To the Graduate Council:

I am submitting herewith a thesis written by Shannon R. Bowden entitled "An Investigation of Bioremediation of Carbon Tetrachloride and Toluene and the Use of a Surfactant to Enhance this Process." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

T W Schultz

Terry W. Schultz, Major Professor

We have read this thesis
and recommend its acceptance:

Anthony V. Palumbo

Thomas Anderson

Walter R. Farmer

Accepted for the Council:

Carolyn Winkler

Associate Vice Chancellor and
Dean of The Graduate School

# AN INVESTIGATION OF BIOREMEDIATION OF CARBON TETRACHLORIDE AND TOLUENE AND THE USE OF A SURFACTANT TO ENHANCE THIS PROCESS

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Shannon Rene' Bowden
May 1996

# DEDICATION

This thesis is dedicated to my husband

Mark Alan Bowden

and

My Parents

Alan Jack Branch and Jo Anna Branch

Who have supported and encouraged my educational pursuits.

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Many people contributed to this endeavor and supported and encouraged me at each step of the way. I am particularly grateful to my thesis committee, Janet Strong-Gunderson, Anthony Palumbo, Terry Schultz, and Walter Farkas. These individuals, spent numerous hours planning, developing and assisting in this research project, and reviewing many copies of the manuscript.

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# ABSTRACT

This study investigated the potential for using microorganisms to degrade carbon tetrachloride and toluene in an aqueous environment. An extensive literature search yielded numerous reports of similar studies in which the investigators met with limited success using various test parameters. These previous results, such as specific nutrient media and oxygen content, were incorporated into this study to further enhance the potential for identifying microorganisms capable of biodegrading carbon tetrachloride and toluene. Results indicate that four out of seven of the bacterial consortia tested were capable of carbon tetrachloride and toluene degradation. In addition, a non-ionic surfactant, Tween-80, was tested to determined if biodegradation would be enhanced by increasing the contaminant solubility and bioavailability. These results show that Tween-80 did increase contaminant partitioning into the aqueous phase, thus potentially increasing the contaminant bioavailability. However, at the concentration tested, there was no measured increase in biodegradation due to the application of Tween-80.

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# PART I

# LOW-CONCENTRATION SURFACTANT APPLICATIONS ENHANCE THE SOLUBILITY AND POTENTIAL BIOAVAILABILITY OF CARBON TETRACHLORIDE AND TOLUENE: TWO VOLATILE ORGANIC COMPOUNDS

## ABSTRACT

This investigation determined the effects of low-concentration applications of Tween-80, a synthetic non-ionic surfactant, on the aqueous-gaseous partitioning of two volatile organic compounds, carbon tetrachloride (CT) and toluene (Tol). The relative concentration of the two contaminants was measured in the gaseous phase (i.e., headspace) by gas chromatography. The partitioning of both CT and Tol from the headspace to the aqueous phase was enhanced by the addition of Tween-80 at concentrations equal to, below, and slightly above the critical micelle concentration (CMC). The partitioning of CT was enhanced the most by addition of Tween-80 at a concentration slightly above the CMC. The partitioning of Tol was most enhanced at the Tween-80 CMC. These results suggest that surfactant applications below the CMC increase contaminant solubility into the aqueous phase, thereby potentially increasing bioavailability and enhancing biodegradation. In addition to surfactant concentration, surfactant-contaminant interactions must be considered as well as the type of contaminated medium (i.e., water, soil, etc.) when deciding on a remediation strategy.


## INTRODUCTION

Carbon tetrachloride (CT) and toluene (Tol) have many industrial and commercial uses such as being solvents and degreasing agents (Anonymous 1992a; 1992b). As a result of improper disposal, volatile organic compounds, including CT and Tol, are commonly found as environmental pollutants and are the most prevalent ground water contaminants (Haley et al. 1991). Carbon tetrachloride, a carcinogen, is listed as a hazardous substance, a hazardous waste, and priority pollutant by the U. S. Environmental Protection Agency (EPA) (Sittig 1985a). In addition, CT is listed with the Resource Conservation and Recovery Act (RCRA) as a Toxicity Characteristic Leaching Procedure (TCLP) hazardous waste and is regulated at the 0.5 ppm leachate concentration

in groundwater (CFR 261.26). Carbon tetrachloride is very stable in the environment with an estimated half-life of 7,000 years in water at a concentration of 1 mg/L (Anonymous 1992a).

Toluene, a depressant of the central nervous system, is listed as a hazardous substance, a hazardous waste, and a priority pollutant by the U.S. EPA (Sittig 1985b). While Tol readily breaks down when exposed to air, there is considerable potential for Tol to persist as a groundwater contaminant (Anonymous 1992b). The U.S. EPA does not currently list Tol as a RCRA-TCLP hazardous waste. The U.S. EPA does regulate Tol in drinking water at the 1 mg/l level (CFR 141).

High concentrations of these contaminants may be removed using conventional remediation treatments such as pump and treat (Haley et al. 1991; Keeley 1989). However, these processes leave residual levels of contaminants which must also be removed to meet preset remediation clean-up levels (Semprini 1995; Haley et al. 1991). Therefore, alternative techniques must be utilized to address the issue of remediating low concentrations of such contaminants. One such alternative, bioremediation, offers a realistic means of dealing with residual contaminants (Gandhi 1995; Semprini et al. 1992). Bioremediation is an attractive option because it has the potential to: (1) permanently eliminate contaminants through biochemical transformation or mineralization; (2) avoid harsh chemical and physical treatment; (3) operate *in situ*; and (4) be cost effective (Sturman et al. 1995). McGrath (1995) reports that bioremediation in general may ultimately prove to be the most cost-effective remedial action.

Biodegradation can be enhanced by increasing contaminant bioavailability (Rosenburg 1986). This increased bioavailability is typically achieved by increasing aquatic solubility (i.e., partitioning the contaminants into the aqueous phase where bacteria populations can gain access to the contaminants for biodegradation). In an effort to enhance aquatic solubility, it is sometimes necessary to employ a solubilization

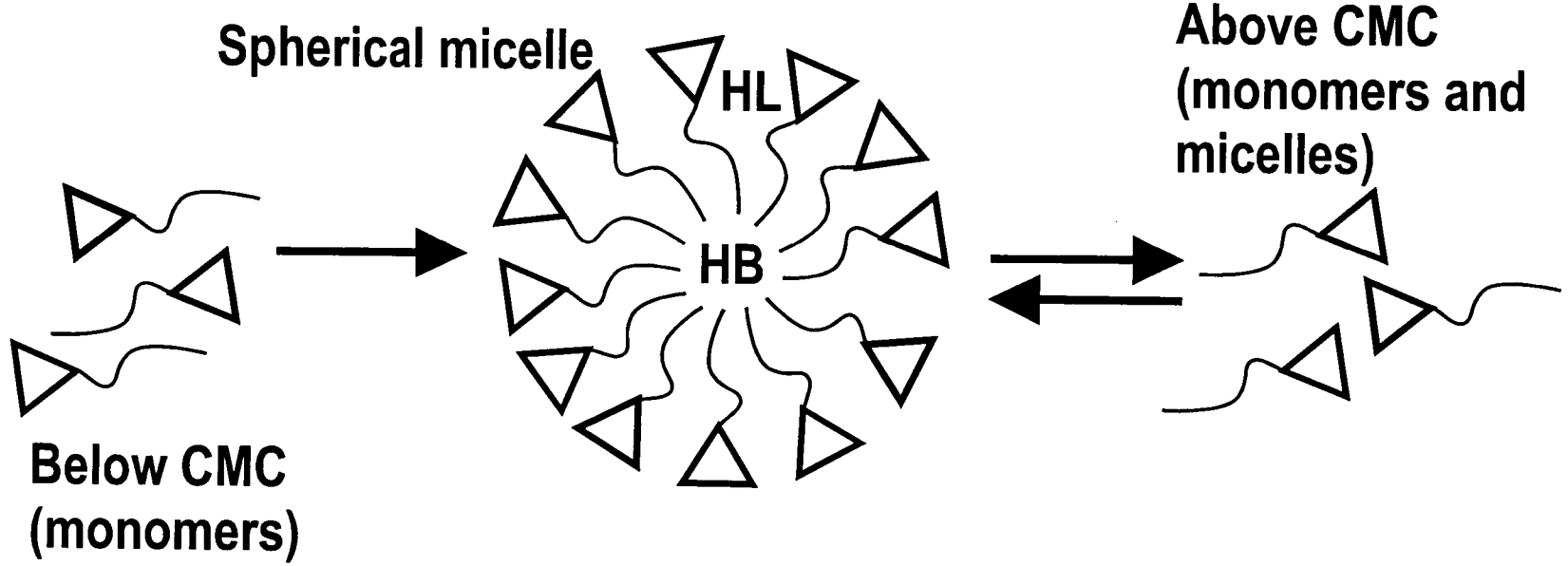
process. One such process is the administration of a surfactant to the subsurface matrix (Aronstein et al. 1991; 1992; Edwards et al. 1991; Fountain 1992; Kile et al. 1989; Park 1993; Shiau et al. 1994).

Surfactants, or surface active agents, are molecules of hydrophobic and hydrophilic moieties which accumulate at surface interfaces (West et al. 1992; Rosenburg 1986). These molecules reduce the surface tension of the aqueous phase, which facilitates the solubilization of organic pollutants. The individual surfactant molecules (monomers) aggregate at a critical concentration to form dynamic 3-D clusters called micelles. This critical micelle concentration (CMC) is unique to each surfactant. The monomers, in an aqueous environment, will orient in a cluster with the hydrophilic head groups in contact with the aqueous phase and the hydrophobic tails oriented towards the interior of the aggregate (Figure 1-1).

Surfactants have been used to solubilize and mobilize organic contaminants in field demonstrations (Fountain 1992; Harwell 1992; West 1992; Fountain et al. 1991). Therefore, when using surfactants it is important to consider the surfactant concentration required to obtain the desired effect. Incorporation of neutral organic compounds into micelle cores of surfactants above the CMC, result in increased solubility (West et al. 1992; Edwards et al. 1991; Jafvert et al. 1994; Kile et al. 1989). However, surfactants used above the CMC may result in the contaminant being less available for microbial degradation. Reduced availability being the result of the contaminant being partitioned into the micelle (Strong-Gunderson et al. 1995). In an alternate view, Laha et al. (1992) suggest that the inhibitory effect of non-ionic surfactants used above the CMC on microorganisms is related to a micellar surfactant-bacteria interaction that results in a reversible interference with cell membrane processes. It would, therefore, be desirable to use surfactants at concentrations below the CMC. Several studies have shown that low-concentrations of surfactants facilitate biodegradation (Aronstein et al. 1991; 1992;

FIG. 1-1 Aquatic Surfactant Micellization. The critical micelle concentration (CMC) is the concentration at which individual surfactant monomers aggregate into dynamic 3-D clusters, with the hydrophobic (HB) moieties oriented into the center of the cluster and the hydrophilic (HL) moieties oriented outward from the cluster.

# Examples of Surfactant Micellization




(Modification of: West, C.C. and J.H. Harwell. 1992. Surfactants and subsurface biodegradation. Environ. Sci. Technol. 26:2324-2330)

Strong-Gunderson et al. 1994; Strong-Gunderson et al. 1995). In addition, it is important to consider other technologies being utilized in the remediation scheme. Surfactants may have a negative effect on conventional remediation methods such as soil venting and air sparging, which are dependent on contaminant volatilization (Marley et al. 1992; Johnson et al. 1993; Reddy et al. 1995; Long 1992). Fountain (1992) reported that air stripping of tetrachloroethylene (PCE) was not as effective after surfactant applications. Air stripper performance is a function of the Henry's law constant for a compound. Since surfactants increase the contaminant solubility which works against the mechanisms of soil venting and air sparging, surfactants should be administered only after gross contamination has been removed.

