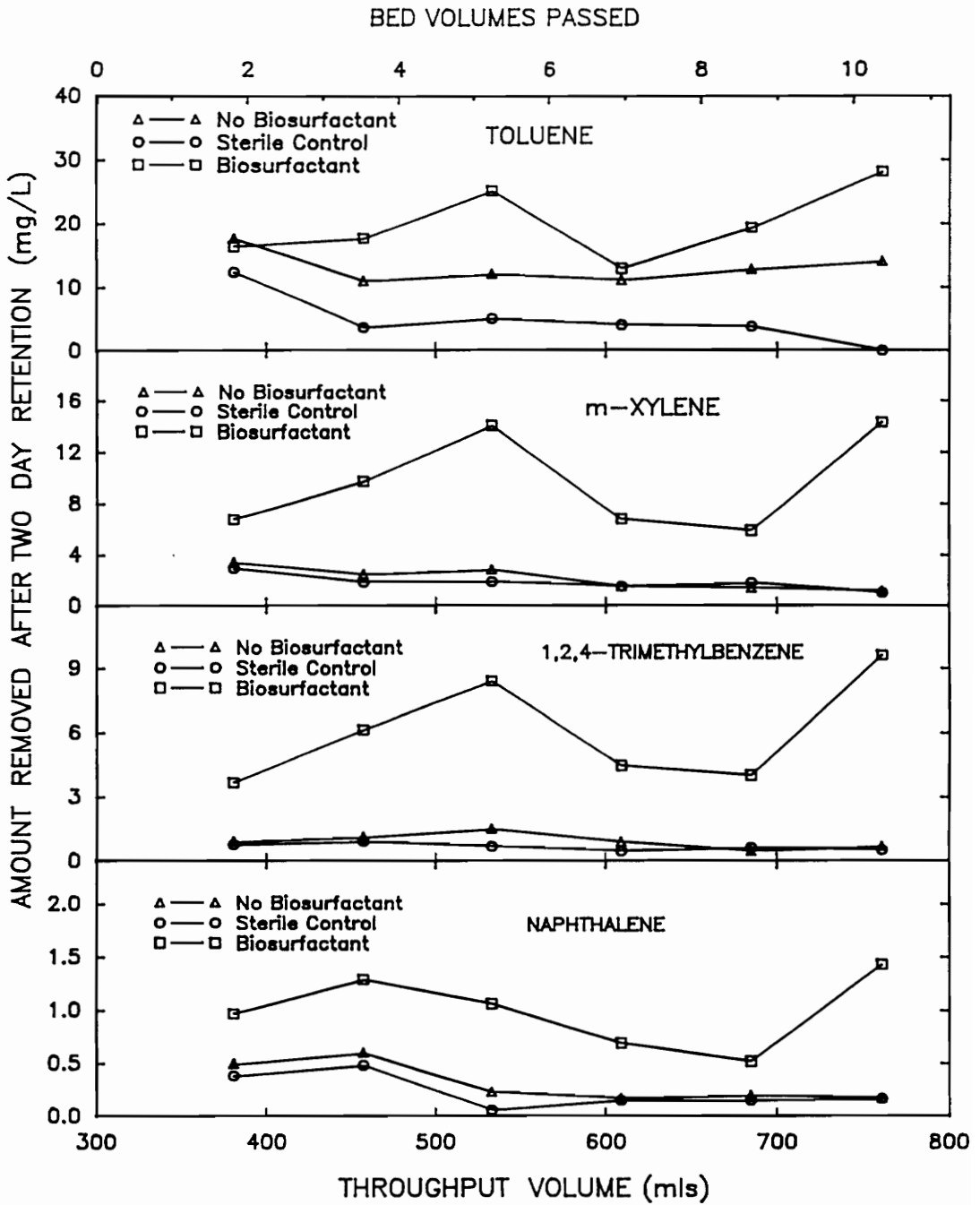


Figure 20. Amount removed during a two day retention in the column as determined by the change in compound concentration between sample points B and D.

## DISCUSSION

### BIOSURFACTANT PRODUCTION

The potential for the use of different types of biosurfactants in groundwater remediation was investigated in this research. The use of biosurfactants as an aid to enhance solubility and therefore removal of hydrocarbons from soils was investigated in both static batch systems and flow through column systems. The use of biosurfactants as an aid to biodegradation was investigated primarily in batch systems.

Two groups of substrates were used to produce the biosurfactants in this study. By using different substrates, it was assumed that the type of biosurfactant produced varied. During production of the biosurfactants, surface tension reduction proceeded at a steady, generally linear rate over a two week period. The steady rate suggests that the production of biosurfactants was associated with growth, and not cell lysis. The two week time period needed for surface tension to stabilize is longer than as reported by some investigators. If the biosurfactant production occurred after the main growth period, it could suggest that the biosurfactant production was more the result of cell lysis (Cooper and Goldenberg, 1987).

Biosurfactant compounds produced from cell lysis would be predominantly from the cell membrane, which is composed of various, somewhat polar lipids (Slydatk and Wagner, 1987). Biosurfactants produced for substrate transfer could include various phospholipids and

glycolipids, such as the rhamnolipid produced by *P. aeruginosa* (Edwards and Hayashi, 1965). These two methods of biosurfactant production are not mutually exclusive. Biosurfactant compounds excreted from the cell membrane to help solubilize and transfer the substrate through the membrane would be of a similar structure as those lipids making up the membrane. Other surfactant compounds present also could include fatty acids which might accumulate during gasoline degradation, or humic acid type degradation byproducts.

