

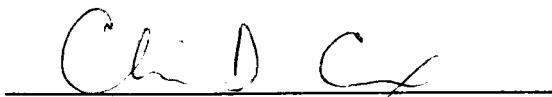
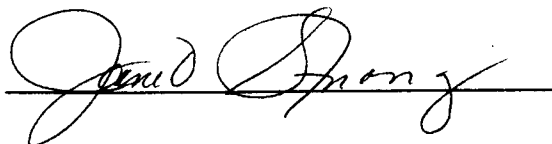
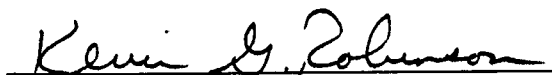
To the Graduate Council:

I am submitting herewith a thesis written by Marie T. Gillespie entitled "An Investigation into the Effects of a Nutrient-Surfactant Compound on the Bioremediation of Trichloroethylene". 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 Environmental Engineering.



Chris D. Cox, Major Professor

We have read this thesis and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of the Graduate School

**An Investigation into the Effects of a
Nutrient-Surfactant Compound on the
Bioremediation of Trichloroethylene**

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

Marie T. Gillespie
August 1997

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Abstract

Batch experiments were performed to determine the effects of BioTreat, a nutrient-surfactant compound, on *Burkholderia cepacia* G4 PR1₃₀₁ and the degradation of TCE by this bacteria. BioTreat was found to have surfactant properties as evidenced by its ability to lower the surface tension of water; an average critical micelle concentration (CMC) of 0.05% was measured. A partitioning coefficient, K_{m2} , analogous to K_m , was calculated on a mass basis rather than a molar basis since the molecular weight of BioTreat is not known by this researcher. Experimental data from abiotic solubilization experiments confirmed that BioTreat could enhance the solubilization of TCE at concentrations greater than the average CMC. BioTreat had no effect on increasing the apparent aqueous solubility of TCE at concentrations less than the average CMC. BioTreat was found to have some nutrient-providing capabilities. BioTreat is partially biodegradable by G4 and can support cell growth. BioTreat, at concentrations greater than the average CMC, inhibited the specific activity of the enzyme responsible for TCE degradation by G4. BioTreat was found to increase TCE remediation at concentrations less than or equal to the average CMC during degradation experiments, possibly due to increased cell numbers made possible by the increased nutrients available from BioTreat. Treatments containing low concentrations of BioTreat were found to have increased cell numbers when compared to treatments containing no BioTreat. BioTreat was found to inhibit TCE degradation at concentrations in excess of the average CMC, possibly from a toxic effect on the bacteria or oxygen limiting conditions.

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Chapter 1

Introduction

Trichloroethylene (TCE), a potential carcinogen, has been widely used as a solvent and as a degreaser. Contamination of soil and groundwater by TCE is a common problem throughout the United States. Its concentrations in these media are regulated by the United States Environmental Protection Agency (5 ppb in drinking water). The fate and transport of TCE in the subsurface is largely dependent on the sorptive capacity of soils. TCE present in contaminated soils can be sorbed to the soil, present as a dense nonaqueous-phase liquid (DNAPL), as a dissolved aqueous-phase contaminant, or as a gas-phase contaminant. Remediation activities have included pump-and-treat methods for groundwater contamination (Fountain et al., 1991; Gandi et al., 1995; Haley et al., 1991; West, 1992) and incineration of contaminated soil (Cohen and Johnson, 1989; Leuser et al., 1989) as well as other physical and chemical treatments such as chemical oxidation and vapor stripping. These methods can be expensive, time consuming, and are often impractical. In situ bioremediation is an attractive alternative for some sites because it can be cost effective, and is able to permanently eliminate the contaminant without the use of harsh chemical or physical treatments (McGrath, 1995; Sturman et al., 1995). Bioremediation can also be accomplished with little disruption to the site (compared to excavation of soil or the construction of new treatment facilities) and offers the advantage of less worker exposure to the contaminant.

Bioremediation can be limited by several factors. Bacteria require an electron donor, an electron acceptor, nutrients, and trace metals for growth and maintenance

activities (Beller et al., 1992; Criddle et al., 1990; Gantzer and Wackett, 1991; Maymou-Gatell et al., 1995; Tartara et al., 1993; Schwartz and Bar, 1995). The bacterium used in this study, *Burkholderia cepacia* G4 PR1₃₀₁, is an aerobic heterotroph. Aerobic heterotrophic bacteria use oxygen as the electron acceptor and organic carbon as the electron donor. Nitrogen, phosphate, magnesium, iron, zinc, and manganese are supplied as nutrients and trace metals. A lack of any of these compounds can result in a failure to initiate biodegradation, lead to incomplete degradation and undesirable transformation product formation, or slower degradation rates. The presence of a more readily degraded carbon source may result in preferential degradation of that carbon source instead of the targeted contaminant (Hyman et al., 1995; Reij et al., 1995; Sturman et al., 1995).

The sorption of the contaminant onto the soil also affects in situ biodegradation. Sorbed contaminants are not available for microbial degradation (Aronstein et al., 1991; Deziel et al., 1996; Harms and Zehnder, 1995; Laha and Luthy, 1992; Lee et al., 1988; Shiao et al., 1994; Strong-Gunderson et al., 1995; Thibault et al., 1996; Volkerling et al., 1995; Zhang and Miller, 1995). Therefore, the bioavailability of the contaminant strongly influences overall remediation rates. In order for a contaminant to be bioavailable, it must be present in the aqueous phase. Surfactants, or surface active agents, can be used to enhance the bioavailability of sorbed contaminants by increased solubilization.

1.1 Bioremediation of Trichloroethylene

TCE is biodegradable by a variety of microorganisms, such as Pseudomonads (Fan and Scow, 1993; Leahy et al., 1996; Munakata et al., 1996; Nelson et al., 1987; Phelps et al., 1990; Shields et al., 1989; 1991; 1992; 1995; Wackett and Gibson, 1988), methanotrophs (Brockman et al., 1995; Chu and Alvarez-Cohen, 1996; Little et

al., 1988; Thompson et al., 1994; Uchiyama et al., 1995) and Nitrosomonads (Arciero and Suk, 1986; Ely et al., 1995) both in the laboratory and in the environment. Most microorganisms capable of degrading TCE do so only in the presence of a co-substrate which induces production of non-specific enzymes. The organisms do not obtain energy for growth from the degradation process.

Recently, microorganisms have been isolated that degrade TCE without an inducing co-substrate (Munakata et al., 1996; Shields et al., 1992, 1995). In situ bioremediation can potentially be achieved by augmenting sites with these known contaminant degraders. Biostimulation, or the addition of nutrients to contaminated sites to stimulate the growth of indigenous microorganisms, can also be used at sites that have been shown to harbor contaminant degraders at low concentrations (Graham et al., 1995; Leavitt and Brown, 1994; Lindstrom et al., 1991; Raymond, 1974; Sveum et al., 1994).

1.2 Role of Surfactants in Enhancing Bioremediation Processes

Remediation of TCE in the subsurface environment is often difficult because of its ability to sorb to soil particles and volatilize in pore spaces, thus rendering it unavailable to microorganisms for biodegradation. TCE present as a DNAPL has a low surface area to volume ratio such that the area that can be contacted and subsequently degraded by microorganisms is low. High TCE concentrations are also toxic to microorganisms. Surfactants can affect the bioavailability of contaminants by enhancing the solubility of hydrophobic contaminants by partitioning these compounds into the hydrophobic core of surfactant micelles (Aronstein and Alexander, 1992; Chaiko et al., 1984; Edwards et al., 1991; Fountain et al., 1991; Jackson, 1993/94; Jafvert et al., 1994; Pennel et al., 1994; West and Harwell, 1992; Yeom et al., 1995).

In addition to the physical parameters changed by surfactant addition, surfactants that have nutritive value to the microorganisms can enhance the bioremediation process by providing elements necessary for growth, such as carbon, nitrogen, or phosphorus (Basseres and Ladousse, 1993; Churchill et al., 1995; Gillespie and Strong-Gunderson, 1997; Lindstrom et al., 1991; Shiao et al., 1994; Venosa et al., 1992). The addition of these elements in substrate-limiting conditions can possibly increase remediation rates by increasing cell numbers.

1.3 Objectives and Scope

The overall purpose of this study was to perform initial laboratory treatability studies in support of a field demonstration. The experiments here describe the effects of a commercial nutrient-surfactant compound (BioTreat, Rem-Tec, Inc.) on the bioremediation of TCE by *Burkholderia cepacia* G4 PR1₃₀₁ (Munakata et al., 1996). This work was confined to aqueous systems only. First, the surfactant capabilities of BioTreat were investigated by the measurement of TCE disappearance from the headspace. The apparent solubility of TCE in the liquid phase (water + micelles) is increased by the partitioning of TCE into micelles. An increase in the apparent solubility will produce a concentration gradient from the headspace into the liquid phase, thus a decrease in TCE headspace concentrations corresponds to an increase in micellar TCE partitioning. A modified partition coefficient for TCE, K_{m2} , was also determined.