The goal of this study was to investigate the effects of a commercial non-ionic surfactant, Tween-80, used at low concentrations (0.01%-0.1%) on the partitioning patterns of the two common groundwater contaminants CT and Tol at low concentrations (<1 mg/l and <5 mg/l, respectively). Tween-80 was selected as the surfactant due to its relatively low cost and low toxicity (Anonymous 1984). Moreover, its nonionic nature makes it suitable for solubilization of the nonionic contaminants selected for study.

## MATERIALS AND METHODS

### Chemicals.

Toluene (99% purity) was obtained from EM Science Chemical Co., Gibbstown, NJ. Methylene Chloride (MeCl; 99% purity) was obtained from Burdick & Jackson Laboratories, Inc., Muskegon, MI. Carbon tetrachloride (99% purity) was obtained from EM Science Chemical Co., Gibbstown, NJ. Tween-80 was obtained from Fisher Scientific Chemical Co., Fair Lawn, NJ. All chemicals used were American Chemical Society reagent grade. All water used in reagent preparation was of 18-MΩ resistance or greater.

Media.

Phosphate buffered saline (PBS) solution contained (per liter of water) 1.2g sodium phosphate diabasic, 2.2g sodium phosphate monobasic, and 8.8g sodium chloride. A volume of 15 ml PBS was added to 40-ml EPA vials with Teflon lined screw caps. Appropriate dilutions of a water saturated CT (800 mg/l, the aqueous solubility of CT) (Verschueren 1991) stock solution was used to prepare a graded concentration series of the following concentrations: 0.5, 0.75, 1.0, 1.25, and 1.5 mg/l CT. Similarly, appropriate dilutions of a water saturated Tol (515 mg/l, the aqueous solubility of Tol) (Anonymous 1992b) stock solution was used to prepare graded concentration series of 0.125, 0.25, 0.5, 0.75, and 1.0 mg/l Tol. Tween-80 was added to final concentrations of 0.01%, 0.03% (i.e., the CMC), or 0.1% (v/v). In addition, for Tol one test was conducted with 0.001% Tween-80. All tests were conducted in triplicate. Vials were placed on a rotary shaker for approximately one hour prior to headspace analyses by gas chromatography.

Analytical Methods.

Surface tensions of Tween-80 solutions were determined with a CSC Scientific Co., DuNouy Interfacial Tensiometer, Fairfax, VA, which conforms to specifications of the American Society for Testing and Materials (ASTM) standard method D1331 for testing solutions of surface-active agents. This instrument operates on the DuNouy principle, in which a platinum-iridium ring is suspended from a torsion balance, and the force (in dynes per centimeter) necessary to pull the ring free from the surface film is measured (Kile et al. 1989). A range of Tween-80 concentrations from $6.25 \times 10^{-4}$% to 1.0% were measured in triplicate. The CMC was determined by measuring the surface tension over a wide concentration range and noting the inflection in the plot of surface tension versus surfactant concentration.

Toluene analyses were performed using a Perkin-Elmer Sigma 2000 gas chromatograph (GC), Norwalk, CT, equipped with a flame ionization detector and a stainless steel 6' X 1/8" outer diameter, 0.1% AT1000 Graphpac packed column. The oven temperature was 200°C, the injector temperature was 200°C, and the nitrogen carrier gas flow rate was 60 ml/min. Headspace samples of 30 µl were injected onto the GC and analyzed for 8 minutes. The retention time for Tol was 2.4 minutes. Standard curves were constructed at the above listed concentrations.

Toluene was extracted from the headspace and aqueous layers by the addition of methylene chloride, added in a single aliquot of 1 ml to the 0.01% Tween-80 spiked vials. Samples were allowed to equilibrate overnight. One microliter of extractant was injected onto the AT1000 column with an initial oven temperature of 80°C, increasing at the rate of 20°C/min to a final temperature of 225°C, and an injector temperature of 200°C. Headspace and aqueous concentrations were determined from standard curves generated from data on the above noted concentrations.

Carbon tetrachloride analyses were performed using a Hewlett Packard 5890 Series II Plus GC, Wilmington, DE, equipped with an electron capture detector and a DB624 megabor 30-m capillary column, with 3 µm film thickness. The temperature program began at an initial oven temperature of 50°C and an injector temperature of 100°C. Following an initial 0.3 min hold, temperature was increased 20°C/min to a final temperature of 120°C which was maintained for 0.3 minutes. A mixture of argon and methane gas was used as a carrier gas with a flow rate of 7 ml/min. Headspace samples of 30 µl were injected onto the GC and analyzed for 4.1 minutes. The retention time of CT was 3.0 minutes and the retention time of chloroform was 2.8. Headspace and aqueous concentrations were determined from standard curves generated from data on the above noted concentrations.

# RESULTS

The Tween-80 surface tension curve in water is depicted in Figure 1-2. The CMC value was determined to be 0.03% (+/- 48 dynes/cm). It was from these data that the surfactant concentrations of 0.01%, 0.03%, and 0.1% were chosen for this study.

Toluene headspace samples were measured in the presence of Tween-80 at the concentrations from 0.125 to 1.0 mg/l (Figure 1-3). Although five concentrations of Tol were tested, due to instrument limitations, linearity could only be achieved with standards from 0.125 mg/l to 0.5 mg/l.

The results indicate that Tol partitioned into the aqueous phase at low concentrations (0.5-1.0 mg/l) in the presence of Tween-80. The addition of Tween-80 to aqueous systems at 0.01% exhibited a 51% reduction of headspace Tol compared to controls. The 0.03% Tween-80 concentration further enhanced partitioning (i.e., solubilization) to yield a 72% reduction in headspace Tol; whereas, the 0.1% Tween-80 concentration yielded a 50% reduction in headspace Tol. In theory, this increased partitioning into the aqueous phase means the Tol is more bioavailable for bacterial degradation.

Methylene chloride extractions performed on test samples with 0.01% Tween-80 yielded a 97% extraction efficiency (Figure 1-4). Thus, the decrease in headspace concentration noted above was not the result of loss from the system. Rather, these results demonstrated that Tween-80 did increase Tol partitioning into the aqueous phase. The ratio of headspace Tol vs. aqueous phase Tol was decreased by 50% by the addition of 0.01% Tween-80. These results are in agreement with the results presented in Figure 1-3, which show a 51% decrease in headspace Tol concentration.

There was no evidence of enhanced Tol partitioning at 0.001% Tween-80 concentration (Figure 1-5). This was tested in an effort to demonstrate that there is a threshold level, below which the surfactant is ineffective at increasing Tol partitioning.

FIG. 1-2 Surface tension standard curve of Tween-80 in an aqueous system.

Measurements were taken in triplicate from 0.000625% to 1.0% Tween-80 in water.

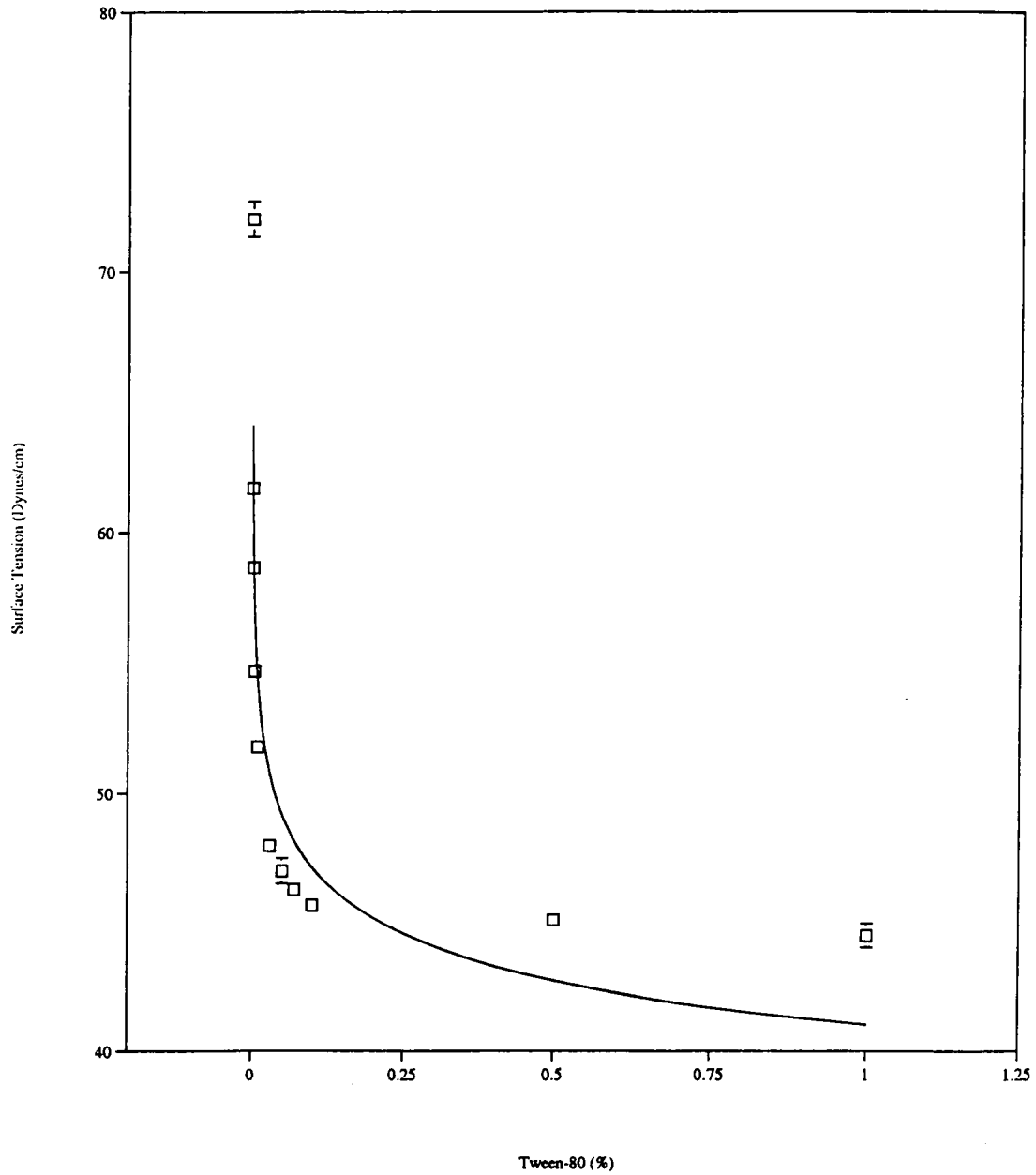

80
70
60
50
40
0
0.25
0.5
0.75
1
1.25
Surface Tension (Dynes/cm)
Tween-80 (%)

FIG. 1-3 Toluene partitioning in the presence of Tween-80. The standard curve for Tol with no Tween-80 yielded a linear regression formula of $Y = 1.82 \times 10^5 X + 8.72 \times 10^2$, $r^2 = 0.998$; with 0.1% Tween-80, $Y = 5.94 \times 10^4 X + 9.75 \times 10^3$, $r^2 = 0.999$; with 0.03% Tween-80, $Y = 8.39 \times 10^4 X + 1.68 \times 10^3$, $r^2 = 0.987$; and with 0.01% Tween-80, $Y = 9.33 \times 10^4 X + 3.50 \times 10^3$, $r^2 = 0.997$. The apparent large standard deviation for the 0.5 mg/l point on the line is actually only ±0.0694 mg/l.