The characterization of the different biosurfactant solutions was completed only to show that the substrates themselves were not present and acting as a second phase organic for the gasoline compounds to partition into. The confirmation of the presence of surfactants was based on the reduction of surface tension in the reactor solution. Using gas chromatography retention times, the times for the biosurfactant organics did not match the times for the substrates.

#### SOLUBILITY ENHANCEMENT

In both the batch and column systems the biosurfactants increased the solubility of the monitored compounds when contacted with gasoline. As with any surfactant compound, this increase in solubility was expected. In addition, as with synthetic surfactants, the biosurfactants increased the solubility more for compounds of low solubility, such as naphthalene, than for compounds of high solubility, such as toluene. The largest increase on a percentage basis was for

1,2,4-trimethylbenzene, followed by naphthalene, m-xylene and toluene. It appeared that the presence of the methyl substituent groups greatly facilitated the increase in solubility of the trimethylbenzene in the presence of the biosurfactant. The trend of the increases in solubility correlates with the increases in octanol/water partitioning coefficients for the compounds.

When contacted with gasoline, at 300 mg/L TOC levels, the glucose and oil biosurfactant increased the solubility of the monitored compounds the most. The other biosurfactant mixes produced by the gasoline utilizing microorganisms usually were not as effective at solubilizing the compounds. The biosurfactant referred to as the gas biosurfactant, was extracted from the total liquid of the reactor, and possessed good solubility enhancement ability. Splitting the reactor liquid into solids and centrate fractions provided the two other biosurfactant mixes from the gasoline reactor. At low TOC levels (300 mg/L), it was found that the gas solids biosurfactant material was better at solubilization of gasoline than the gas centrate biosurfactant. At higher TOC levels, and a five week growth period in the batch reactor, the gas centrate biosurfactant and the gas solids biosurfactant had approximately equal ability for solubilizing the gasoline compounds. It is unclear exactly why this occurred, but it may be due to different growth patterns resulting from the older cell age in the batch reactor of gasoline utilizing microorganisms, as well as an increased acclimation to the larger, less volatile compounds.

The higher TOC value in the supernatant was achieved through a

longer growth period which gave a longer period for the release of biosurfactant compounds from the cells. The longer growth period and larger reactor size required an increase in the air supply to the reactor. This may have resulted in an increased volatilization rate and a larger proportion of non-volatile, relatively non-polar compounds remaining in the reactor. Microorganisms growing on non-polar compounds of low solubility are more likely to produce biosurfactants and non-polar cellular material. The cellular or biomass material, which likely makes up the solids for the gas solids biosurfactant, may have acted more like the biosurfactants produced by the glucose and vegetable oil utilizing microorganisms, and therefore not be as effective against a strong non-polar organic phase like the mineral oil. The non-polar biosurfactant compounds would partition strongly into the non-polar mineral oil. A more polar biosurfactant would remain present dissolved in the water and be available to solubilize compounds from the organic phase.

When the biosurfactant solutions were contacted with mineral oil spiked with the four monitored compounds, the gas solids biosurfactant (at low TOC levels) increased the solubility of the compounds the most. The biosurfactant produced from growth on glucose and oil was relatively ineffective against the mineral oil. This was shown in Figures 6 & 7. The effectiveness of different types of surfactants at increasing solubility or emulsifying a second organic phase is usually attributed to the hydrophile-lipophile balance (HLB) value of the surfactant. The HLB value indicates the degree of attraction the two ends of the



surfactant molecule have for the polar solvent (water) and the nonpolar solvent (gasoline or mineral oil). If the lipophilic end is strongly nonpolar, more so than the hydrophilic end is polar, the surfactant will have a limited solubility in water, and a strong attraction for the non-polar solvent. If the hydrophilic end has a polar attraction overriding the lipophilic end group, the surfactant has a strong polar attraction, and is more water soluble.

The biosurfactant produced from glucose and oil was found to have a limited solubility in water. Excess biosurfactant was removed as a clear, viscous, immiscible liquid. Cooper and Paddock (1984) also found that the biosurfactant produced by *T. bombicola* when grown on glucose and oil was insoluble at high concentrations. Boulton and Rutledge (1987) noted that the substrate often dictated the characteristics of the biosurfactants produced. It would appear that the biosurfactant produced from glucose and oil would have a strong non-polar tendency. The biosurfactants produced from gasoline may tend to be more polar due to the presence of relatively polar or soluble compounds present in gasoline. The gasoline produced solids biosurfactant, most likely composed of cellular lipids, may still have a limited aqueous solubility. This was evident in the slow rate at which the gas solids biosurfactant was redissolved in water for use in this study. The gas concentrate biosurfactant extracted from the supernatant could be the most polar of the biosurfactant compounds obtained. It was easily dissolved at a higher concentration after the extraction.

The differences in the degree of polarity of the biosurfactants,

typically characterized in surfactants as the HLB value, could account for the differences in effectiveness of the biosurfactants in the presence of different organic phases. In comparing mineral oil to gasoline, mineral oil contains compounds of lower polarity than gasoline. The biosurfactant produced from glucose and mineral oil may have had a greater affinity for the mineral oil, and partitioned into it, leaving only a small amount in aqueous solution to solubilize the spiked compounds. When contacted with gasoline, the glucose and oil biosurfactant would partition between the relatively polar gasoline and water, and would still provide a strong non-polar micelle present in solution to solubilize compounds. The gas biosurfactants, being more polar, would also partition into the gasoline, but some would still remain in solution to solubilize compounds. The interactions occurring between the biosurfactants and the second phase organic liquid are most likely more complex than as described, but the basic concepts still apply. The differences in the ability of the biosurfactants to solubilize compounds is consistent with the idea that organics will try to partition into a similar organic phase or solvent.