Second, the nutritive value of BioTreat was confirmed by monitoring an increase in cell numbers. Its effect on the activity of the enzyme responsible for TCE degradation by *B. cepacia* was also investigated. Various concentrations of BioTreat were tested for their ability to enhance the degradation of low levels of TCE in aqueous only systems.

Chapter 2

Literature Review

2.1 Significance of Trichloroethylene as a Pollutant

Trichloroethylene (TCE) is used as a solvent for fats, waxes, oil, resins, rubber, paints, varnishes, lacquers, adhesives, cellulose esters and ethers, and as a dry cleaning fluid. It is also used as a degreaser and in the manufacture of organic chemicals and pharmaceuticals. Estimates for its use in western Europe, Japan and the United States in 1990 was 225 thousand tons (IARC 1995). It is a clear, colorless, nonflammable liquid with a sweet odor resembling that of chloroform (Merck Index, 1976).

TCE has been detected in air, water, soil, food, and animal tissues. It enters the environment by industrial discharges, municipal waste treatment plant discharges, spills, or leakage from underground storage tanks.

Acute health effects of TCE exposure to humans include irritation and burning of the skin, eyes, nose, throat, and lungs; severe exposure can even lead to death by ventricular fibrillation. Ingestion of 7,000 mg per kg body weight has been reported to be fatal to humans (ATSDR/TP-88/24, 1989). Estimated excess lifetime cancer risks from continuous exposure to TCE contaminated water are less than one in one million (1×10^{-6}) at average contamination levels found in groundwater (7.7×10^{-6} - 7.7×10^{-4} mM). At the highest concentrations observed in drinking water (0.27 mM), the excess lifetime cancer risk is estimated at one in one thousand (1×10^{-3}) based on the assumption that exposure is continuous over a lifetime (EPA-440/4-85-019, 1981).

TCE has also been shown to be toxic to microorganisms. Wacket and Gibson (1988) reported that TCE is toxic to *Pseudomonas putida* F1 as observed by a cessation of TCE degradation after exposure to 320 mM, and by decreased degradation rates at 15 mM, presumably from the formation of toxic degradation products. Chang and Alvarez-Cohen (1995) investigated the toxic effects of TCE on mixed methane, propane, toluene and phenol cultures. It was determined that the toxic effect was not due directly to TCE exposure, but from transformation products. Oldenhuis et al. (1991) reported that TCE toxicity in *Methylosinus trichosporium* OB3b was from the nonspecific reaction of TCE conversion products, such as TCE epoxide, with cell components. In a study by Hyman et al. (1995) the ammonia oxidizing capability of *Nitrosomonas europea* was decreased after TCE cometabolism. The rate of recovery was dependent on the synthesis of damaged cell proteins required to oxidize ammonia.

2.2 Bacteria Capable of Trichloroethylene Biodegradation

TCE is recalcitrant to degradation because of its highly oxidized state. However, several species of bacteria have been identified in recent years that can aerobically degrade TCE. Selected heterotrophs and methanotrophs capable of TCE biodegradation will be the focus of review. Specifically, *Burkholderia cepacia* G4 PR1₃₀₁ will be examined in detail.

Burkholderia cepacia G4 is a heterotrophic bacteria that was isolated from a industrial waste treatment lagoon at Pensacola Naval Air Station (NAS) in 1986 (Nelson et al., 1986). G4 is capable of aerobic degradation of TCE to carbon dioxide and chloride. At the time of isolation, its genus and species was unknown and it was referred to only as G4. Testing of G4 revealed that it would only degrade TCE if the NAS wastewater was present. Analysis of TCE degradation studies showed that a

component in the wastewater disappeared during degradation. In 1987, the mystery component was identified as phenol (Nelson et al., 1987).

Phenol was identified as the necessary component for TCE degradation based on gas chromatograph retention time and by mass-spectral analysis. Phenol addition to distilled water could replace the NAS wastewater in degradation studies and was also identified as the growth substrate. Further experiments revealed that toluene, *o*-cresol, and *m*-cresol also functioned as growth substrate and inducers for TCE degradation (Table 1).

Table 1. Chemicals that induce TCE degradation by G4.

Chemical Added (1 mM)	TCE remaining (mM) from 3.5 mM addition (24 hr degradation period)
None	3.35 $\pm$ 0.26
Phenol	0.04 $\pm$ 0.0
Toluene	<0.02
<i>o</i> -Cresol	<0.02
<i>m</i> -Cresol	0.07 $\pm$ 0.09

(Modification of Table 2 by Nelson et al., 1987.)

The specific rates of oxidation of phenol and toluene were similar (455 nmol O₂/min per mg protein for phenol, 543 nmol O₂/min per mg protein for toluene when induced by phenol; 202 nmol O₂/min per mg protein for phenol, 151 nmol O₂/min per mg protein for toluene when induced by toluene). Chloride was produced by G4 during TCE degradation at a linear rate of 3.6 nmol/min. This corresponded to a TCE degradation rate of 1.2 nmol/min. Catechol and 3-methylcatechol, aerobic degradative intermediates of toluene and phenol degradation, were also degraded at similar rates (data not shown by Nelson et al., 1987). The *meta* fission pathway for G4 oxidation

of phenol and toluene was indicated by the production of α -hydroxymuconic semialdehyde (a yellow product), which is a meta fission product of catechol. The failure of G4 to oxidize benzoate indicated that the TOL pathway, of which benzoate is an intermediate, is not used. The TOL pathway degrades toluene to catechol with toluene side-chain monooxygenase enzymes. Therefore, Nelson et al. (1987) concluded that G4 degrades toluene first by a mono or dioxygenase attack to form catechol, then proceeds by *meta* fission of the catechol to form α -hydroxy-*cis,cis*-muconic semialdehyde. These results were extrapolated to TCE degradation. TCE would be degraded by an initial attack by a mono or dioxygenase to form TCE epoxide which would decompose to form nonchlorinated compounds such as CO₂ and cellular carbon.

In 1989, Shields et al. further reported on the enzymes and pathway used by G4 to degrade toluene. *o*-Cresol, *m*-cresol, and 3-methylcatechol were identified in the culture supernatants after toluene degradation experiments by high pressure liquid chromatography and by ultraviolet-visible spectrum analysis. The presence of *o*-cresol indicated there was also an ortho fission pathway. The uptake of heavy ¹⁸O₂ confirmed monooxygenation. Therefore, it was concluded that the pathway for toluene catabolism by G4 proceeds by 2 sequential monooxygenations. First, the aromatic ring is hydroxylated at the *ortho* position, then at the *meta* position (Figure 1). It was not determined whether this occurred by the action of one or two enzymes.

The degradation pathway of TCE by G4, now identified as *Pseudomonas cepacia* G4, is reported by Shields et al. (1991). Mutants that could not degrade toluene were identified in order to find the enzyme responsible for TCE degradation by comparison with wild type cells that degrade TCE and toluene.

The oxidation of TFMP (*m*-trifluoromethylphenol) was used as an indicator of ring cleavage by oxygenase enzymes. TFMP is transformed to TFMC (3-