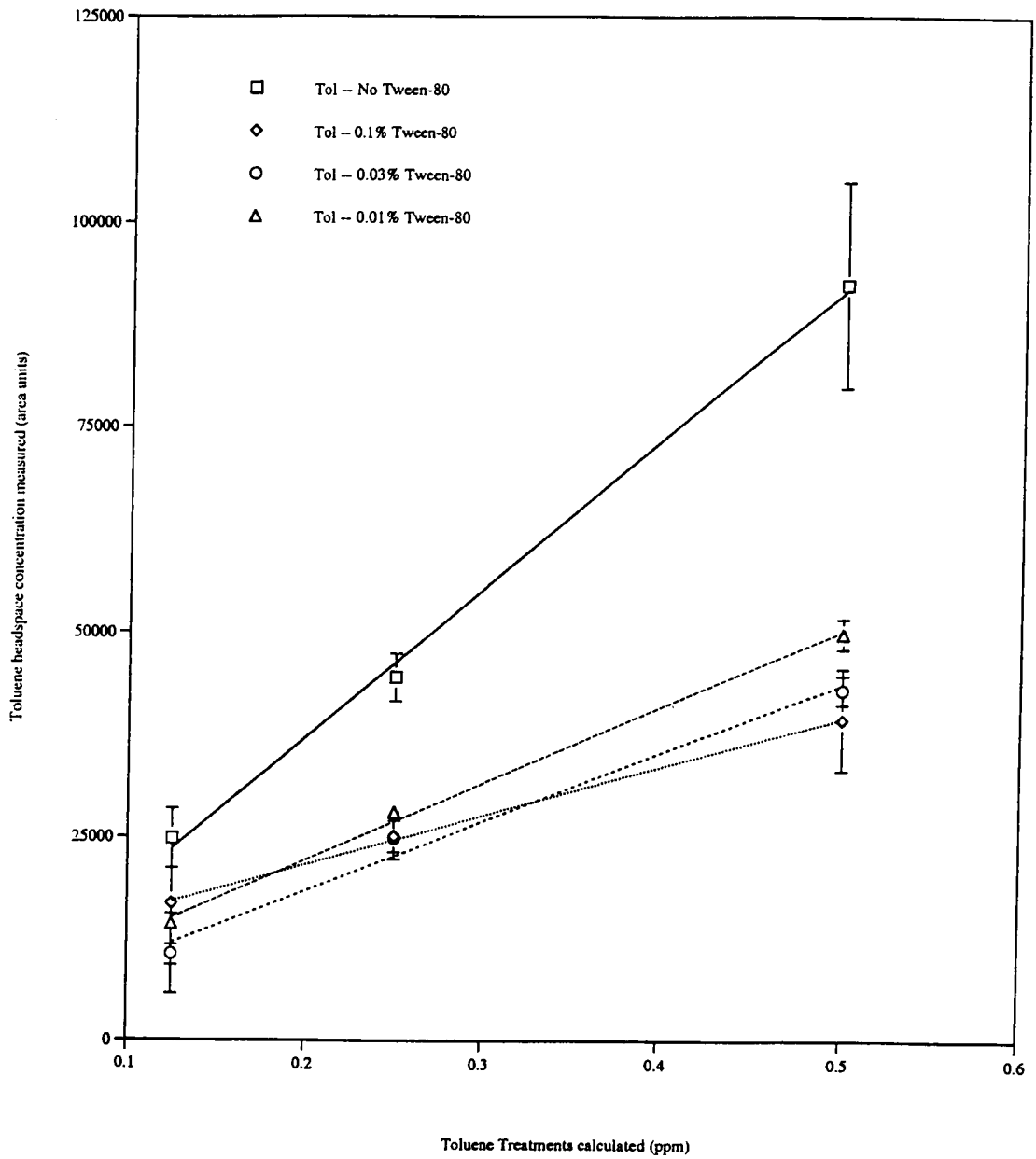

125000
100000
75000
50000
25000
0
0.1
0.2
0.3
0.4
0.5
0.6
Tol – No Tween-80
Tol – 0.1% Tween-80
Tol – 0.03% Tween-80
Tol – 0.01% Tween-80
Toluene headspace concentration measured (area units)
Toluene Treatments calculated (ppm)

FIG. 1-4 Methylene chloride extractions of toluene in the presence of 0.01% Tween-80. The linear regressions of these data yielded $r^2$ values of 0.999 for the controls and 1.000 for the samples with Tween-80.

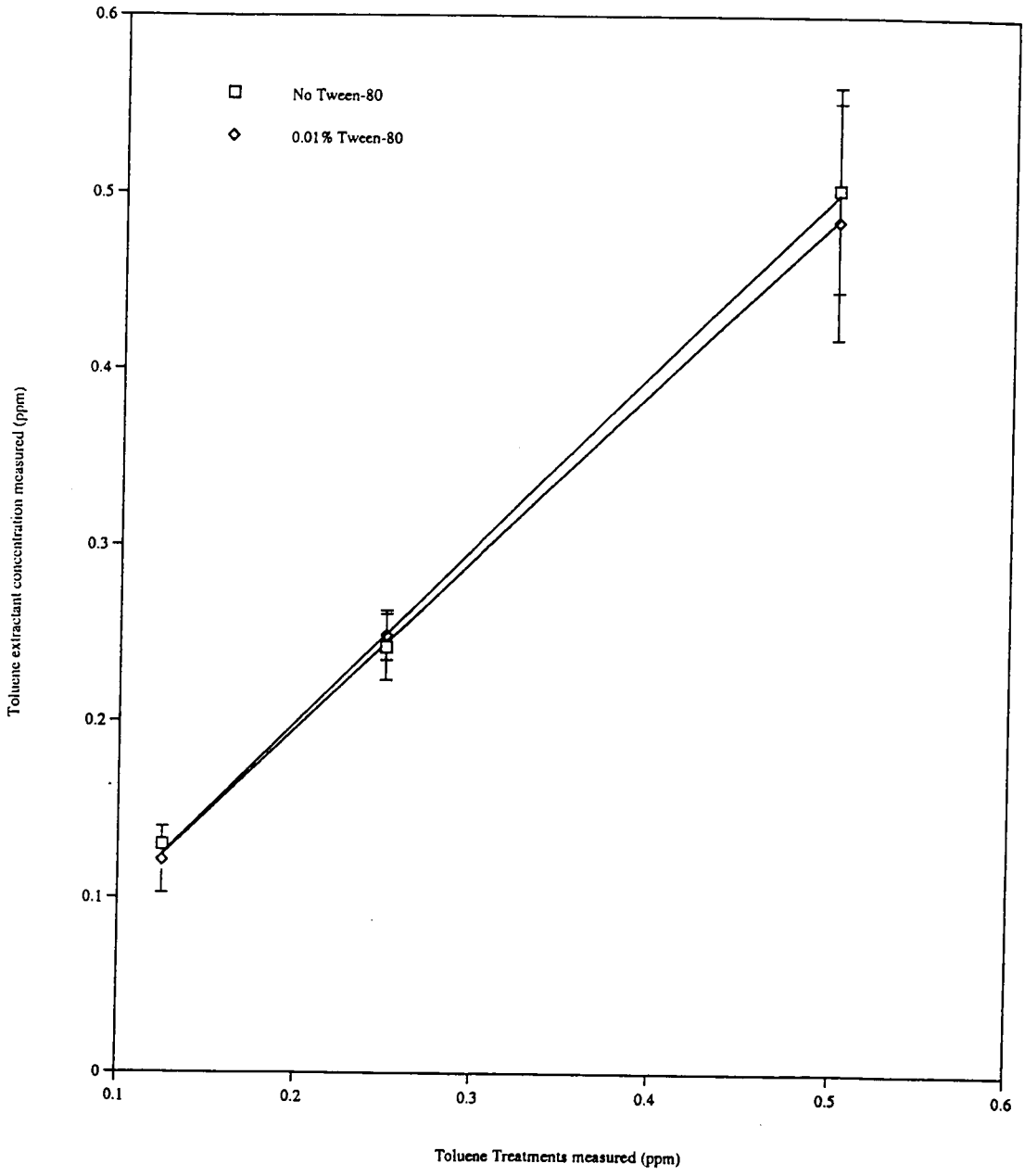
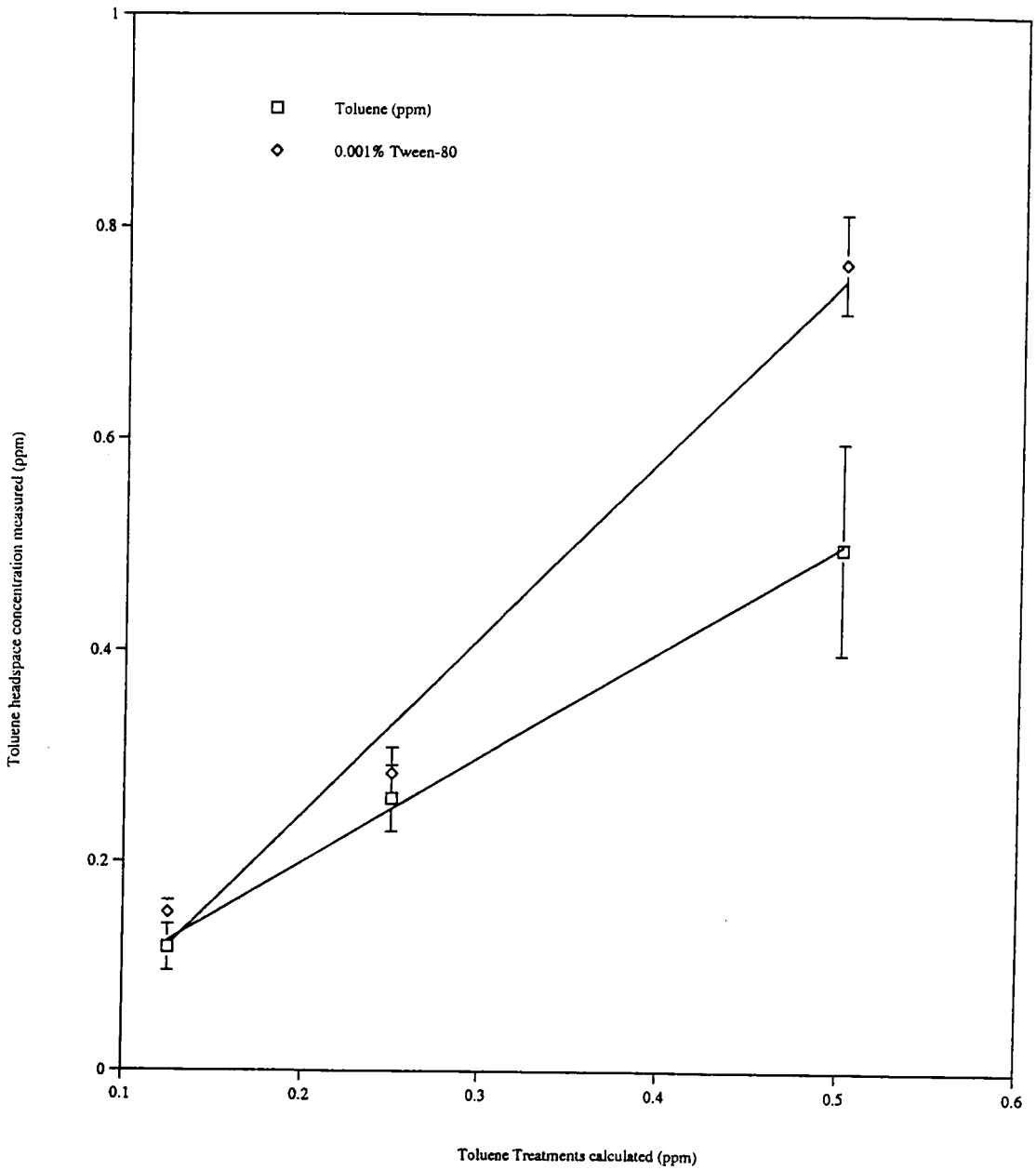

No Tween-80
0.01% Tween-80
Toluene extractant concentration measured (ppm)
Toluene Treatments measured (ppm)
0
0.1
0.2
0.3
0.4
0.5
0.6

FIG. 1-5 Toluene partitioning in the presence of 0.001% Tween-80. The $r^2$ values of 0.998 and 0.984 were obtained for the controls and samples with Tween-80, respectively.