If a biosurfactant partitions strongly into a second phase organic, it is not necessarily rendered ineffective. The biosurfactant would not increase the solubility of the organics until there was enough to saturate the second phase liquid. It would however, lower the surface tension of the organic liquid, or the interfacial tension between the organic and water, making it easier to remove in the subsurface through hydraulic pumping. The degree to which the

biosurfactants partitioned into the separate organic phase was not investigated in this research.

Enhanced solubility or mobilization of gasoline in flow through columns was tested with the glucose and oil biosurfactant. The levels of increased solubility in the flow through system were at similar levels as the batch system. Enhanced mobilization and flow of second phase gasoline could also increase the surface area of the gasoline, multiplying the increase caused by the biosurfactant. This effect was not observed however, as there may be a maximum solubility level that was reached, or the gasoline and biosurfactant solutions had not reached an equilibrium level of solubility.

Adsorption of the biosurfactant to the sand in the column was not extremely large, but large enough to cause a lag time in the breakthrough of increasing solubility. As shown in Figure 17, 3.13 bed volumes passed through the column before levels of solubilized gasoline increased and the biosurfactant passed through. It was assumed that the increase in the concentration of the gasoline compounds in the effluent coincided with the breakthrough of the biosurfactant. This gave a retardation factor 3.13 based on the pore volume passed. A finer grained porous media or one with clay present would most likely result in a higher retardation factor, coinciding with an increase in the surface area.

## BIODEGRADATION

Biodegradation of the four monitored compounds was observed by sacrificial sampling of microcosms containing the biosurfactants with either whole solubilized gasoline or spiked with the four monitored compounds. Toluene biodegradation was least affected by the biosurfactants. Degradation of the other compounds was inhibited more than toluene. Toluene was often degraded in the presence of the biosurfactants, while the other compounds were not. Toluene has the highest aqueous solubility, and the lowest octanol-water partitioning coefficient. Because of this, it would be the compound most likely to remain in solution, unassociated with biosurfactants or other organics present. The other compounds are progressively less soluble and have higher partitioning coefficients going from m-xylene to naphthalene, and 1,2,4-trimethylbenzene. Their tendency to partition into or remain bound to organics or biosurfactants is therefore also progressively greater from m-xylene to 1,2,4-trimethylbenzene.

Degradation of the four compounds in the presence of whole solubilized gasoline was at times much slower than the degradation when only the four compounds were present. This may have been due to the presence of additional substrates from the whole solubilized gasoline. The presence of numerous short chain alkanes, considered the most easily degraded gasoline compounds, may have caused a lag phase in the degradation of toluene and the other compounds by being degraded preferentially. Later microcosm studies were completed with the addition of only the four monitored compounds.

While toluene was the most degradable compound when contacted

with the biosurfactants, the different biosurfactants inhibited degradation of the three other compounds differently. Initial studies showed that the gasoline-produced biosurfactant caused the least interference in the degradation of all compounds, while the glucose and oil produced biosurfactant caused the most. The gasoline produced biosurfactant gave different results in different microcosm studies, with the compounds sometimes degrading quickly and completely, and sometimes degrading slowly or not at all. Initially, the gasoline produced biosurfactant was extracted from the whole broth/mixed liquor from the reactor. In the microcosm studies shown in Figures 14 through 17, the gasoline-produced biosurfactant was extracted differently. The centrate and the solids material were separated and then extracted with the solvent mix. These two biosurfactants were tested and it was determined that the solids material caused the greatest interference with degradation. The biosurfactant from the centrate extraction did not appear to inhibit degradation. It appears that in previous studies, different results were obtained depending on the ratio of the centrate and solids fractions.

The solids or cellular material, possibly polar lipids, usually increased the solubility of the compounds more so than the gas centrate biosurfactant, but once the compounds were attached or bound to the gas solids biosurfactant, they were much less available for degradation. The supernatant extracted gas centrate biosurfactant, at low TOC levels, was generally the weakest biosurfactant based on its ability to increase the solubility of the compounds, but provided the least interference

with degradation. In the microcosm study shown in Figures 16 and 17, the four compounds present in the microcosms with the gasoline centrate biosurfactant were fully degraded at the same rate as those microcosms without any biosurfactant being present. When the gas solids biosurfactant was present, degradation of the four compound was completed to only 50-75% of the total present. It is likely that only 25-50% of the compounds were bound with the biosurfactant and unavailable for degradation, the remainder were still free in solution and available for degradation.

Biological degradation in the column system was difficult to measure. In order to determine what was degraded in the presence of the biosurfactant, a sterile control with the biosurfactant was required. The only sterile control that was run was for the column without the biosurfactant present. It was observed that toluene was the only monitored compound being degraded. This was most likely due to, as in the batch systems, the presence of other substrates in the gasoline. The columns were useful in demonstrating the physical and chemical interactions that might occur in a subsurface system, but the biological degradation was not well defined.

## SUMMARY AND CONCLUSIONS

The link between a biosurfactant's ability to increase solubility and subsequent degradation is likely due to the chemical characteristics of the biosurfactants. These characteristics might include the relative polarity of the end groups, the nature of the substrate utilized to produce the biosurfactants, and the overall solubilization strength of the biosurfactants. The results of the microcosm studies indicate that some types of biosurfactants will increase the solubility of hydrophobic compounds, but can inhibit degradation of these same compounds. Other biosurfactants increased solubility to a lower degree, but will not interfere with degradation.

All the biosurfactants tested in this research increased the solubility of gasoline compounds into aqueous solutions. Proportionally the increases in solubility were greatest for compounds with the lowest solubility. This solubilization ability is similar to that of synthetic surfactants, and therefore the biosurfactants could typically be used in conditions where synthetic surfactants might be used.