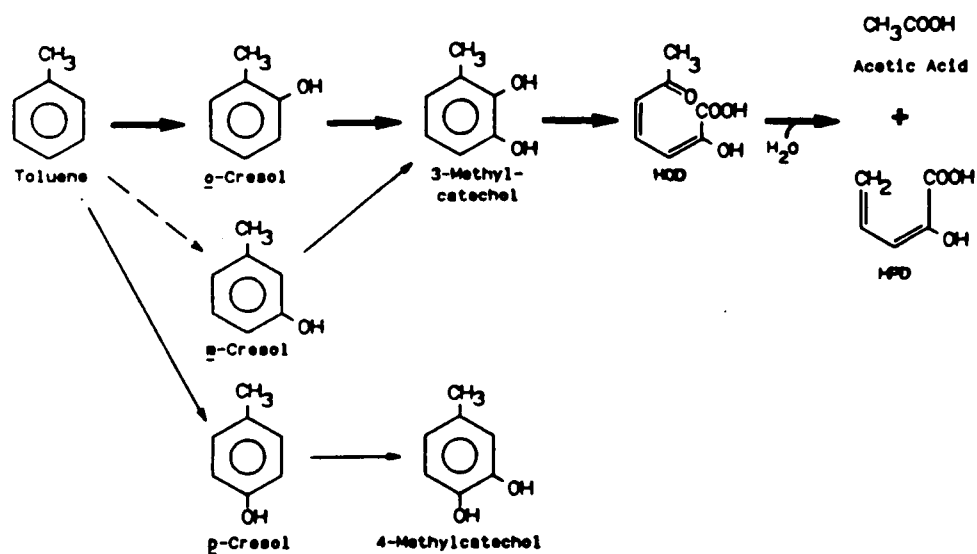


Figure 1. Toluene catabolism pathway of G4. (Source: Shields et al. 1989)

trifluoromethylcatechol) by addition of oxygen, and is cleaved at the *ortho* position to form TFHA (7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid) (Figure 2). TFHA is a yellow product and cannot be metabolized further. Cells sprayed with TFMP that were capable of oxygenation turned yellow within 5 minutes. Cells that could not oxidize TFMP were isolated using the TFMP oxidation assay method, and were checked for TCE, toluene, phenol, *o*-cresol, and *m*-cresol degradation. Mutants that were defective in toluene, phenol, *o*-cresol, and *m*-cresol degradation were designated as TOM A⁻. TOM A⁻ mutants were incapable of TCE degradation. Therefore, it was concluded that the enzymes required for TCE degradation were the same enzymes required for the degradation of toluene, phenol, *o*-cresol, and *m*-cresol. Thus, TCE was shown to be degraded in all cases in which an oxygenase is expressed that adds molecular oxygen to the aromatic nucleus of toluene. It was proposed that a new designation, *tom*, be used for the toluene *ortho*-monooxygenase enzyme responsible for TCE degradation.

A mutant of *Pseudomonas cepacia* G4 was developed in 1992 by Shields and Reagin that constitutively expressed *tom* and did not require aromatic induction of the TOM pathway enzymes for TCE degradation. To develop the strain, TOM⁻ mutants were obtained from a triparental mating between two *E. coli* strains and *P. cepacia* G4. TOM⁻ mutants were characterized by their inability to oxidize TFMP when grown with lactate provided as the sole carbon source and subsequently exposed to toluene. In other words, TOM⁻ mutants did not turn yellow when tested with the TFMP oxidation assay, indicating that they could not oxidize TCE even though they were grown in the presence of an inducing compound (toluene). These mutants were grown with phenol as their sole carbon source to identify revertant colonies that could again oxidize phenol and toluene. These colonies were assayed for TFMP oxidation activity. TFHA production over the growth curve for both wild type G4 and a revertant mutant, 5223-

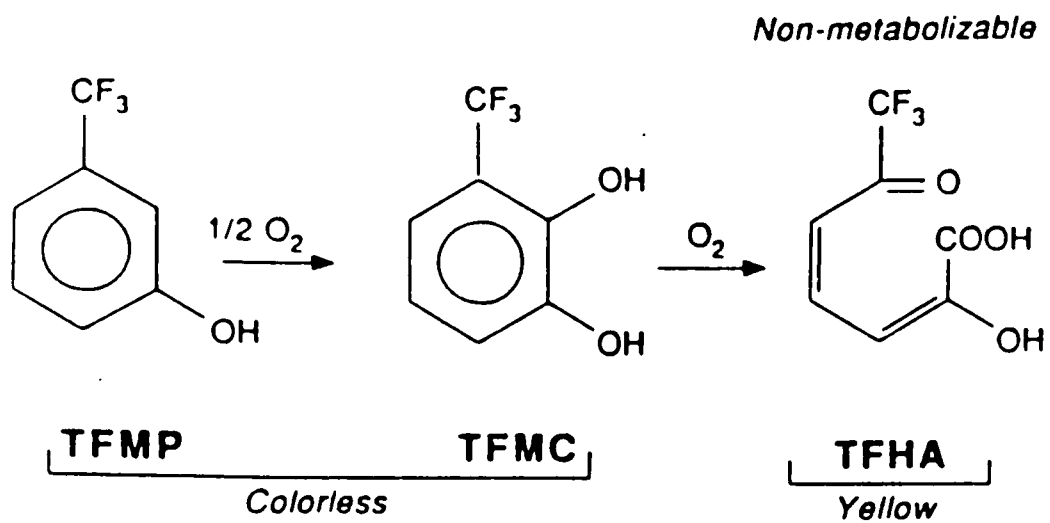


Figure 2. TFMP oxidation pathway. (Source: Shields et al. 1991.)

PR1 was assayed. 5223-PR1 was found to metabolize TFMP at a fairly constant rate over its growth cycle even without the presence of inducers (8 nmol TFHA per min per mg protein). G4 reached nearly the same rate of TFMP oxidation as 5223-PR1 (6.5 nmol TFHA per min per mg protein), but the rate decreased rapidly corresponding to a decrease in inducing compound concentration (Figure 3).

The growth of 5223-PR1 and G4 was tested in the presence of varying TCE concentrations (35, 210, 530, 3260 mM). To observe the effects of TCE metabolism toxicity, wild type G4 was not induced and was unable to degrade TCE. No effect on the growth of 5223-PR1 or G4 was detected at 35 and 210 mM TCE. However at 530 mM TCE, the growth of 5223-PR1 was depressed, while G4 (not induced) was unaffected. Since the uninduced wild type G4 was not affected, the toxicity experienced by 5223-PR1 was attributed to TCE metabolism. Slightly depressed growth rates were detected in both strains at 3260 mM TCE. The maximum rate of TCE degradation by 5223-PR1 was 1 nmol TCE per min per mg protein.

The effects of pH, temperature, and O₂ availability were also determined for 5223-PR1. Optimum environmental conditions were determined as follows: pH = 7, temperature = 30°C, and approximately 3 mg/L O₂ were required for degradation.

Mating, cloning, and hybridization techniques were used by Shields et al. (1995) to determine that the TCE degradative capability of G4 (now classified as *Burkholderia cepacia*) is found on the TOM plasmid. Details of the methods will not be discussed in this review.

Munakata-Marr et al. (1996) were the first to isolate *B. cepacia* G4 PR1₃₀₁. Unlike 5223-PR1, which was obtained using recombinant DNA techniques to incorporate additional genetic information such as antibiotic resistance and is subject to

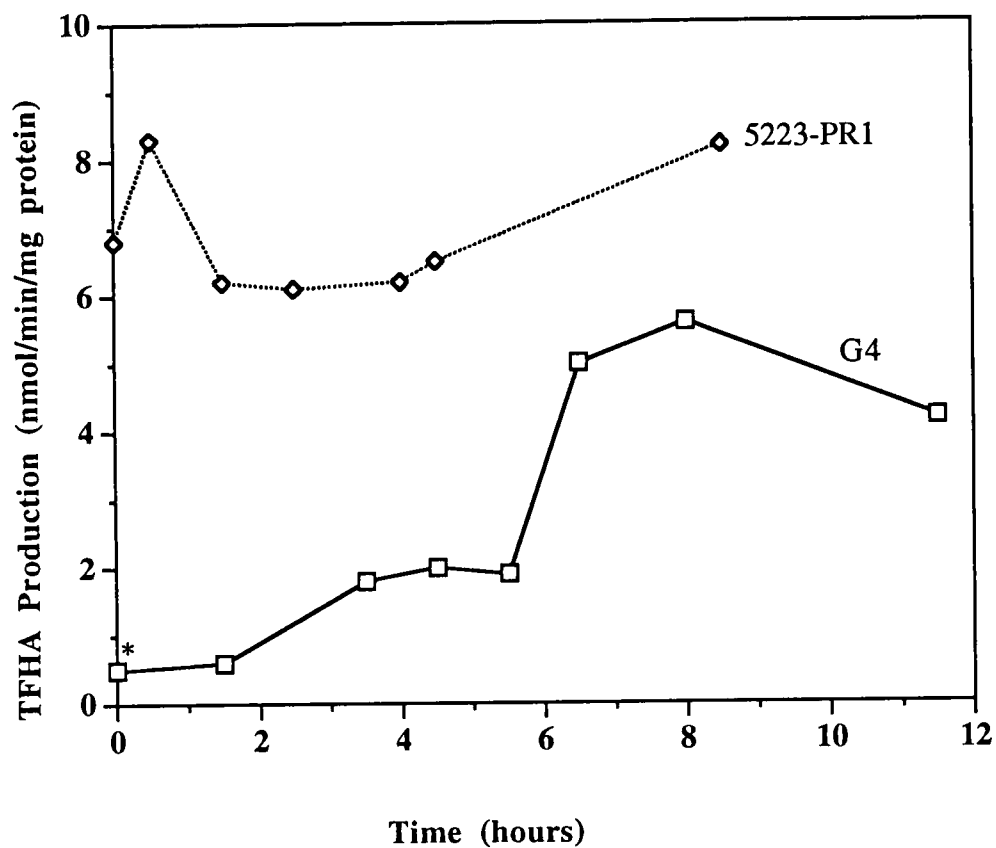


Figure 3. TFHA production rates from TFMP during batch growth of *P. cepacia* G4 and 5223-PR1. * 2 mM phenol added to G4 culture. (Source: Shields et al., 1992).

more severe regulatory approval, PR1₃₀₁ was obtained through NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) mutagenesis. NTG mutants are created by exposure to NTG, which disrupts the DNA sequence of the cell and causes mismatches of base pairs. The cell repairs itself, resulting in a new DNA sequence; thus, NTG mutagenesis is not considered recombinant technology and NTG-derived mutants are not classified as genetically engineered microorganisms. Mutants that could not metabolize phenol, toluene, and TCE were discovered that spontaneously reverted back to TCE⁺, tol⁺, and phe⁺. Thus, G4 PR1₃₀₁ is capable of noninduced constitutive TCE degradation.