Toluene (ppm)
0.001% Tween-80
Toluene headspace concentration measured (ppm)
Toluene Treatments calculated (ppm)
0
0.2
0.4
0.6
0.8
1
0.1
0.2
0.3
0.4
0.5
0.6

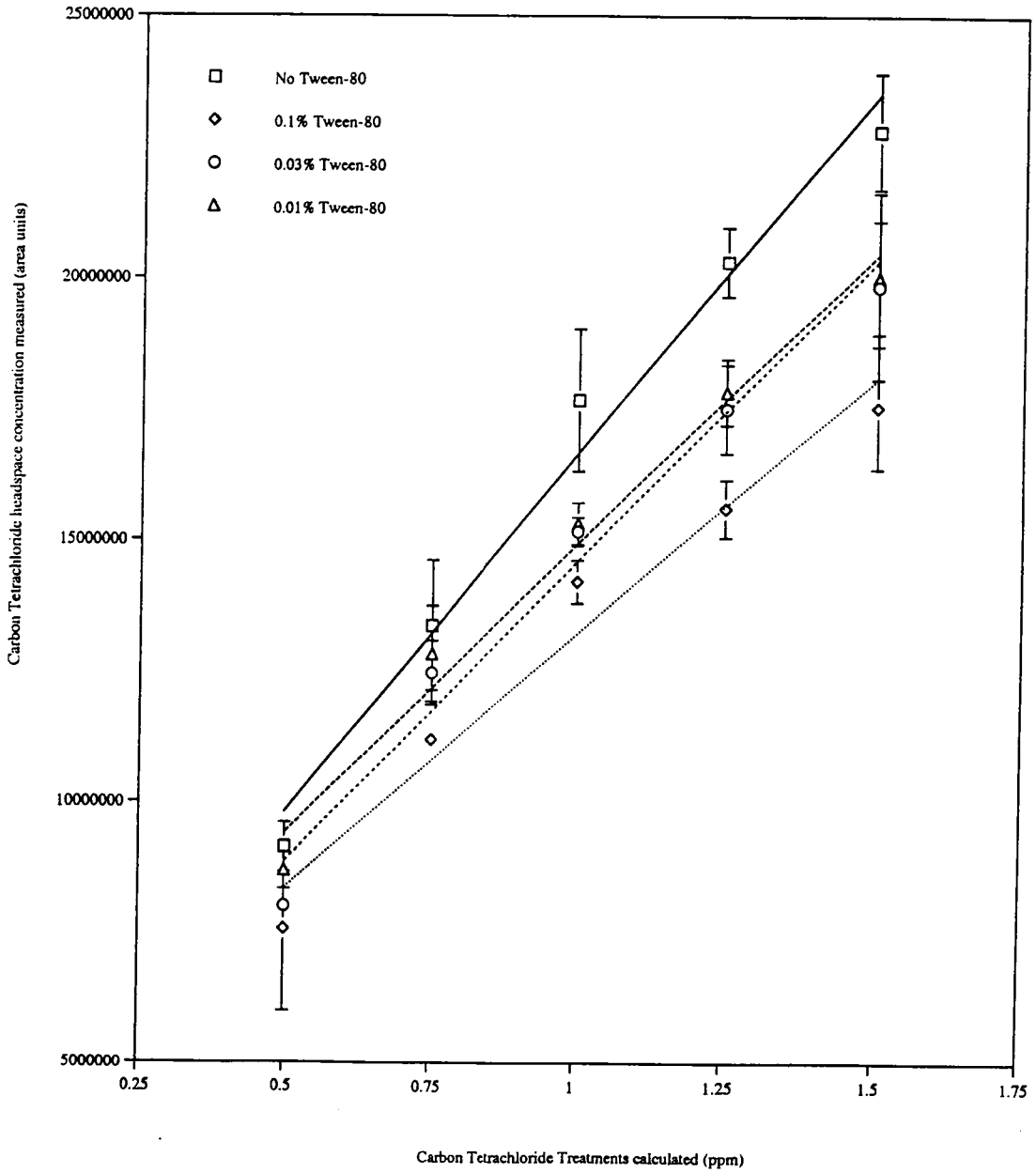
Carbon tetrachloride partitioning was similarly enhanced by the addition of low concentrations of Tween-80 (Figure 1-6) when headspace samples were measured in the presence of Tween-80 at initial concentrations from 0.5 to 1.5 mg/l. However, this effect was less pronounced than with Tol. Specifically, there was an 11% decrease in headspace CT concentration following surfactant treatments at 0.01%; 15% decrease with 0.03% surfactant treatments; and 25% decrease with 0.1% surfactant treatments. Comparison of CT and Tol solubilities in the presence of Tween-80 shows that 40% more Tol than CT was solubilized by 0.01% Tween-80, 57% more Tol than CT with 0.03% Tween-80, and 25% more Tol than CT with 0.1% Tween-80.

## DISCUSSION

The results presented above indicate that surfactants are effective at enhancing contaminant solubility at low concentrations. Strong-Gunderson et al. (1995) showed that addition of Tween-80 at low concentrations to soil slurries containing Tol or naphthalene increased the bioavailability of these compounds. Similarly, Kile et al. (1989) reported that 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane (DDT) exhibited significant solubility enhancements in the presence of several molecularly nonhomogeneous surfactants below the CMC. Aronstein et al. (1991; 1992) also reported improved biodegradation of phenanthrene resulting from the addition of two nonionic surfactants at low levels.

Results show that for CT and Tol, concentrations of Tween-80 just below the CMC (0.01%) increased partitioning into the aqueous phase, where biodegradation occurs. Solubilization by surfactants below the CMC occurs for two reasons. First, for surfactant mixtures (i.e., commercial grade surfactants) no absolute CMC value exists. Monomers of similar chain length tend to self associate; therefore, micelles exist below the operationally defined CMC value. Second, monomers, *per se*, may enhance aqueous

FIG. 1-6 Carbon tetrachloride partitioning in the presence of Tween-80. The standard curve for CT with no Tween-80 yielded a linear regression formula of $Y = 1.37 \times 10^7 X + 2.96 \times 10^6$, $r^2 = 0.983$; with 0.1% Tween-80, $Y = 9.73 \times 10^6 X + 3.49 \times 10^6$, $r^2 = 0.966$; with 0.03% Tween-80, $Y = 1.15 \times 10^7 X + 3.13 \times 10^6$, $r^2 = 0.978$; and with 0.01% Tween-80, $Y = 1.10 \times 10^7 X + 3.89 \times 10^6$, $r^2 = 0.983$.


25000000
20000000
15000000
10000000
5000000
No Tween-80
0.1% Tween-80
0.03% Tween-80
0.01% Tween-80
Carbon Tetrachloride headspace concentration measured (area units)
0.25
0.5
0.75
1
1.25
1.5
1.75
Carbon Tetrachloride Treatments calculated (ppm)

concentrations of hydrophobic organic compounds (HOCs). This enhancement is correlated to the HOC hydrophobicity (Jafvert et al. 1994). West (1992) concurs that solubilization of HOCs is a two step process, first by soluble monomers below the CMC, and second by dispersed micelles above the CMC. The smaller partitioning effect of CT with Tween-80 below the CMC (relative to Tol) is attributed to its greater water solubility, which in essence reduces its partitioning efficiency with the dilute organic fraction of the dissolved surfactant. Furthermore, the larger octanol-water partition coefficient ($K_{ow}$) of Tol (relative to CT) explains the greater partitioning results observed with Tol and surfactants above the CMC compared to CT. West et al. (1992) reports that the larger the $K_{ow}$ of a solute, the greater will be its tendency to concentrate inside the micelle.

The results of this study suggest that surfactant applications below the CMC increase contaminant solubility into the aqueous phase, thereby potentially increasing bioavailability and enhancing biodegradation. Using the minimum concentration of a surfactant to assist bioremediation will potentially reduce the cost of remediation efforts: (1) by decreasing the quantity of surfactant purchased; (2) by creating the optimal conditions for bioremediation via enhanced contaminant solubility into the aqueous phase; and (3) by creating less surfactant residuals in soil to deal with after remediation is completed. The effectiveness of a surfactant in enhancing the removal of a contaminant from the subsurface can be expected to be a function not only of the surfactant's interaction with the contaminants, but also of the surfactant's interactions with the aquifer media at the conditions in the aquifer (Harwell 1992). In addition, surfactant-contaminant interactions must be considered along with the type of contaminated medium (i.e., water, soil, etc.) when deciding on a remediation strategy. This research was performed in a strictly aqueous environment. An important parameter that we did not address in this study was the effect of surfactant sorption to soil particles.

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# PART II

# BIODEGRADATION OF CARBON TETRACHLORIDE AND TOLUENE BY FACULTATIVE ANAEROBIC BACTERIAL CONSORTIA IN AN AQUEOUS ENVIRONMENT

## ABSTRACT

A study was conducted to evaluate the biodegradation potential of several bacterial consortia utilizing toluene (Tol) as the primary carbon and energy source, with carbon tetrachloride (CT) degraded cometabolically. Two consortia were identified that were capable of concomitant CT and Tol degradation under oxygen and iron limiting conditions, without the production of chloroform as an end-product. Additionally, it was determined that the use of a nonionic surfactant, which enhanced contaminant bioavailability by increasing aquatic solubility, did not enhance the overall rate of biodegradation.


## INTRODUCTION

Bioremediation, the process of using microorganisms to chemically change harmful compounds into less or non-harmful chemicals, may ultimately prove to be the most cost-effective remedial action in the battle against environmental pollutants (McGrath 1995). Bioremediation is attractive because it has the potential to: (1) permanently eliminate contaminants through biochemical transformation or mineralization; (2) avoid harsh chemical and physical treatments; (3) operate *in situ*; and (4) be cost effective (Sturman et al. 1995). Common methods of groundwater remediation, such as pump and treat, have been somewhat effective; however, they are costly, they may require surface treatments, and they have resulted in the transfer of contaminants to other environmental compartments (Semprini 1995). Bioremediation may be most effective at removing trace contaminants after the bulk contamination has been removed by other methods (Lee et al. 1988). Gandhi et al. (1995) reported that Tol and benzene are mainly removed by pump-and-treat processes; however, they recommend that bioremediation be employed as a polishing step to remove trace contaminants not readily removed by the flushing process.

Chlorinated solvents, such as CT, are the most frequently observed groundwater contaminants in the United States (Semprini 1995). Many of these chlorinated aliphatic hydrocarbons (CAHs) can be biodegraded; however, microorganisms generally cannot obtain energy for growth from the transformation (Vogel et al. 1987). Therefore, the presence of a cosubstrate as a carbon and energy source can lead to aerobic and anaerobic transformations through interactions of the CAHs with enzymes and cofactors produced by microbes for other purposes (Semprini 1995). Furthermore, CT, and other chlorine substituted organic compounds, make excellent electron acceptors, and are, therefore, prone to reductive transformation, which is facilitated in anaerobic environments (Liang et al. 1993).