The biological degradation of gasoline compounds in contact with the biosurfactants indicated, that in relation to enhancing degradation of generally insoluble substrates, they may be substrate and/or microorganism specific. Biosurfactants produced during growth on gasoline increased the solubility of the compounds, and did not greatly inhibit the degradation of gasoline. Biosurfactants produced from

growth on an insoluble substrate other than gasoline, did increase the solubility of the compounds, but also inhibited degradation of the compounds once they were associated with the biosurfactant. Whether the biosurfactants are substrate specific or microbe specific was not determined from this investigation.

The application of biosurfactants to groundwater systems as an aid to in situ biological degradation or enhanced hydraulic removal would appear to be limited by the degree of adsorption to the soil. Adsorption of the biosurfactant would greatly increase in a soil with a large amount of silt and clay, corresponding to an increase in the surface area of the soil. The biological degradation of the biosurfactant could also occur, creating an additional oxygen demand in a system already low in oxygen. However, if biosurfactants were identified as being produced in situ during biological remediation of groundwater contaminated with hydrocarbons, if properly used, their presence could be beneficial to enhancing hydrocarbon removal.

Based on data from the batch and column experiments, the specific conclusions of this investigation are:

- 1) The biosurfactants produced during microbial degradation of a variety of organics could increase the solubility of the hydrophobic compounds into an aqueous solution in a manner similar to synthetic surfactants.
- 2) The activity of the biosurfactants appeared to be either substrate



or microbe specific, with some inhibiting degradation while others did not.

- 3) Based on results from the column system, it appears that adsorption of the biosurfactant to the soil, and low oxygen levels would be limitations for the application of biosurfactants for soil and groundwater remediation.

#### LITERATURE CITED

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## APPENDIX A

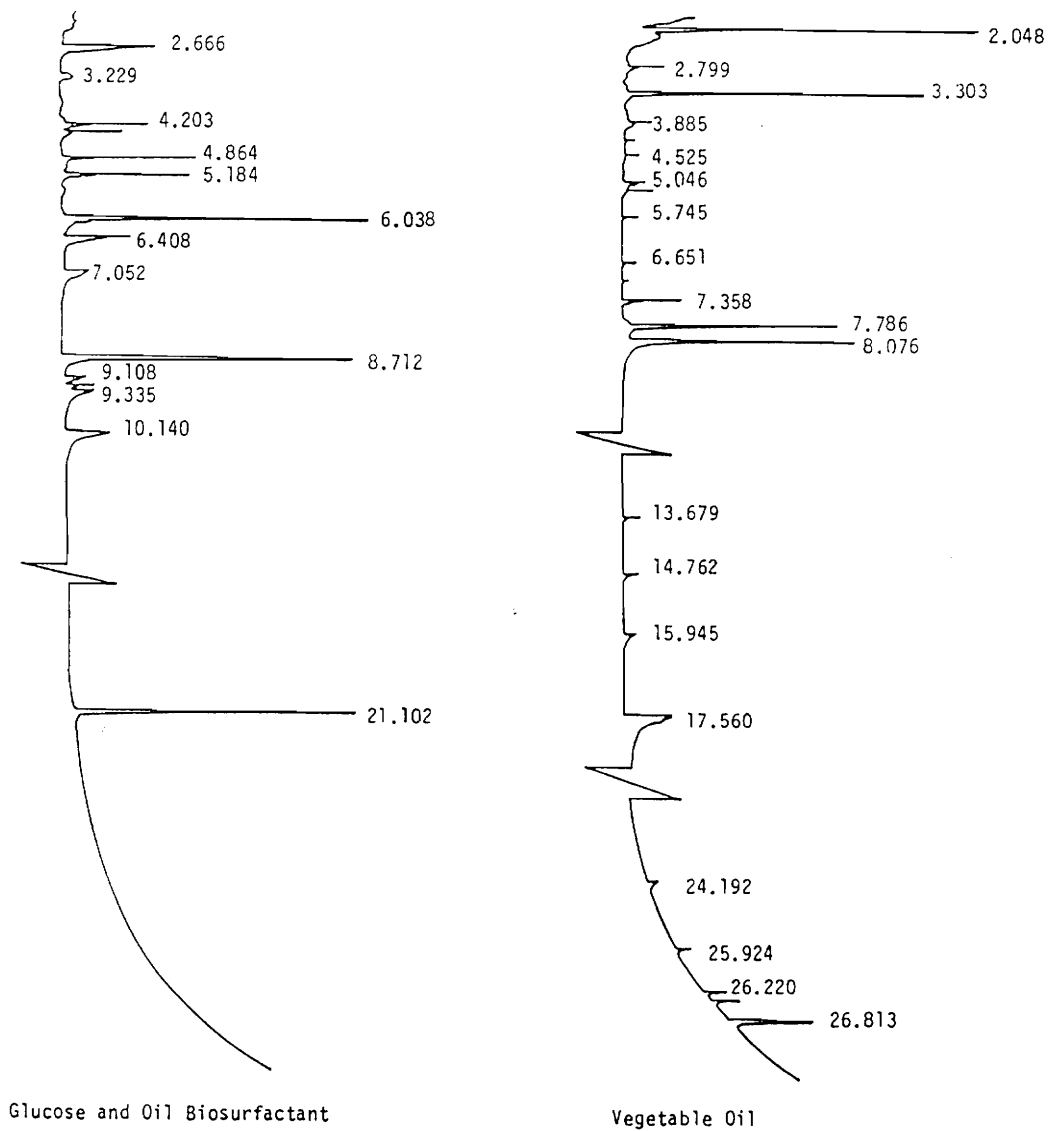


Figure A-1. Comparison of the chromatograms produced by vegetable oil and the glucose and oil biosurfactant.