The degradation of TCE by *B. cepacia* G4 was tested by Leahy et al. (1996). A maximum specific growth rate, μ , of G4 grown on lactate = 0.17/hr was obtained. A 20.8% inhibition effect was observed when G4 was grown on lactate in the presence of TCE (μ = 0.14/hr). A specific TCE degradation rate for cells induced by toluene = 3 nmol/mg/min was measured.

The inhibition effects of high substrate concentrations on phenol degradation by *P. cepacia* G4 were investigated by Folsom et al. (1990). At low substrate concentrations, phenol degradation proceeds at a first order rate. However, at higher phenol concentrations, growth is inhibited. This can be modeled by the Haldane expression, which is a modification of the Michaelis-Menton expression that incorporates an inhibition term, K_s . The initial reaction rate is written as follows:

$$v_o = \frac{V_{\max} [S]}{[S] + \frac{1}{K_{si}} [S]^2 + K_s} \quad \text{(Haldane expression)}$$

where v_o = initial reaction rate

$V_{\max}$ = maximum reaction rate

[S] = substrate (phenol) concentration

K_{SI} = inhibition term

K_s = substrate-binding affinity.

At concentrations < 50 mM, phenol was degraded according to first order kinetics. However, at concentrations above 50 mM, phenol degradation was inhibited according to the Haldane expression. The experimental values for K_s , V_{max} , and K_{SI} were 8.5 mM, 466 nmol/min per mg protein, and 454 mM, respectively.

Since TCE degradation by *P. cepacia* G4 is a cometabolic process in which phenol or toluene is the substrate, phenol degradation is expected to be inhibited by the presence of TCE. The effects of TCE on phenol degradation were modeled according to the Dixon plot (Figure 4), which enables determination of K_I from the slope of the line obtained when $1/v_o$ is plotted versus concentration of inhibitor.

$$\frac{1}{v_o} = \frac{[S] + K_s}{V_{max}[S]} + \frac{1}{V_{max}} \cdot \frac{K_s}{K_I} \cdot \frac{[I]}{[S]}$$

where K_I = inhibitor binding affinity

$[I]$ = inhibitor concentration (TCE).

Values of K_I < 25 mM were estimated by Folsom et al. (1990) from experimental data. TCE degradation kinetics were also determined: K_s = 3 mM, and V_{max} = 8 nmol/min per mg protein. Since the affinity for phenol and TCE are similar (K_s = 8 mM, 3 mM), there is a large inhibition of phenol degradation by the presence of TCE. A 50% reduction in phenol degradation was observed with equal initial concentrations of TCE and phenol (data not shown by Folsom et al.). Similar results are reported by Landa et al. (1994) for inhibition of toluene degradation by *P. cepacia* G4 (K_I = 30 mM). Thus, TCE is degraded much more slowly than the growth substrate (V_{max} phenol = 466 nmol/min per mg protein, V_{max} TCE = 8 nmol/min per mg protein) and competitive

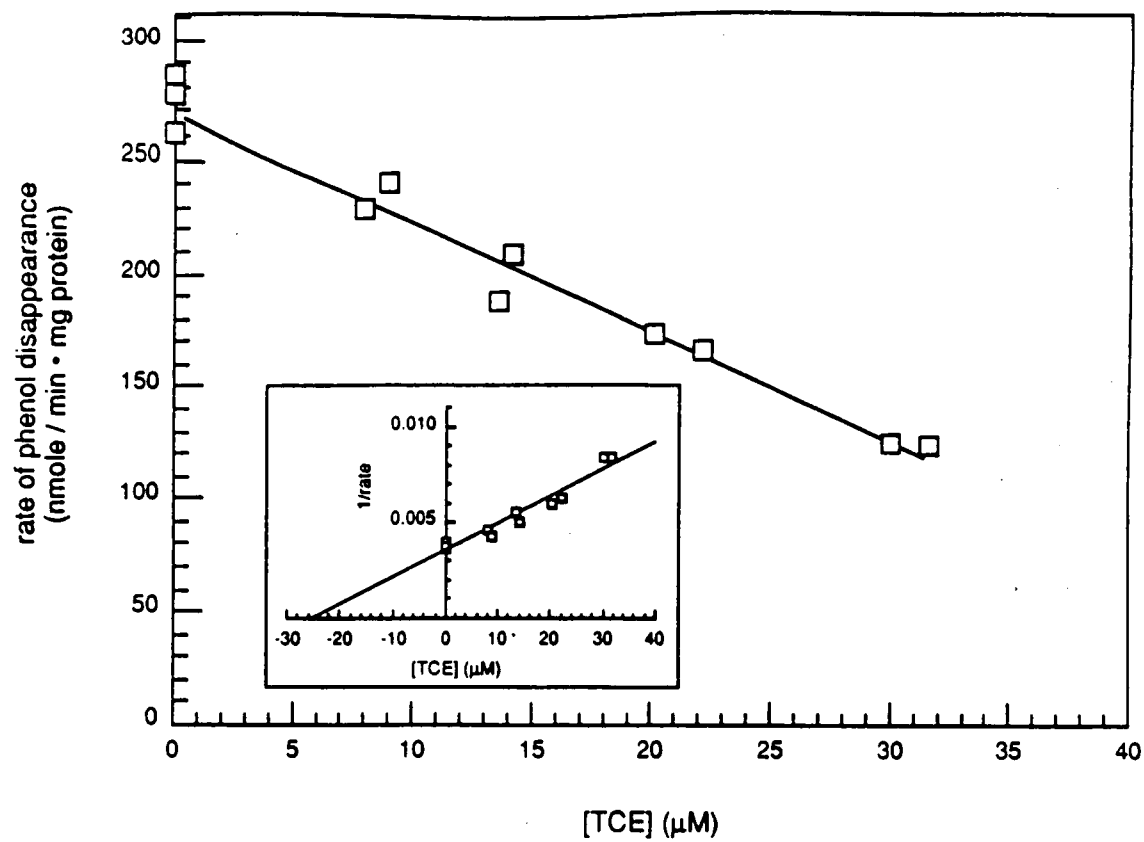


Figure 4. Inhibition of phenol degradation by TCE (Dixon plot inset). (Source: Folsom et al., 1990).

inhibition of the enzyme has been shown to occur based on calculations of K_i .

Other heterotrophs have been shown to degrade TCE when grown on a variety of substrates. Heterotrophic enrichment cultures were obtained from subsurface sediments at the Savannah River Plant (Aiken, SC.) by Fliermans et al. (1988). Methanol, methane, propane, and Trypticase plus yeast extract were supplied as energy sources. From the 400 enrichments and incubation mixtures, 11 cultures were shown to degrade 3.8×10^{-4} M TCE to levels 98 - 99.5% below controls (data not shown by Fliermans et al.). However, incubations in excess of two weeks were required. TCE degradation occurred only after late stationary phase was achieved, thus no cell growth occurred during TCE degradation periods. No evidence was obtained that TCE degradation yielded any benefit to the microorganisms, such as energy formation or new cell growth. This is commonly expected since aerobic TCE degradation is a cometabolic process in which no energy gain has ever been identified. These mixed heterotrophic cultures were able to withstand TCE exposures up to 2.3×10^{-3} M in the contaminated environments in which they were obtained. No bacteria were isolated from subsurface samples containing TCE at concentrations greater than the 2.3×10^{-3} M threshold.