Alkylbenzenes, such as Tol, are the second most common groundwater contaminants and can be degraded under both aerobic and anaerobic conditions by subsurface microorganisms (Sewell et al. 1991). These aromatic hydrocarbons are good electron donors and could potentially assist in the reductive dechlorination of CAHs. Sewell et al. (1991) showed that Tol served as the initial source of reducing potential for the reductive dechlorination of tetrachloroethylene (PCE). Trichloroethylene (TCE) was also shown to be cometabolized by microorganisms in the presence of Tol (Fan et al. 1993).

The purpose of this study was to determine if CT would be degraded in the presence of Tol utilizing bacterial consortia obtained from various field and laboratory sites. Aerobic as well as anaerobic aqueous environments were simulated, and different nutrient media were tested. In addition, the use of a non-ionic surfactant, Tween-80, was evaluated for its potential as a solubilization mechanism to enhance biodegradation.

# MATERIALS AND METHODS

Chemicals.

Toluene (99% purity) and CT (99% purity) were obtained from EM Science Chemical Co., Gibbstown, NJ. Tween-80 was obtained from Fisher Scientific Chemical Co., Fair Lawn, NJ. All chemicals used were American Chemical Society reagent grade. All water used in reagent preparation was of 18-MΩ resistance or greater.

Media.

A minimal salts media (Little et al. 1988) was added in 15 ml aliquots to 40-ml EPA vials with Teflon lined screw caps for the initial screening assay. Subsequent tests used a modification of the above described media, lacking copper and iron, which was added in 14 ml aliquots to 15-ml EPA vials with Teflon-lined screw caps. A 0.01% solution of yeast extract in phosphate-buffered saline was added as a vitamin source. Cell suspensions were made to a final optical density of approximately 2.0 at 600 nm on a Gilford Response UV-Vis spectrophotometer, Oberlin, OH. This bacterial suspension was then added in 10 µl aliquots to the test vials. Carbon tetrachloride was added at an appropriate dilution of a water saturated CT (800 mg/l, the aqueous solubility of CT) (Verschueren 1991) stock solution for a final concentration of 1 mg/l CT. Toluene, similarly was added at an appropriate dilution of a water saturated Tol (515 mg/l, the aqueous solubility of Tol) (Anonymous 1992) stock solution for a final concentration of 5 mg/l Tol. Tween-80 was added at a final concentration of 0.03% (v/v), the previously determined aqueous critical micelle concentration (CMC). In addition to 0.03%, a 5% Tween-80 solution was tested with the NO92 consortium at a CT concentration of 1.0 mg/l and a Tol concentration of 5.0 mg/l. The vials were incubated on a rotary shaker at room temperature for approximately 25-30 days prior to headspace analyses by gas chromatography.

Analytical methods.

Carbon tetrachloride analyses were performed using a Hewlett Packard 5890 Series II Plus gas chromatograph (GC), Wilmington, DE, equipped with an electron capture detector and a standard DB624 megabor 30-m capillary column, with 3 μm film thickness. The temperature program began at an initial oven temperature of 50°C and an injector temperature of 100°C. Following an initial 0.3 minute hold, temperature was increased 20°C/min to a final temperature of 120°C which was maintained for 0.3 minutes. A mixture of argon and methane gas was used as a carrier gas with a flow rate of 7 ml/min. Headspace samples of 30 μl were injected onto the GC and analyzed for 4.1 minutes. The retention time of carbon tetrachloride was 3.0 minutes and the retention time for chloroform was 2.8 minutes.

Toluene analyses were performed using a Hewlett Packard 5890 Series II Plus GC equipped with a flame ionization detector and a 0.320 mm diameter/30-m capillary column. The oven temperature was 175°C, the injector temperature was 200°C, and the nitrogen carrier gas flow rate was 3.5 ml/min. Headspace samples of 30 μl were injected onto the GC and analyzed for 3.0 minutes. The retention time of Tol was 0.9 minutes.

Analysis of variance was calculated using Statistical Analysis Software (SAS), SAS Institute, Cary, NC. The student *t*-test multiple comparison test was used to determined statistically significant differences in CT degradation.

Bacterial Characterization.

The BIOLOG system, Cambridge Technology, Inc., Watertown, MA, was used to identify members of each consortium tested and to differentiate among the consortia. This system utilizes standard 96-well microplates which are pretreated with a dried film consisting of ninety-five different carbon sources; a complex, low-concentration nutrient medium; and a tetrazolium violet redox dye. The cell's metabolism of the test substrates results in the formation of reduced nicotinamide adenine dinucleotide (NADH), which in

order to be reoxidized, passes electrons to an electron transport chain. The tetrazolium dye taps electrons from this flow, reducing it to an irreversible purple formazan. The resulting pattern of purple and colorless wells forms a "metabolic fingerprint" for each organism. Using a special species-matching algorithm, the MicroLog software compares this fingerprint to a database of such patterns and computes an identification of the test culture.

Bacteria.

The bacteria consortia were obtained from several locations listed in Table 1. The "fingerprints" of the consortia were used to differentiate among them and to detect similarities but was not used for identification purposes.

Table 1. Consortia Demographic Information.

| No. | Consortia Name | Consortia Origin | Isolate Member Description |
|---|---|---|---|
| 1a | NO92 | Lab waste stream, ORNL*, TN | White |
| b | | | Yellow |
| c | | | Mucus/peach |
| 2a | GBS | Lab waste stream, ORNL, TN | Yellow |
| b | | | White |
| 3a | 391 | Water-saturated TCE, ORNL, TN | White |
| b | | | Yellow |
| 4a | HC5 | Hanford Soil, WA | White |
| b | | | Yellow |
| 5a | S25 | Yacama Barricade Soil, WA | White |
| b | | | Yellow |
| 6a | S17 | Yacama Barricade Soil, WA | White |
| b | | | Yellow |
| c | | | Bacillus/white |
| 7a | 299 | Hanford Soil, WA | White |
| b | | | Yellow |

*ORNL - Oak Ridge National Laboratory

# RESULTS

Only one consortium, NO92, was identified as a possible CT degrader when run in test systems containing unmodified minimal salts media, Tol, and 25 ml of headspace (Figure 2-1). High standard deviations for three other consortia, 299, 391, and GBS, made it difficult to positively determine their potential for CT degradation. Toluene degradation was complete for all the consortia tested (Figure 2-2).

Four of the seven consortia tested were positive CT degraders (Figure 2-3) when initial conditions and media were changed. In an effort to reduce the oxygen content in the test environment, the second test system was assembled with less than 1 ml of headspace. In addition, a modified nutrient medium was used to eliminate the presence of reduced iron and copper, again in the presence of Tol. Statistical analyses of the data show that consortia NO92, S17, and HC5 significantly reduced CT in the headspace, and that consortium GBS was the most effective at reducing CT in the headspace. A secondary peak for air could be identified at a retention time of 1.1 minutes. Comparison of these air peaks [$O^2$] from the GC analyses showed all the consortia tested had less oxygen in the headspace than the controls, with GBS having the least amount of oxygen present (Figure 2-4). Due to an inherent impurity in the CT stock, there was a baseline concentration of chloroform present in all the standards and samples. Careful examination of the chromatograms and comparison with chloroform standard peaks, indicate that there was no chloroform present at concentrations above the background in these vials. The chloroform standard curve was linear down to 0.31 mg/l. The highest chloroform level detected in any of the test vials was 2.7 times less than this, and consortia GBS and NO92 averaged 75 times less chloroform. Toluene degradation was complete for all consortia except 299 and S17, which showed Tol degradation below 1 mg/l (data not shown).

FIG. 2-1 Carbon tetrachloride degradation with 25 ml headspace and unmodified nutrient media. N=2.

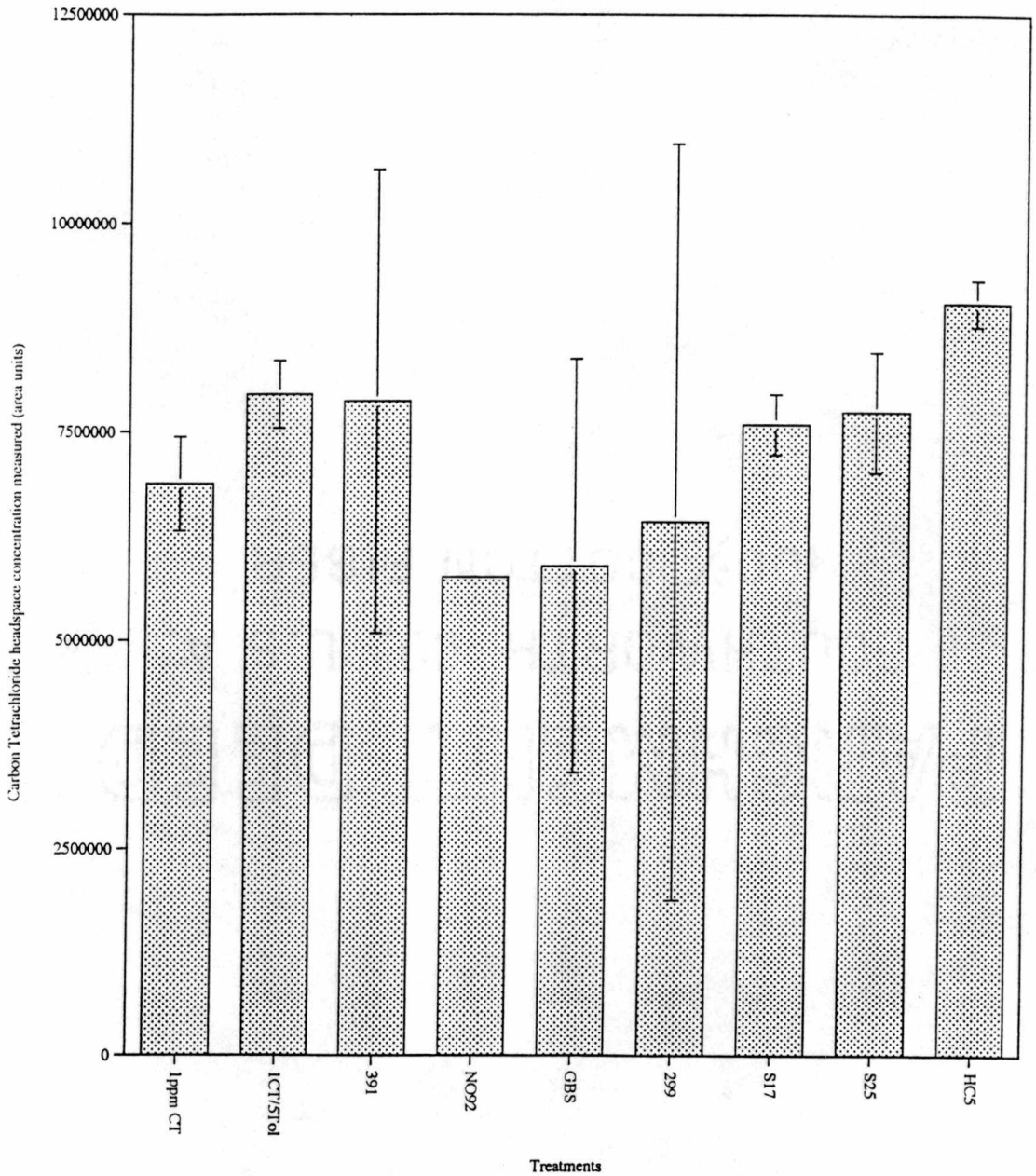

12500000
10000000
7500000
5000000
2500000
0
Carbon Tetrachloride headspace concentration measured (area units)
1ppm CT
1CT/5Tol
391
NO92
GBS
299
S17
S25
HC5
Treatments

FIG. 2-2 Toluene degradation with 25 ml headspace and unmodified nutrient media. N=2.