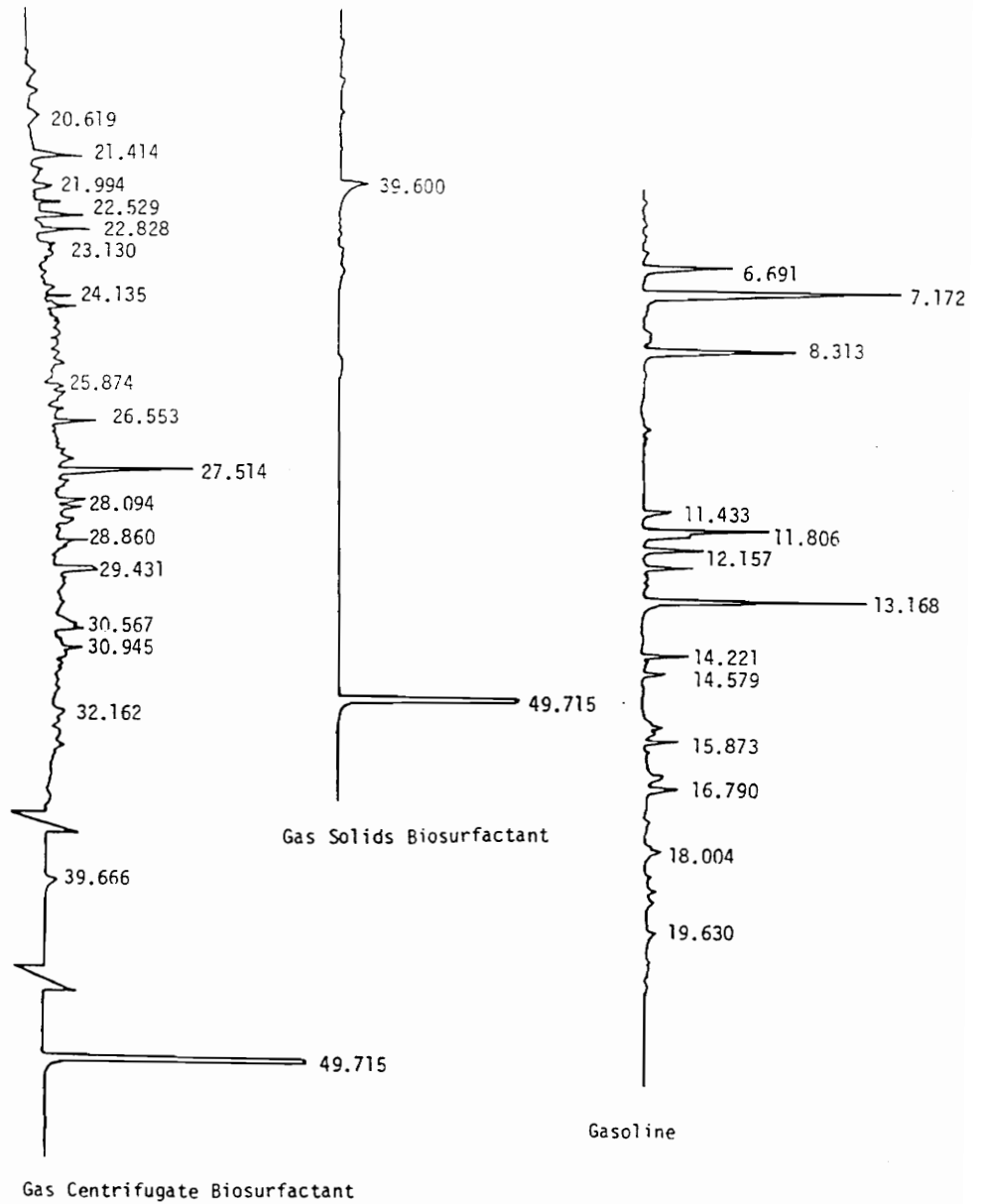


Figure A-2. Comparison of the chromatograms produced by gasoline and the gas centrifugate and solids biosurfactants.



## Appendix B

Four biosurfactant solutions were compared initially for their ability to solubilize the gasoline compounds. These solutions with the solubilized gasoline were then used in a batch biodegradation study. The four solutions along with distilled water were seeded with gasoline utilizing microorganisms and degradation was observed as described in the methods section previously. The concentrations plotted against time for the different microcosms are shown on Figures B1 and B2. Controls were not used on this study and therefore the results are only tentative. This test indicated that once the gasoline produced biosurfactant solubilized the compounds, they were not bound up enough to interfere with subsequent degradation. This did not appear to be the case with the other biosurfactants produced. The other biosurfactants increased the solubility of the other compounds to a larger degree, but the solubilized compounds were not completely degraded.