TCE degradation was evaluated in situ at the Moffet Field Site (Mountain View, CA; Hopkins and McCarty, 1995). Phenol and toluene were provided as primary substrates for indigenous microorganisms. TCE (1.9×10^{-3} M) was injected into horizontal groundwater wells and its disappearance was measured over time. TCE removal efficiencies were similar regardless of whether toluene or phenol were used for induction (93% for toluene feed experiments, 94% for phenol feed experiments). It was determined that the same microorganisms were responsible for TCE degradation during phenol and toluene fed experiments due to the fact that no lag period was observed when the growth substrate was switched from phenol to toluene. *o*-Cresol

was detected after the substrate change, providing evidence that TOM was the oxygenase responsible for degradation by the indigenous bacteria. This is the same enzyme system used by *B. cepacia* G4. It was proposed that higher removal efficiencies could be obtained if greater biomass could be maintained. However, addition of growth substrate to produce more biomass would increase the dissolved oxygen requirements.

A pure culture, *Pseudomonas putida* F1, capable of TCE degradation was reported by Wacket and Gibson (1988). No kinetic data was provided; however, a 68% removal of TCE from a 20 mM TCE solution after 1 hr was reported. At concentrations > 320 mM TCE degradation ceased. This data suggests TCE metabolic by-product toxicity.

Xanthobacter sp. strain Py2 grown on propene was shown to degrade TCE (Reij et al., 1995). Maximum degradation rates for TCE were much slower than for propene (TCE $V_{\max} = 16$ nmol/min/mg, $K_m = 116$ mM; propene $V_{\max} = 75$ nmol/min/mg, $K_m = 0.62$ mM). A transformation capacity (defined by Reij et al. as the total amount of TCE that could be degraded if no other substrates were present) for TCE (0.03 g TCE/g biomass) was measured. This organism was determined not to be a good candidate for TCE degradation by Reij et al. because of its low specific degradation rates and low affinity for TCE.

Methanotrophs are bacteria which use methane as their sole carbon and energy source. Methanotrophs are divided into 3 classes: I, II, and X on the basis of intracytoplasmic membrane structure, DNA base content, and phospholipid fatty acid content. Methanotrophs have been found to degrade TCE, mainly by the action of the MMO enzyme (methane monooxygenase), which is synthesized in two forms: soluble (sMMO) and particulate (pMMO). MMO catalyzes the addition of molecular oxygen to

methane to form methanol. However, this nonspecific enzyme can also oxidize many other chemicals, such as chlorinated compounds.

The first report of a pure methanotroph culture able to degrade TCE was by Little et al. (1988). Two strains, 46-1 and 68-1, were isolated from a well at a waste disposal site near Oak Ridge, Tennessee where direct dumping of chlorinated solvents occurred from 1970 - 1981. Both strains were found to be type I methanotrophs. In batch culture degradation experiments with methane-limited conditions, up to 40% of the initial TCE concentration was degraded in 20 days. Approximately 15% of the TCE was converted to stable water soluble breakdown products (glyoxylic acid and dichloroacetic acid), while 11% was converted to CO₂, as determined by radiolabel studies. Glyoxylic and dichloroacetic acid were postulated to originate from the spontaneous decomposure of TCE epoxide, which resulted from the monooxygenation of TCE by methane monooxygenase. The bacteria were unable to metabolize TCE completely to CO₂ and Cl⁻; no metabolic benefit was provided to the microorganisms because it was a cometabolic process by the nonspecific enzyme activity of methane monooxygenase.

Methylosinus trichosporium OB3b has been studied by many researchers for its TCE degrading capabilities. It is a type II methanotroph that expresses sMMO under copper limiting conditions (Stanley et al., 1983). Tsien et al. (1989) reported maximum degradation rates of TCE by OB3b at 1.2 mmol/hr per g cells dry weight. This rapid rate of degradation only occurred when sMMO was present. However, this rate decreased over time, corresponding to a depletion of sMMO and reducing power (NADH).

Speitel and Leonard (1992) investigated the effects of enzyme competition between chloroform (another chlorinated solvent) and methane on the degradation of TCE by OB3b and reducing power availability by the use of a sequencing biofilm

reactor. TCE degradation by this methanotroph is expected to behave in the same manner with respect to enzyme inhibition since the same enzyme is used. Pseudo first-order kinetics are followed at low chlorinated solvent concentrations, where the degradation rate, r , is defined as follows:

$$r = -k_1 \times S$$

where r = degradation rate, mg/L day

k_1 = pseudo-first order rate degradation rate constant, L/mg TSS day

X = cell concentration, mg TSS/L

S = chloroform concentration, mg/L.

However, in the presence of methane, competitive inhibition of the enzyme occurred, and degradation rates decreased significantly. A methane concentration of 3.5×10^{-5} M decreased the rate constant by 93%. In order to observe the effects of methane and chloroform on the degradation rates by MMO, the bioreactor was operated in alternating cycles of growth and degradation. In the degradation mode, chloroform was supplied along with formate (to supply NADH) and 2.5×10^{-4} M oxygen. During degradation cycles, the bacteria were starving, and internal energy stores of reducing power (such as NADH) and sMMO are depleted over time. Thus, the bioreactor was cycled to growth mode in which methane was supplied to stimulate new cell growth and production of sMMO. Oxygen was supplied during both modes because 6.25×10^{-5} - 1×10^{-4} M O_2 per 6.25×10^{-5} M CH_4 oxidized were required. When formate was supplied during degradation cycles, approximately 80% of the chloroform was removed during the first 2 days of bioreactor operation, as opposed to 50% chloroform removal when no formate was supplied. The maximum rate constant, k_1 , for formate treatments = 0.0075 L/mg TSS-day was observed. Bioreactor runs without formate

reached a maximum rate constant = 0.0018 L/mg TSS-day. However, after the first few days of operation, formate had no beneficial affect on chloroform degradation; thus, reducing power was not limiting in this design. These results were compared to continuous flow reactor operation, in which both methane and chloroform were supplied continuously. A rate constant of 0.0006 L/mg TSS day (one order of magnitude lower than that obtained with sequencing operation) was observed for continuous flow operation. An average of 7% chloroform removal was obtained with the continuous flow design. This was attributed to a much smaller biomass concentration present in the bioreactor because methane was supplied in the aqueous phase at 2.5×10^{-4} M as opposed to the 4.4×10^{-3} M methane supplied as gas during sequencing operation. From these results, it was determined that the sequencing biofilm reactor was a better design for chloroform removal, and that decreased degradation rates over time were not due to the lack of reducing power. It was proposed that enzyme inactivation, or metabolic intermediate toxicity was responsible for decreased degradation rates for reactor operation times greater than two days.

Fox et al. (1990) reported that sMMO could degrade several chlorinated aliphatics: ethylene, vinyl chloride, *cis*-dichloroethylene, *trans*-dichloroethylene, vinylidene chloride, and TCE. PCE (perchloroethylene) was not oxidized and was used to determine the nature of the enzyme inhibition. At low PCE concentrations, the effect of TCE and substrate inhibition was noncompetitive; both the inhibitor and substrate bind to different sites on the enzyme. However at higher PCE concentrations, the inhibition was found to be competitive; the inhibitor and substrate bind at the same site on the enzyme. Enzyme inactivation was also found to be time-dependent as a function of TCE oxidation. Each molecule of hydroxylase could turn over 200 molecules of TCE before complete inactivation. However, enzyme inactivation was not due directly to the presence of TCE oxidation intermediates such as

TCE epoxide as previously thought, but rather due to some other labile intermediate, possibly from the isomerization of the TCE epoxide or some non enzyme-catalyzed hydrolysis product. Similar results were reported by Oldenhuis et al. (1991). Oldenhuis et al. used radiolabeled $^{14}\text{C}[\text{TCE}]$ to observe the toxicity caused by nonspecific binding of TCE degradation products with cellular components, including sMMO.

A summary of degradation rates for the various bacteria discussed here are presented in Table 2.

Table 2. Summary of maximum TCE degradation rates for the bacteria discussed.

Organism	Inducer	Max TCE degradation rate
G4	phenol/toluene	3 nmoles/min/mg
<i>P. cepacia</i> G4 PR1	none	1 nmoles/min/mg
<i>B. cepacia</i> G4	toluene	3 nmoles/min/mg
<i>Xanthobacter</i>	propene	16 nmoles/min/mg
OB3b	methane	20 nmoles/min/mg

A direct comparison of kinetic data for the bacteria is not possible due to the fact that different methods were used by different researchers and complete kinetic data was not reported for each strain. However, a comparison of $V_{\max}$ values for the bacteria discussed here reveals that the strains capable of cometabolic degradation degraded TCE at the fastest rates. The constitutive TCE degrader, *B. cepacia* G4 PR1, degraded TCE at the slowest rate. A disadvantage of using cometabolic strains is the increased worker exposure to the inducing compound, which in most cases is a hazardous chemical in itself. Also, residual inducer concentrations must be considered if the remediation activity fails or the bacteria does not degrade the inducer or the targeted contaminant. An advantage of using a constitutive TCE degrader is that there is no

increased risk due to chemical exposure and no residual concentrations of inducer are possible.