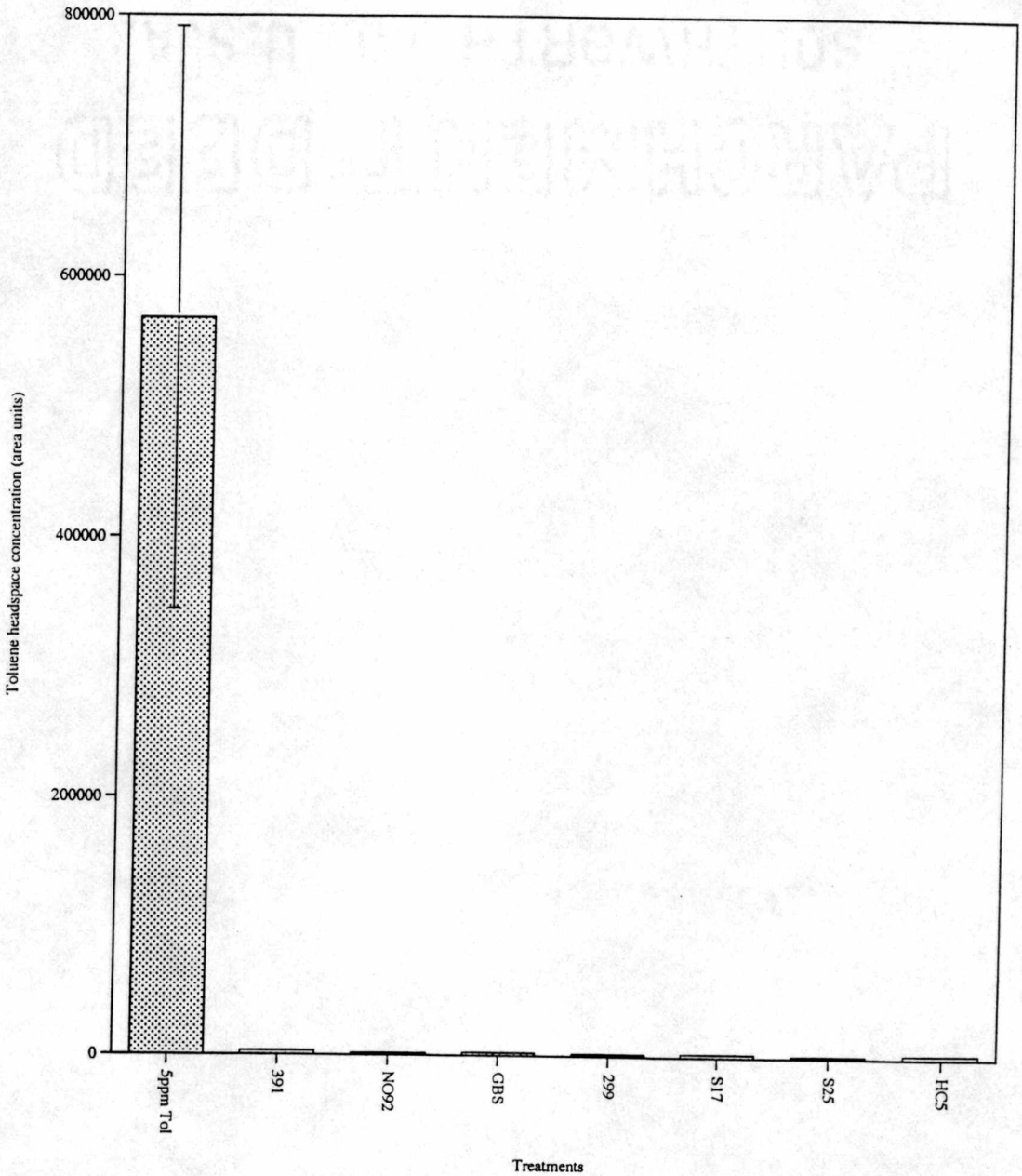

800000
600000
400000
200000
0
Toluene headspace concentration (area units)
5ppm Tol
391
NO92
GBS
299
S17
S25
HC5
Treatments

FIG. 2-3 Carbon tetrachloride degradation with less than 1 ml headspace and modified nutrient media. Letter designations A-D represent statistically significant relationships between consortia based on degree of CT degradation.

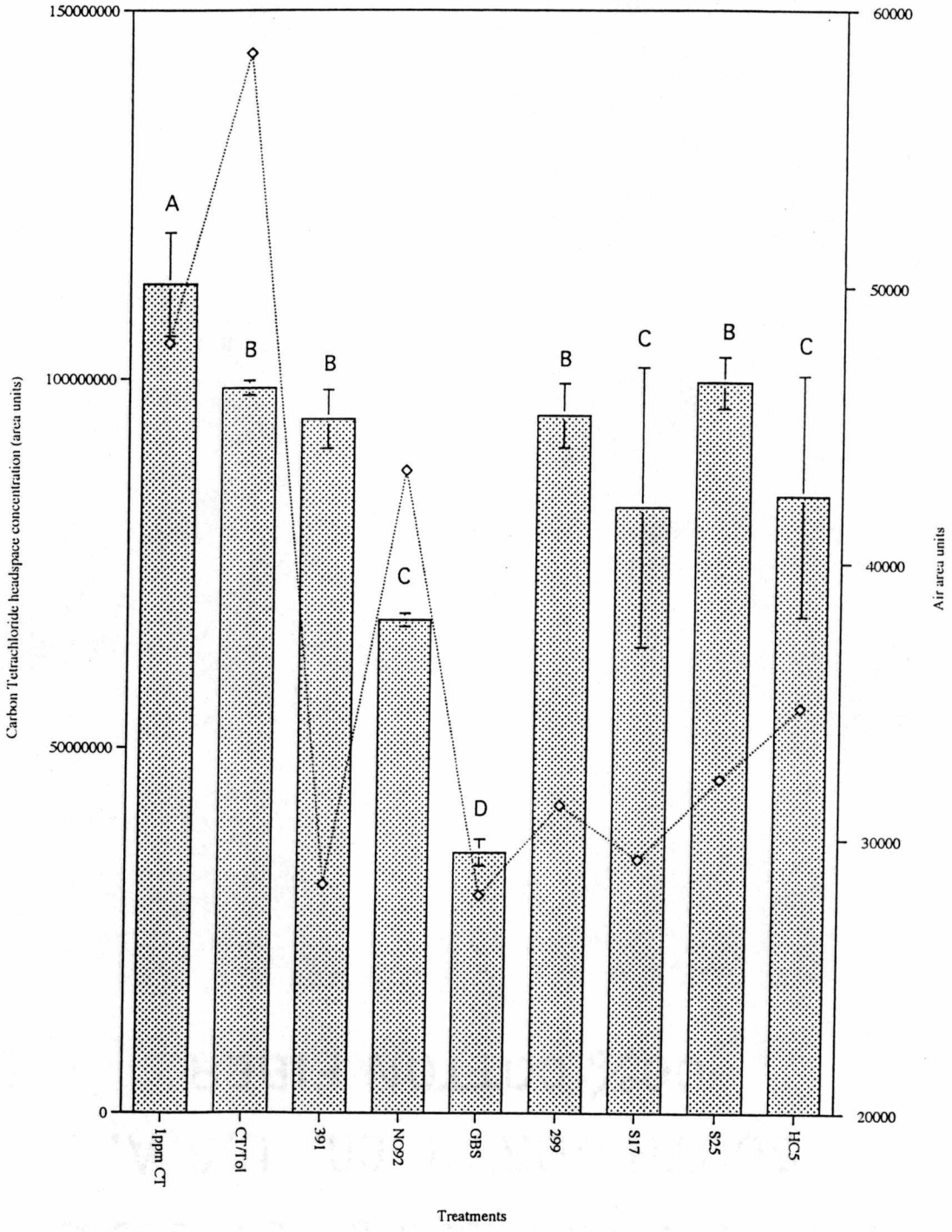
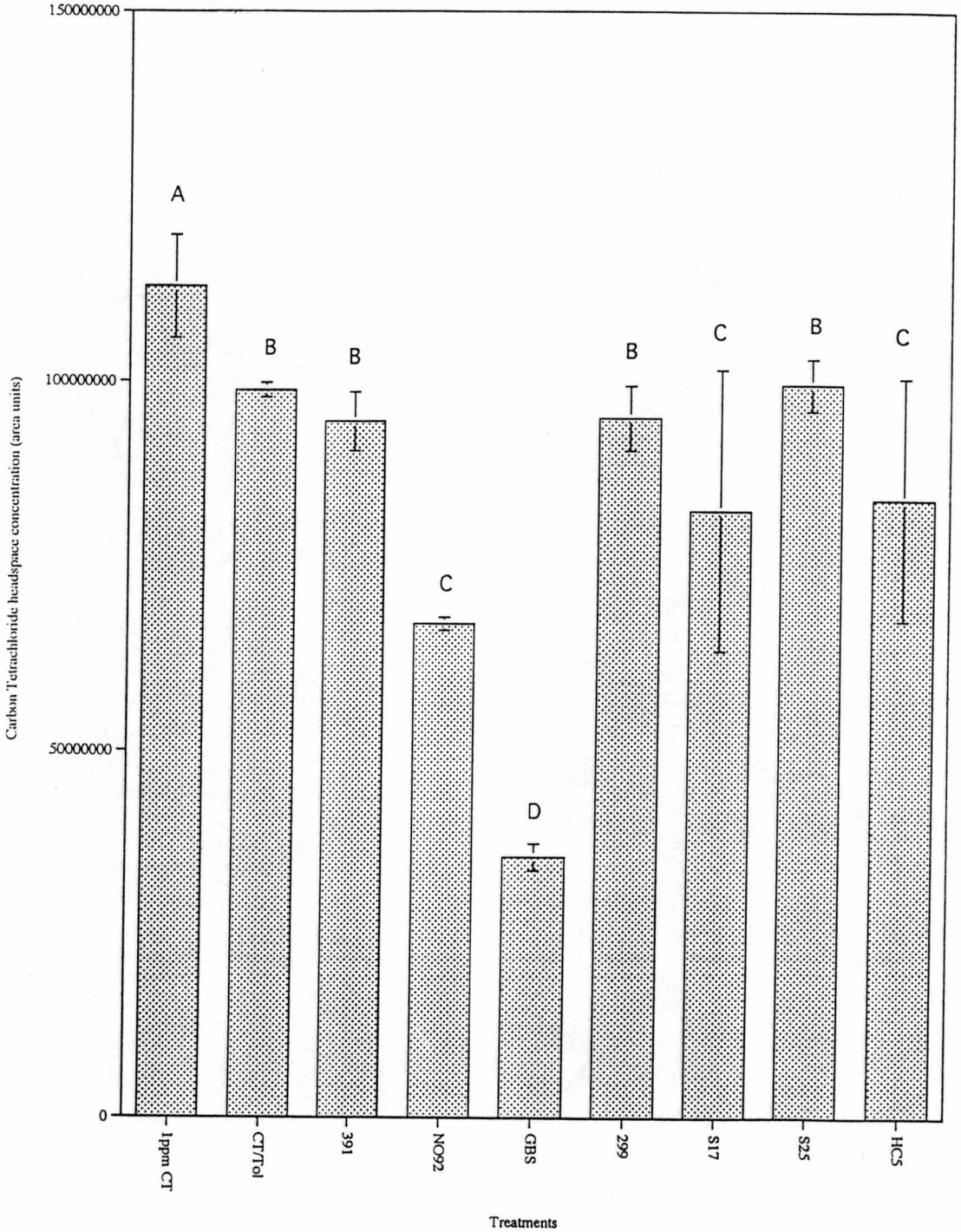

150000000
100000000
50000000
0
60000
50000
40000
30000
20000
A
B
B
C
D
B
C
B
C
Carbon Tetrachloride headspace concentration (area units)
Air area units
1ppm CT
CT/Tol
391
NG92
GBS
299
S17
S25
HC5
Treatments

FIG. 2-4 Carbon tetrachloride degradation with less than 1 ml headspace and modified nutrient media, with oxygen peaks to demonstrate reduced oxygen content in test vials. The oxygen peaks are unique per treatment and are not related as illustrated with the line graph.