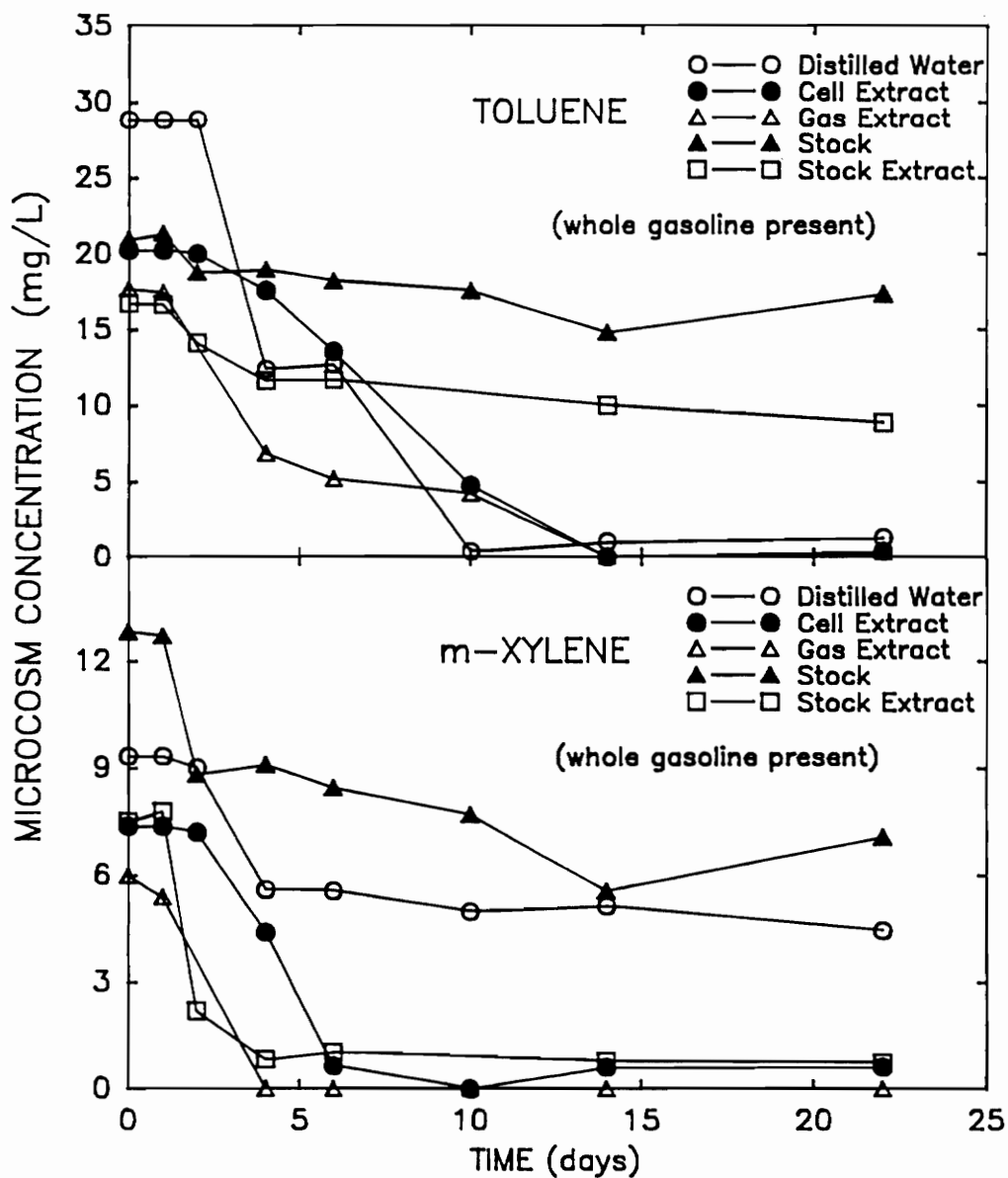


Figure B1. Biological degradation of toluene and m-xylene in the presence of various biosurfactants.