2.3 Surfactant Enhanced Remediation

The use of surfactants to increase bioremediation rates is a topic of interest to scientists and engineers in the environmental field. In order for a contaminant to be biodegraded by microorganisms, it must be bioavailable, or present in the aqueous phase. Remediation is difficult when the contaminants are not bioavailable; for example, contaminants can be sorbed to soil particles, present as a non-aqueous phase liquid (NAPL), or volatilized within the pore spaces. Surfactants can enhance contaminant remediation rates by two mechanisms, solubilization and mobilization.

Surfactants are characterized by their ability to lower interfacial tensions at liquid-liquid and liquid-gas interfaces. A surfactant is a surface active compound and will concentrate at these interfaces. A surfactant monomer is amphiphilic, having both a hydrophobic tail and a hydrophilic head group. This characteristic enables surfactants to dissolve in water as monomers, form micelles, or adsorb to a surface. The charge on the head group determines the surfactant classification. A positively-charged head group indicates a cationic surfactant, a negatively-charged head group indicates an anionic surfactant, and a nonionic surfactant has no charge. It is also possible for the head group to carry both positive and negative charge; in this case it is called zwitterionic (West and Harwell, 1992). Above a certain concentration known as the critical micelle concentration (CMC), surfactants self-associate into clusters called micelles. A micelle consists of surfactant monomers oriented with their hydrophobic tails to the inside of the aggregation and the hydrophilic heads to the outside. The CMC for each surfactant is different.

Mobilization of contaminants is achieved by lowering the interfacial tensions at the liquid-liquid interface (Jackson, 1993/94; Volkering et al., 1995; West and Harwell, 1992). This mechanism is applicable for releasing contaminants trapped by capillary forces in sediments. Surfactants at concentrations many times in excess of the CMC may reduce the interfacial tension to ultra-low levels (< 1 dyne cm, the surface tension of water is approximately 75 dynes/cm) and cause mobilization of these compounds (Fountain et al., 1991). This technique has been used to increase pump and treat efficiencies, but it is not without its own dangers. DNAPLs (dense nonaqueous phase liquids) migrate downward through the subsurface under the force of gravity until all of the contaminant is immobilized by adsorption onto the soil or the DNAPL is stopped by a layer of impermeable soil. A DNAPL pool then spreads laterally. The addition of excess quantities of surfactant with the intent of mobilization can lower the interfacial tension enough to allow the surfactant to permeate the previously impermeable layer and subsequently contaminate the clean soil beneath (Fountain et al., 1991).

The solubility of hydrophobic contaminants can be enhanced by the presence of surfactants. Typically, solubilization is discussed in terms of contaminant partitioning into the hydrophobic core of the micelle. Solubilization begins at the CMC and increases linearly with increasing surfactant concentration. As more micelles are formed with increasing surfactant concentration, more contaminant can be partitioned into the hydrophobic cores. Figure 5 depicts a typical plot of the relationship between the concentration of contaminant solubilized and surfactant concentration in the presence of excess hydrophobic contaminant. The slope of the line obtained is the MSR, or the molar solubilization ratio (Attwood and Florence, 1983). From this relationship, it can be seen that solubilization occurs as a function of the concentration of surfactant in the micellar phase.

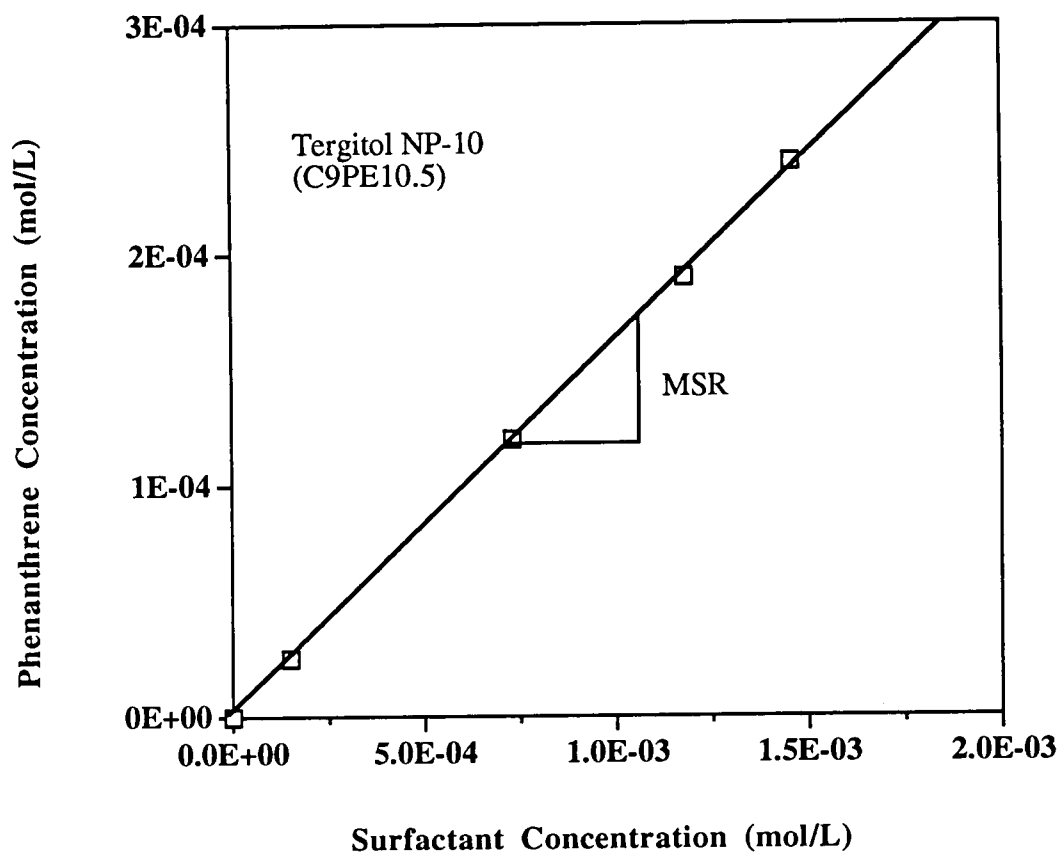


Figure 5. Typical plot of contaminant solubilization by a surfactant.
(Source: Edwards et al., 1991).

Several researchers have demonstrated solubility enhancement for hydrophobic contaminants above the CMC. Kile and Chiou (1989) found that micellar solubilization is the primary mechanism for solubilization of 1,1-bis(*p*-chloro-phenyl)-2,2,2-trichloroethane (DDT). Edwards et al. (1990) reported that polycyclic aromatic hydrocarbon solubility (at bulk aqueous concentrations) increased linearly as the surfactant concentration increased above the CMC. Laha and Luthy (1992) have shown that surfactant concentrations greater than the CMC are required to enhance solubilization of phenanthrene from soil. Park and Jaffe (1993) demonstrated that the partitioning of nonionic compounds increased directly proportionally to the mass of the micelles present in solution. The solubility of chlorinated organics such as PCE, TCE, and dichloroethylene (DCE) was also shown to increase with surfactant concentration above the CMC (Shiau et al., 1994).

Solubilization can also occur below the CMC. Jafvert et al. (1994) found that solubilization below the CMC occurred for 2 reasons. The first pertains to commercial grade surfactants which consist of mixtures with hydrophobic tails of different lengths. Surfactant monomers with longer tails are more hydrophobic and tend to form micelles at lower concentrations than monomers with shorter tails. Therefore, a CMC may be poorly defined and surfactant micelles may occur over a wide range of concentrations. One report by Kuneida et al. (1995) states that mixtures of surfactants are actually better solubilizers. Secondly, monomers themselves can enhance the apparent solubility of hydrophobic contaminants by hydrophobic tail-contaminant interactions. Yeom et al. (1996) proposed that surfactant monomers penetrate coal tar and cause swelling of the matrix, thereby increasing matrix diffusivity and the rate of solubilization.

Researchers have shown enhanced biodegradation rates as an indicator of enhanced solubility of hydrophobic compounds with surfactant concentrations below the CMC. Aronstein et al. (1991) reported that two nonionic surfactants at low

concentrations increased the biodegradation of phenanthrene. It was proposed that the instantaneous rate of removal of the contaminant from the soil was increased due to the presence of surfactant, even though the equilibrium concentrations of contaminant in solution remained constant. Therefore, the overall solubilization of the contaminant was not increased at concentrations below the CMC, but the dynamic exchange of contaminant between the aqueous phase and the soil was increased; a greater amount of substrate was made available to the microorganisms for biodegradation per unit time. In 1992, Aronstein and Alexander reported that low concentrations of two nonionic surfactants stimulated the biodegradation of sorbed phenanthrene and biphenyl by enhanced partitioning of the contaminants.