150000000
100000000
50000000
0
Carbon Tetrachloride headspace concentration (area units)
A
B
B
C
D
B
C
B
C
1ppm CT
CT/Tol
391
NC92
GBS
299
S17
S25
HC5
Treatments

Subsequent laboratory tests focused on consortia NO92 and GBS, due to the significant potential shown by these consortia to degrade CT. In an effort to facilitate biodegradation by enhancing contaminant solubility, CT degradation was measured in the presence of 0.03% and 5% Tween-80 (Figure 2-5). Carbon tetrachloride was again degraded by both GBS and NO92 consortia without the presence of Tween-80. The presence of Tween-80 at 0.03% or 5% had no enhanced effect on CT degradation. Toluene degradation was complete for each of the consortia without the presence of Tween-80 (Figure 2-6). Toluene degradation was complete for bacterial consortium GBS in the presence of 0.03% Tween-80; however, Tol degradation appears to be hindered by the presence of 0.03% Tween-80 for consortium NO92. Furthermore, there was no enhanced rate of Tol degradation by NO92 following the addition of 5% Tween-80.

BIOLOG analyses were performed in an attempt to differentiate the seven bacterial consortia that were evaluated for their potential for CT degradation, and to identify the consortia members. A comparison of the BIOLOG results for the seven consortia (Figure 2-7) shows that consortium GBS is closely related to consortia S17, HC5, and S25. Consortium 391 is the next closest related consortium, followed by 299 and finally NO92. A similar comparison of the individual consortia members (Figure 2-8) shows that the NO92 peach isolate and the GBS white isolate are not closely related to any of the other consortia members. This may indicate that these two strains are responsible for the CT degradation seen with the GBS and NO92 consortia.

## DISCUSSION

The results presented above indicate that CT is biodegradable with Tol present as a cosubstrate and energy source by four bacterial consortia isolated from field and laboratory sites. It has been shown by numerous authors that Tol and other aromatic

FIG. 2-5 Carbon tetrachloride degradation in the presence of 0.03% and 5% Tween-80.

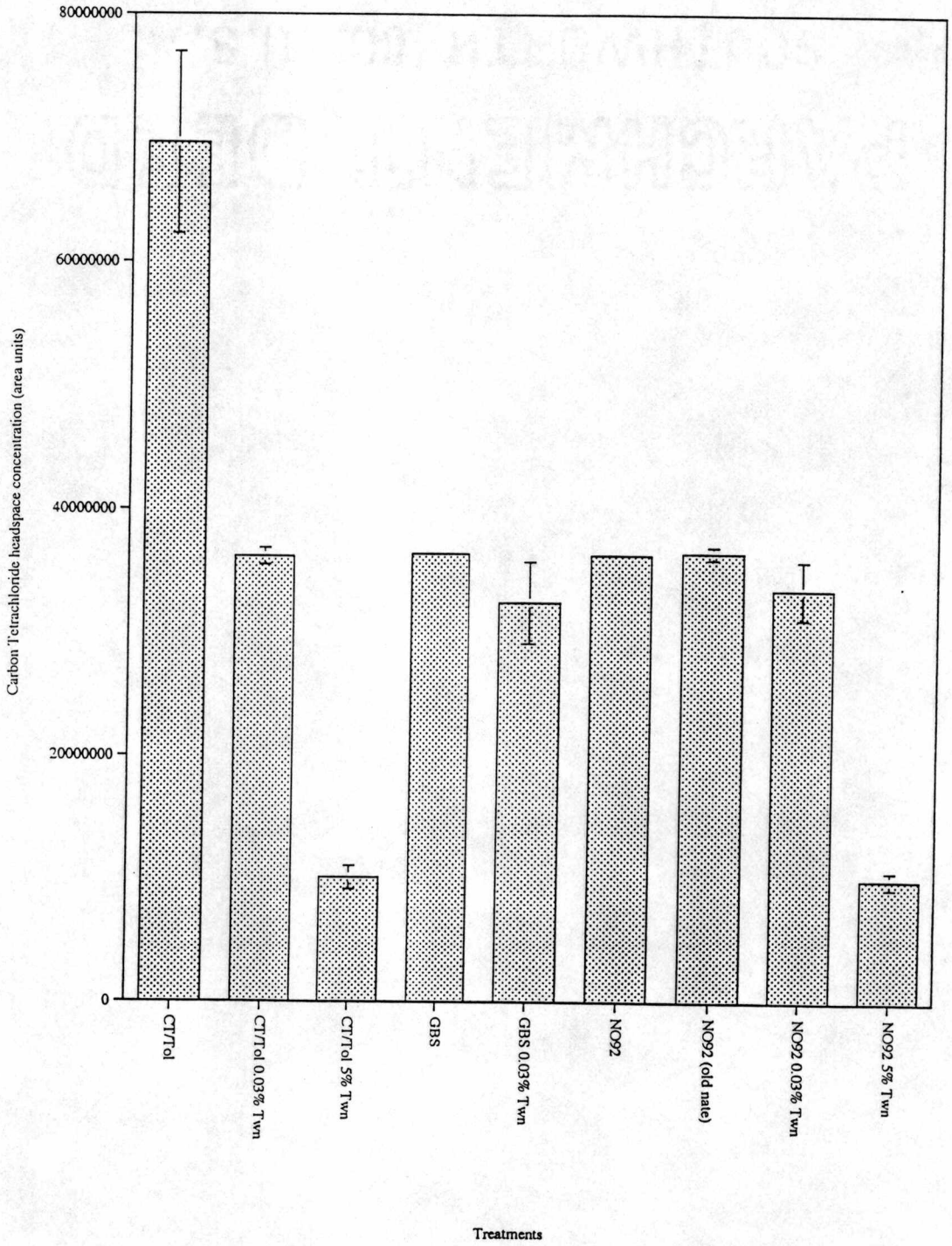

80000000
60000000
40000000
20000000
0
Carbon Tetrachloride headspace concentration (area units)
CT/Tol
CT/Tol 0.03% Twn
CT/Tol 5% Twn
GBS
GBS 0.03% Twn
NO92
NO92 (old nate)
NO92 0.03% Twn
NO92 5% Twn
Treatments

FIG. 2-6 Toluene degradation in the presence of 0.03% and 5% Tween-80.

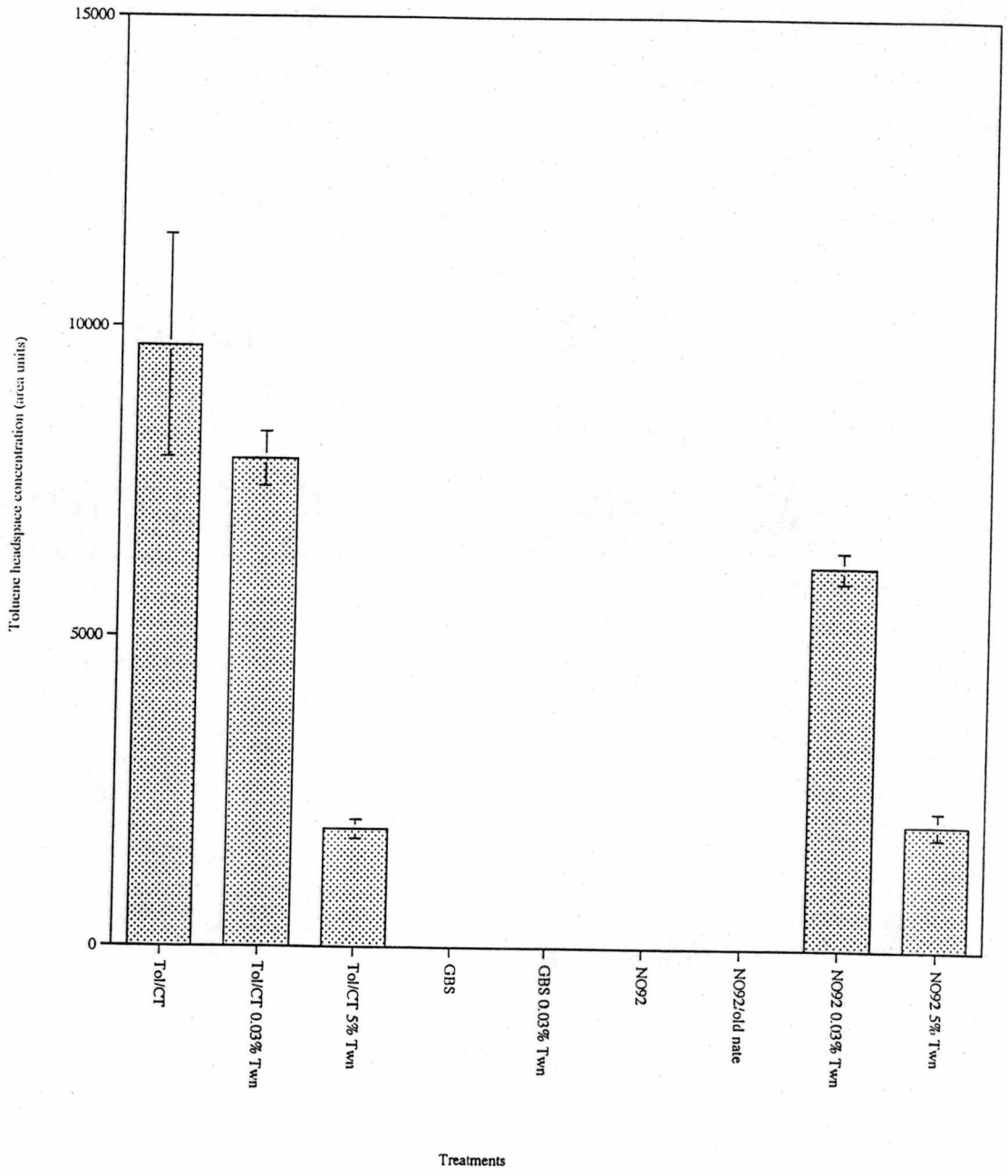

15000
10000
5000
0
Toluene headspace concentration (area units)
Tol/CT
Tol/CT 0.03% Twn
Tol/CT 5% Twn
GBS
GBS 0.03% Twn
NO92
NO92/old nate
NO92 0.03% Twn
NO92 5% Twn
Treatments

FIG. 2-7 Biolog results for consortia.