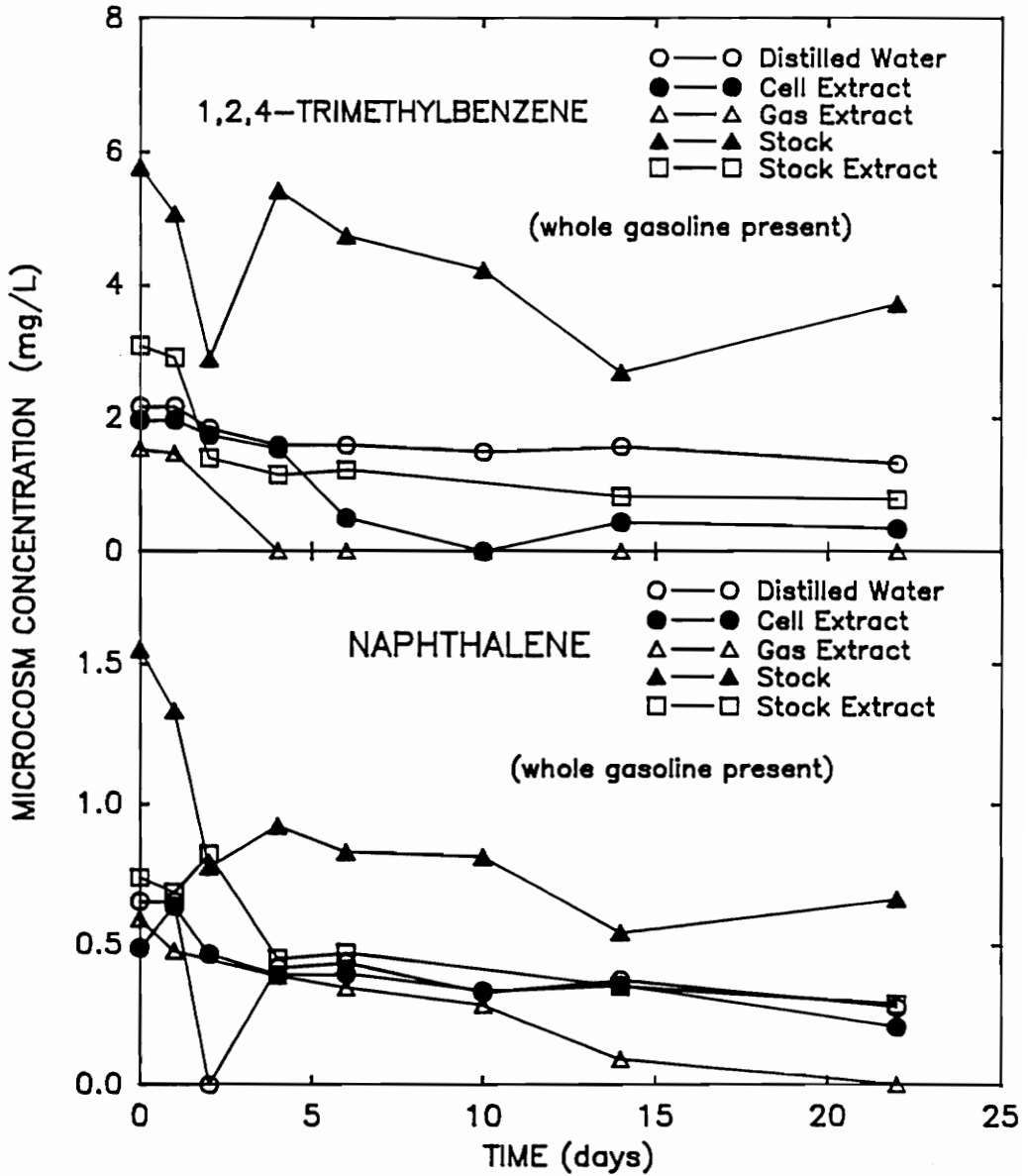


Figure B2. Biological degradation of toluene and m-xylene in the presence of various biosurfactants.

## Appendix C

A second microcosm study was completed using the same biosurfactant mixes noted previously but also using a synthetic surfactant, N-95, at two different concentrations. In this study, toluene was generally degraded in the presence of most of the biosurfactants. The other compounds with lower solubility were apparently not degraded in the presence of a biological or synthetic surfactant, except for the one produced by the gasoline utilizing microorganisms.

The changes of the microcosm concentration levels with time are shown on Figures C1 and C2. Only one control was used in this experiment, and it was observed that the extraction efficiency for the more insoluble compounds decreased with time. This may be due to binding of the gasoline compounds to the biosurfactants or other organic matter. A change in the extraction procedure was used, increasing the ratio of methylene chloride to water sample to a 2:1 ratio on the 18th day, and a 3:1 ratio on the 20th day sample. Subsequent tests showed that increasing the ratio beyond that level did not improve recovery, and only increased the error in the analysis. It is unclear how this extraction problem affected the previous tests. The recorded concentrations of the compounds decreased slowly, and with the 3:1 extraction ratio, the initial compound levels were obtained in some biosurfactant mixes but not in the others.

It should be that the difference between the initial and final

concentration is the amount degraded. If so, this study again indicates that the gasoline biosurfactant did not greatly inhibit the degradation of the four compounds monitored. The other surfactants apparently did not inhibit the degradation of toluene, but did inhibit the degradation of the other compounds. The other compounds have lower aqueous solubility, and therefore would partition more into other organics present, such as the surfactants. Whatever the reason, m-xylene, 1,2,4-trimethylbenzene and naphthalene remained at their initial concentrations when in contact with all the solutions except the distilled water and the gasoline biosurfactant.