Strong-Gunderson et al. (1995) proposed that adding surfactants at concentrations below the CMC ahead of the path of a contaminant plume may prevent contaminant sorption to soil by surfactant sorption to soil binding sites and facilitate contaminant removal by soil washing and pump and treat activities. The addition of Tween 80, a nonionic surfactant, below the CMC had no effect on increasing toluene desorption from soil, but the addition of surfactant before toluene was added to the soil increased the bioavailability.

The MSR (molar solubilization ratio) can be used to determine the effectiveness of a surfactant at solubilizing a particular contaminant. The MSR is defined as the moles of contaminant solubilized per mole of surfactant in solution. When contaminant concentration is plotted against surfactant concentration, the resulting slope is the MSR (see figure 5). In the presence of excess TCE, the MSR can be calculated as follows (Dzombak and Luthy, 1984):

$$\text{MSR} = \frac{S_{\text{mic}} - S_{\text{CMC}}}{C_{\text{surf}} - \text{CMC}} \quad (2-1)$$

where S_{mic} = total apparent solubility of TCE in moles per liter at a surfactant concentration greater than the CMC

S_{CMC} = apparent solubility of TCE in moles per liter at the CMC

C_{surf} = surfactant concentration at which S_{mic} is determined

CMC = critical micelle concentration.

The excess contaminant method is limited by the fact that it does not describe the effects of contaminant concentration on partitioning between the aqueous and micelle pseudophases (Gadelle et al., 1995; Rouse, et al. 1995; Smith et al., 1989). The aqueous contaminant concentration is held constant because it is supplied in excess above the saturation concentration. However, this relationship can also be applied for low TCE concentrations. In this case, $S_{mic} - S_{CMC}$ in equation 2-1 is replaced by C_{mic} where C_{mic} = concentration of contaminant in the micelle.

The molar partition coefficient, K_m , can also be used to determine the solubility enhancement of a contaminant with the use of a surfactant. K_m is defined as the ratio of the mole fraction of the contaminant in the micellar pseudophase to the mole fraction of contaminant in the aqueous pseudophase (Edwards et al., 1990).

$$K_m = \frac{X_m}{X_a} \quad (2-2)$$

where X_m = mole fraction of TCE in the micellar pseudophase

X_a = mole fraction of TCE in the aqueous pseudophase.

$$X_m = \frac{\text{moles TCE}_{micelles}}{\text{moles TCE}_{micelles} + \text{moles surfactant}_{micelles}} \quad (2-3)$$

$$X_a = \frac{\text{moles TCE}_{in \text{ aqueous pseudophase}}}{\text{moles TCE}_{in \text{ aqueous pseudophase}} + \text{moles water}} \quad (2-4)$$

K_m can also be calculated in terms of MSR (Edwards et al., 1990).

$$K_m = \frac{\frac{MSR}{1 + MSR}}{X_a} \quad (2-5)$$

In the research presented here, only low concentrations of TCE were investigated. The surfactant used was a proprietary commercial product, and the molecular weight was not known by this researcher. Therefore, the number of moles of surfactant could not be determined. However, an alternative partitioning coefficient, K_{m2} , was calculated based on the mass of surfactant in solution, rather than the moles of surfactant. Development of the theory and the calculations involved in obtaining K_{m2} are included in Appendix I.

An important consideration in choosing a surfactant for remediation activities is surfactant-soil and surfactant-cell interactions. Preliminary studies must be performed to determine if the surfactant being considered is compatible not only with the site characteristics, but also with the microorganisms that will be used. Surfactant losses due to sorption, precipitation, liquid crystal formation, and partitioning into the DNAPL must also be considered (Jackson, 1993/94).

Surfactants, depending on molecular structure and concentration, have been found to be toxic to microorganisms. In a study by Ankley and Burkhard (1992), the toxicity of the effluent from a publicly owned treatment works that received no secondary treatment was tested. It was found that the majority of the toxicity to the test organisms was due to the presence of surfactants, not to the residual chlorine concentrations. Aronstein et al. (1991) found that a nonionic surfactant at twice the CMC was toxic to phenanthrene degraders. Larson and Maki (1982) reported that microbial exposure to 5 mg/L LAS (linear alkylbenzene sulfonate), an anionic

surfactant, depressed rates of glucose metabolism in a microbial ecosystem. Glucose metabolism is an indirect measure of the chemical's toxicity to the microorganisms. Surfactants can also increase toxicity to the bacteria by enhancing solubilization, leading to toxic levels of the contaminant in contact with the bacteria (Thibault et al., 1996). Contrary results were found by Volkering et al. (1995). Volkering suggested that the toxicity of naphthalene decreased in the presence of micelles. Nonionic surfactants are found to be generally less toxic than ionic surfactants (Laha and Luthy, 1992; Volkering et al., 1995). For example, Volkering et al. (1995) found that a nonionic surfactant was not toxic to the microorganisms under study, even at concentrations up to 10 g/L.

Surfactant persistence should also be taken into consideration. Residual surfactant concentrations after the remediation activity is complete is undesirable for environmental reasons and from a regulatory standpoint. Surfactants resistant to physical or biological degradation may persist in the environment. Biodegradable surfactants may also stimulate the growth of indigenous microorganisms. Certain compounds that are marketed as nutrient complexes have also been found to have surfactant-like properties such as increased contaminant solubility and mobilization.

The addition of nutrient solutions has been shown to increase remediation rates. Inipol EAP22 is an oleophilic fertilizer containing nitrogen and phosphorus that was applied to beach sediments in the clean up of the Exxon Valdez oil spill (Lindstrom et al., 1991). Inipol EAP22 also has surfactant properties, in that it significantly lowers surface tensions. It has been hypothesized that the surface-active components of Inipol EAP22 contributed to the enhanced mineralization rates of contaminants present after the oil spill (Churchill et al., 1995). Basseres and Ladousse (1993) found that the oleic acid present in Inipol EAP22 was degradable by indigenous bacteria at the oil spill site. Inipol was speculated to have not only increased the bioavailability of the

hydrocarbons, but also may have acted as a carbon and nutrient source for the bacteria, which lead to increased cell numbers and increased remediation rates. Shiau et al. (1994) investigated the use of edible food-grade surfactants. It was found that these surfactants are as effective in solubilizing hydrophobic compounds as the non-edible surfactants investigated by West (1992), as indicated by their similar K_m values. It was suggested that it may be easier to obtain regulatory approval for the use of such surfactants over non-edible types. It can be hypothesized with reasonable assurance that food-grade surfactants would also be biodegradable by microorganisms.

Surfactants have also been found to decrease remediation rates. Partitioning of contaminants into micelles can decrease the aqueous-phase concentration and lower bioavailability. Volkering et al. (1995) explained the inhibition of naphthalene degradation due to the partitioning of contaminant into the micelles and the decreased contaminant concentration in the aqueous phase. In a study by Grimberg et al. (1996), micelles were treated as a separate phase. Micellarized phenanthrene was not directly available for biodegradation. Zhang and Miller (1995) reported that treatments with inhibited degradation rates were correlated with the appearance of large aggregates 1 - 2 mm in diameter in the culture solution. The aggregates were hypothesized to be surfactant-cell-hydrocarbon interactions which decreased the uptake and degradation of hydrocarbon. Laha and Luthy (1992) tested phenanthrene degradation at high surfactant doses. Phenanthrene mineralization was inhibited at surfactant concentrations > 0.10%. Low exit rates of phenanthrene from the micelles and surfactant interactions with microbial cell membranes and proteins were proposed. Experiments showed that such micellar surfactant-bacteria interferences were reversible. Cell membranes are composed of a phospholipid bilayer which could be disrupted by interactions with the hydrophobic tails of surfactants.

In summary, TCE is a hazardous chemical that can be degraded by several species of bacteria. This study focused on degradation by a constitutive TCE degrader, *Burkholderia cepacia* G4 PR1₃₀₁. The literature reveals that surfactants may enhance remediation rates by increasing contaminant solubilization or by providing supplemental nutrients to the bacteria. Possible enhanced degradation by the presence of a nutrient-surfactant compound, BioTreat, was investigated. BioTreat could increase TCE degradation by enhancing solubilization into surfactant micelles, or by increasing cell numbers which may be made possible by the nutrients provided by BioTreat.