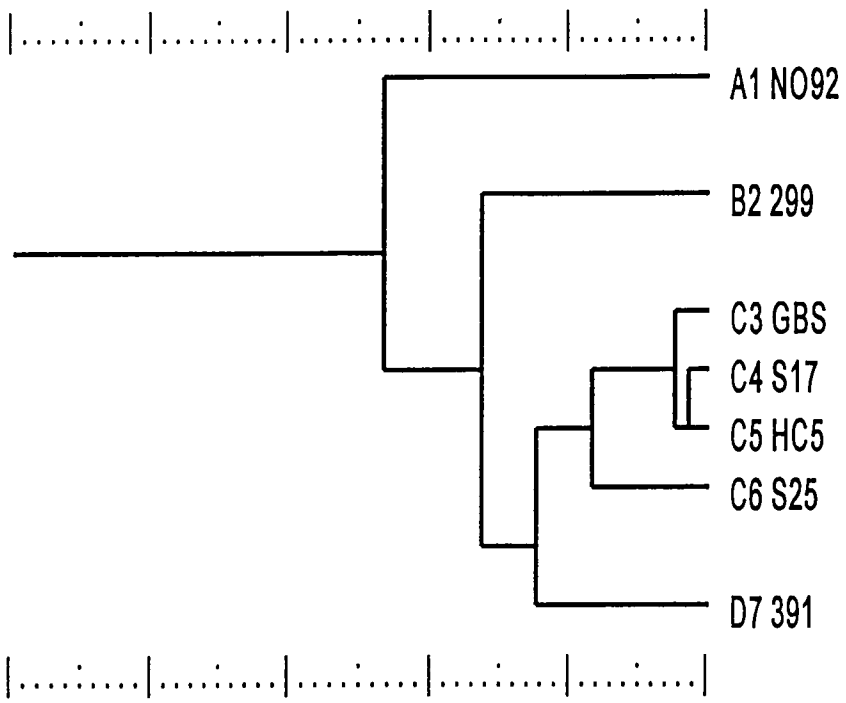
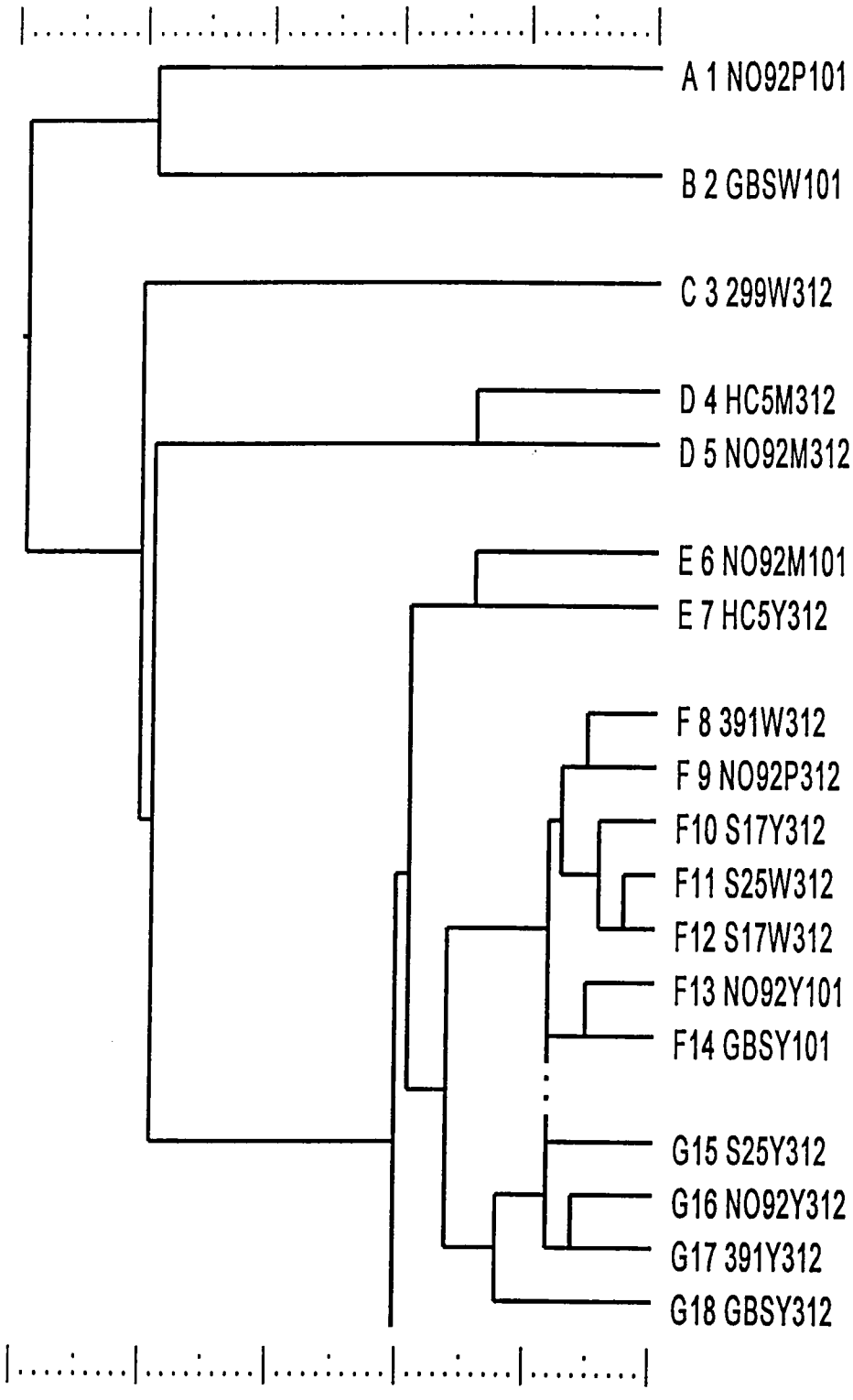

A1 NO92
B2 299
C3 GBS
C4 S17
C5 HC5
C6 S25
D7 391

FIG. 2-8 Biolog results for consortia members. The last four characters in the titles represent phenotype of isolate and date of analysis. For example, NO92P101 denotes the NO92 consortia, peach isolate, tested on October 1, 1995. W = white; M = mucus; Y=Yellow. 312 = March 12, 1995.


A 1 NO92P101
B 2 GBSW101
C 3 299W312
D 4 HC5M312
D 5 NO92M312
E 6 NO92M101
E 7 HC5Y312
F 8 391W312
F 9 NO92P312
F10 S17Y312
F11 S25W312
F12 S17W312
F13 NO92Y101
F14 GBSY101
G15 S25Y312
G16 NO92Y312
G17 391Y312
G18 GBSY312

hydrocarbons can serve as cometabolites for biodegradation of chlorinated solvents. Sewell et al. (1991) showed that Tol served as the initial source of reducing potential for the reductive dechlorination of tetrachloroethylene (PCE). Similarly, Liang et al. (1993) reported that the simultaneous anaerobic degradation of Tol and reductive dechlorination of PCE suggests that Tol served as an electron donor for the dechlorination process. In addition, Fan et al. (1993) reported that rapid and complete trichloroethylene (TCE) biodegradation required the concurrent biodegradation of Tol or phenol. Prior studies also linked TCE degradation to the aromatic biodegradative pathway of Tol (Nelson et al. 1987; Wackett et al. 1988).

Although anaerobic conditions in general exhibit a greater transformation potential for compounds with higher degrees of chlorine substitution (Semprini 1995), a variety of environmental conditions have been shown to support CT degradation including aerobic, denitrifying, methanogenic, and fumate respiring conditions. Criddle et al. (1990a) showed reductive dechlorination of CT under aerobic (low oxygen content only) conditions and fumate respiring conditions by *Escherichia* coli K-12. Semprini et al. (1992) reported enhanced *in situ* transformation of CT under denitrifying conditions using indigenous bacterial populations in a shallow sandy aquifer. In another study, Criddle et al. (1990b) demonstrated complete and rapid transformation of CT in groundwater aquifer solids by a denitrifying *Pseudomonas* sp. (strain KC) with the major end product being carbon dioxide. Carbon tetrachloride transformation was also shown using methanogenic mixed cultures with acetate as a primary substrate (Bouwer et al. 1983).

Enhanced CT biodegradation was achieved by reducing the available headspace in the test system (i.e., oxygen concentration) and by modifying the original minimal salts media to exclude reduced iron and copper. These results are in agreement with other studies in the current literature. Criddle et al. (1990a) showed that CT transformation

occurred in low oxygen samples using *Escherichia* coli K-12 but that no transformation occurred from cultures with high levels of oxygen, suggesting that high oxygen levels prevent metabolism of CT by out-competing CT for available electrons. Lewis et al. (1993) investigated the physiological factors affecting CT dehalogenation by *Pseudomonas* sp. strain KC and determined that low (<10 μM) additions of dissolved iron (II), iron (III), and cobalt (II) inhibited CT transformation. Furthermore, by removing iron from the trace metals solution used in media preparation and limiting copper to trace amounts of 1μM, increased CT transformation was achieved (Tartara et al. 1993).

Laboratory studies utilizing Tween-80, a non-ionic surfactant, to enhance degradation of CT and Tol by the GBS and NO92 bacterial consortia resulted in no enhanced effect from the addition of the surfactant on CT degradation and in one case, decreased degradation of Tol. Previous work indicated that CT and Tol aqueous solubilities were increased by the application of Tween-80 at low concentrations (0.03%), implying that bioavailability could be potentially increased. These results are supported by numerous authors (Aronstein et al. 1991; Aronstein et al. 1992; Edwards et al. 1991; Kile et al. 1989; Park et al. 1993; Shiau et al. 1994; Strong-Gunderson et al. 1994). One possible explanation for the lack of enhanced CT and Tol biodegradation using Tween-80 is surfactant toxicity to the microorganisms; however, turbidity in test systems and viability of bacteria after exposure to high concentrations of Tween-80 argue against it. Larson et al. (1982) reported in a study on the toxicity of linear alkylbenzene sulfonate (LAS) on microbial populations, that even at high doses the microbial structure was unaffected. Another plausible theory is that the bacteria preferentially degrade the surfactant as a carbon and energy source over CT and Tol. However, Laha et al. (1992) demonstrated that relatively high concentrations of surfactant would be required before there was substantial microbial inhibition due to preferential substrate utilization. In the same

study, they report that ten nonionic surfactants tested at concentrations above the aqueous CMC in the presence of soil showed inhibition of phenanthrene biodegradation. In contrast, sub-CMC surfactant doses had little or no effect on the biodegradation of phenanthrene. Their data suggests that a micellar surfactant-bacteria interaction may result in a reversible interference with cell membrane processes, thus inhibiting biodegradation. These results indicate that nonionic surfactant aqueous solubilization of CT and Tol from the gaseous phase may not be beneficial for the concomitant enhancement of groundwater remediation.

Results from this study indicate that CT can be biodegraded aerobically in the presence of Tol as a cometabolite under oxygen and iron limiting conditions. Chloroform was not found to be a major byproduct of the transformation process. The question remains regarding the specific bacterial isolate(s) responsible for CT degradation and is currently being investigated via 16S-RNA analyses. Preliminary results indicate the presence of a number of Rhodococcus species in the consortia (J. Zhou, personal communications). In order to extend these results into practical applications, it will be important to investigate the biodegradation rates of CT and Tol in soil matrices as well. In addition, it will be necessary to ascertain the specific end products produced in the CT transformation.

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# VITA

Shannon Bowden was born in Long Beach, California on June 6, 1968. She attended schools in the public system of Pope County Arkansas, where she graduated from Pottsville High School in May, 1986. She entered Arkansas Tech University, Russellville, Arkansas during August, 1986 where in May, 1992 she received the Bachelor of Science in Biology with a minor in secondary education. She began working at Lockheed Martin Energy Systems (formerly Martin Marietta Energy Systems), Oak Ridge, Tennessee immediately following graduation. She entered the Master's program in Environmental Toxicology at the University of Tennessee, Knoxville in August, 1992. In January, 1995 she began working at Oak Ridge Associated Universities in the Center for Epidemiologic Research as a research associate in occupational epidemiology. The master's degree was received May, 1996.