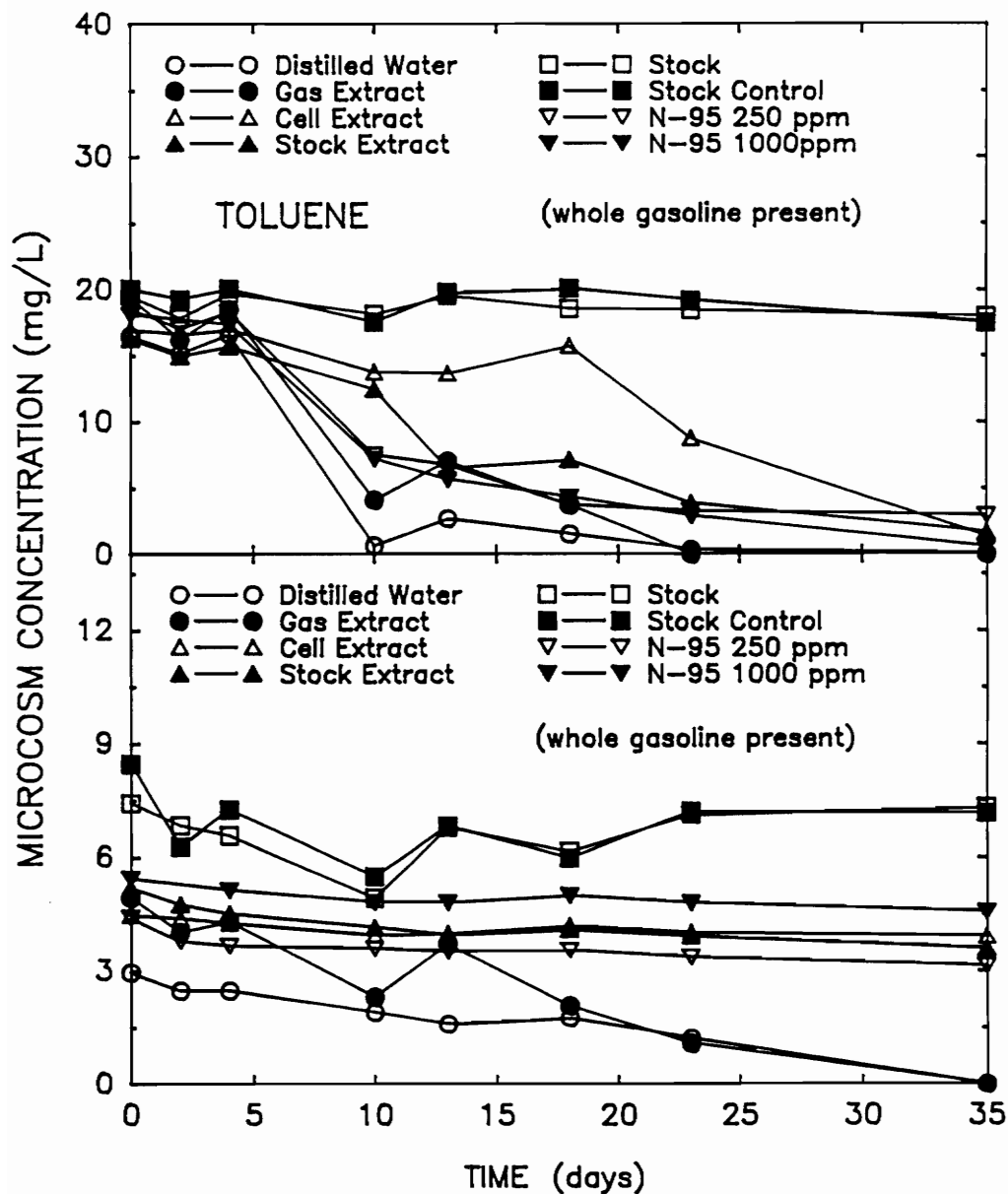


Figure C1. Biological degradation of toluene and m-xylene in the presence of various biological and synthetic surfactants.

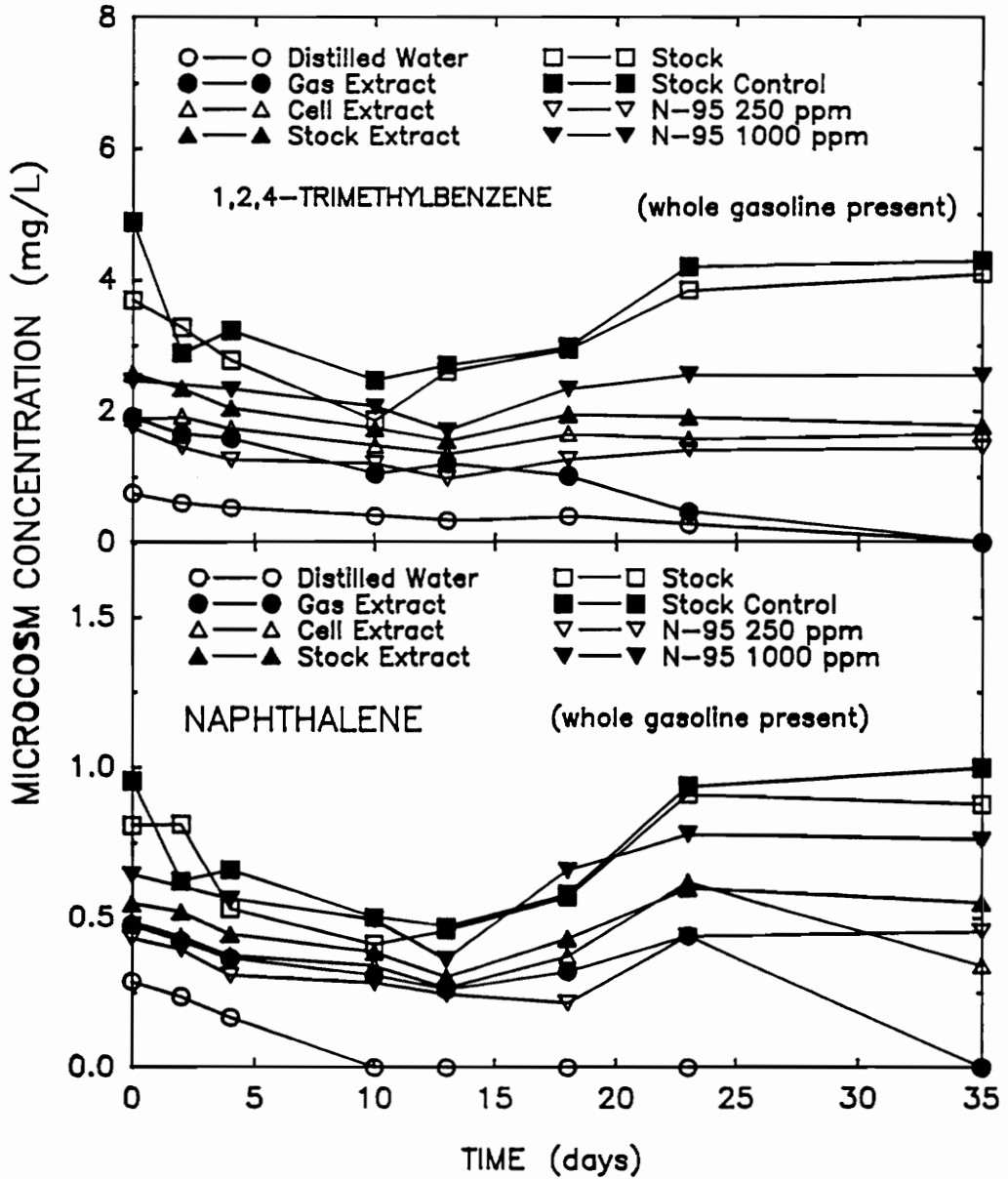


Figure C2. Biological Degradation of 1,2,4-trimethylbenzene and naphthalene in the presence of various biological and synthetic surfactants.

## VITA

David Michael Falatko was born on January 28, 1961, in Washington, D.C. He lived in Bethesda, Maryland until he graduated from high school there in 1979. He was employed in a variety of areas before obtaining a B.S. degree in geology from the University of Maryland at College Park in 1985. He worked as a hydrogeologist in Pennsylvania and Maryland prior to attending graduate school at Virginia Polytechnic Institute and State University. He received his M.S. degree in environmental engineering in January, 1991.

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