Chapter 3

Materials and Methods

3.1 Bacteria Strain, Culture Conditions, and Enzyme Activity

Burkholderia cepacia G4 PR1₃₀₁ (referred to as G4 throughout) was obtained from Dr. Malcolm Shields at the University of West Florida (Shields et al., 1989). The bacteria were maintained on agar plates made from basal salts media (BSM, Hareland et al., 1975) and 1.7% w/v purified agar (Becton Dickinson Microbiology Systems, Cockeysville, MD), with the addition of 20 mM sodium lactate (syrup, approximately 60%, Fisher Scientific Co., Fair Lawn, NJ) as the sole carbon source. Liquid cultures were started from plates by scraping several colonies and resuspending in 10 ml BSM + 20 mM glucose (J.T. Baker, Phillipsburg, NJ) in a 15 ml sterile centrifuge tube (Corning Inc., Corning, NY). After a 2 day incubation on a rotary shaker (250 rpm) at ambient temperature, 1 ml was withdrawn and the optical density (OD) was measured at 600 nm (Gilford Response UV-Visible spectrophotometer, Oberlin, OH). The optical density after this incubation period was typically between 0.2 and 0.5. The remaining 9 ml were transferred to 91 ml of BSM + 20 mM glucose in a 250 ml Erlenmeyer flask and returned to the shaker for 2 days or until OD > 2. All media for culturing the bacteria was sterilized by autoclaving at 16 psi and 120°C for 10 minutes per liter. All glassware used in these experiments was cleaned by washing with detergent, rinsing with tap water, and rinsing an additional 3 times with distilled water before ash sterilizing in a 500°C oven for 5 hr. Glucose and sodium lactate 1 M stock solutions were prepared in deionized water and filter sterilized through a 0.8 - 0.2 µm filter (Nalge Co., Rochester, NY). The sterility of these solutions was verified

periodically by streaking on nutrient agar plates (Becton Dickinson Company, Cockeysville, MD) and observing for growth.

After the liquid culture reached an OD > 2, the specific activity of the enzyme was measured using the TFMP (trifluoromethyl phenol or m-hydroxy benzotrifluoride) oxidation assay (Shields et al., 1991). The expression of the enzyme responsible for TCE degradation, ortho-monooxygenase, is measured by the rate of production of TFHA (7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid) from TFMP. This rate of production correlates to the potential rate of TCE degradation by the enzyme (Shields et al., 1992). TFHA is a yellow product that can be quantified by its absorbance at 386 nm. The assay can be performed as follows (Shields, 1995). The OD of the culture was measured at 600 nm. One milliliter of culture was pelleted by centrifuging in a microcentrifuge tube (Microfuge E, Beckman Instruments, Palo Alto, CA) at 13,750 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1 ml of 10 mM Tris-Cl pH 8.5 (Tris(hydroxymethyl) amino-methane, 99.8%, Aldrich Chemical Co., Milwaukee, WI) plus 1 μ M TFMP (α,α,α -trifluoro-m-cresol, 99%, Aldrich Chemical Co., Milwaukee, WI). The resuspension was incubated at 30°C for 20 minutes in a 25 ml Erlenmeyer flask. After incubation, the solution was pelleted again and the supernatant transferred to micro-cuvettes for determination of absorbance. The absorbance was measured at 386 nm and 600 nm. The absorbance of a dilute cell suspension (OD < 0.1 at 600 nm) was also determined at 386 and 600 nm. The enzyme specific activity was determined from the following series of calculations (Shields, 1995):

The differential response of the spectrophotometer (C) to suspended cells in solution at 386 nm relative to 600 nm is the ratio of the absorbances at these wavelengths for a dilute cell suspension.

$$C = A_{386}/A_{600} \quad \text{when } A_{600} < 0.1$$

where A_{386} is the absorbance at 386 nm
 A_{600} is the absorbance at 600 nm.

The yellow product, TFHA, absorbs strongly at 386 nm. The absorbance at 386 nm must be corrected to account for the diffractive loss which contributes to absorbance.

$$A_{386 \text{ corrected}} = A_{386} - [A_{600} \times C]_{\text{diffractive loss contributing to absorbance}}$$

The concentration of protein in mg/ml can be calculated by the approximation that each absorbance unit at 600 nm contains 0.290 mg of protein/ml.

$$\text{mg protein / ml} = A_{600 \text{ dense culture}} \times 0.290$$

The concentration of TFHA can be calculated as follows:

$$c = A_{386} / \epsilon$$

where c = TFHA concentration, mol/L
 ϵ = molar extinction coefficient, 26900 L cm/mol .

By converting the concentration of TFHA to the amount of TFHA produced per minute in 1 ml of culture, the specific activity can be obtained as follows:

$$\text{Specific Activity} = (\text{nmoles TFHA/min}) / (\text{mg protein/ml}).$$

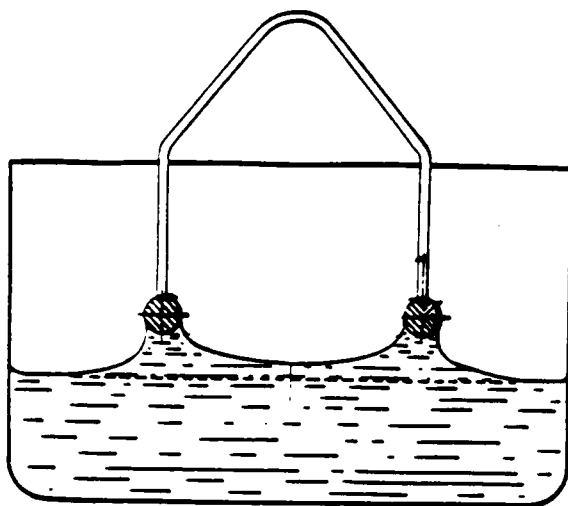
The enzyme specific activity in all cultures used in the degradation experiments described below was ≥ 3.0 (nmoles TFHA/min) / (mg protein/ml).

A growth curve for G4 grown on glucose was constructed by taking OD measurements hourly over a 12 hr period. Two cultures were started by taking a 10% inoculum from a previously grown culture at $OD \geq 2$. One flask was inoculated at $t = 0$

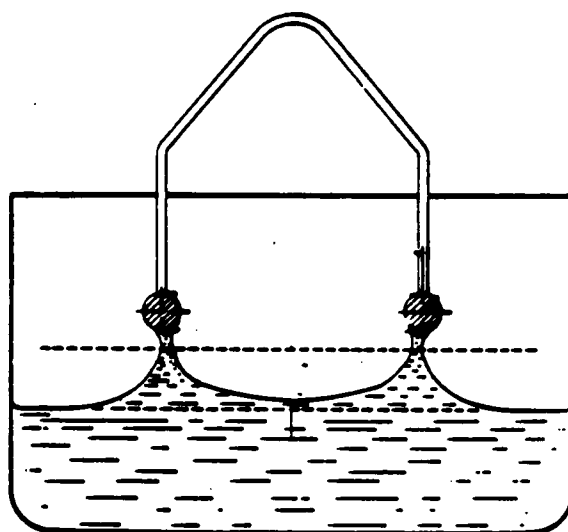
hr; another flask had been inoculated 12 hours earlier so that measurements could be taken over a 24 hr growth period. Optical density measurements were performed by withdrawing 1 ml of culture and recording the absorbance at 600 nm on a spectrophotometer. Optical density measurements from 0 - 12 hr and 12 - 24 hr were used to construct the growth curve from 0 - 24 hr.

3.2 Nutrient-Surfactant Property Characterization of BioTreat

The surfactant characteristic of BioTreat was observed by measuring its ability to lower the surface tension of a liquid. BioTreat was supplied by Rem-Tec Group Inc., Winston-Salem, NC (see Appendix II for Material Safety Data Sheet). A stock solution of 10% BioTreat in deionized water was filter sterilized (0.8 - 0.2 μ m filter, Nalge Co., Rochester, NY). All experimental surfactant solutions were prepared from the 10% stock. Surfactant solutions of varying concentrations (0.001, 0.003, 0.01, 0.05, 0.1, 0.3, 0.5%) were measured in 5 ml aliquots into 60 x 15 mm sterile petri dishes (Becton Dickinson Labware, Lincoln Park, NJ). The surface tension was measured at ambient temperature ($25^{\circ}\text{C} \pm 1^{\circ}\text{C}$) using a DuNUOY ring tensiometer (CSC Scientific Co., Inc., Fairfax, VA). The surface tension of a solution is measured as the energy per unit area required to break the tensiometer ring free from the solution in dynes/cm (Figure 6a. and 6b.). The tensiometer was calibrated according to the manufacturer's instructions and cleaned prior to each measurement by rinsing with deionized water for several seconds and blotting with lint-free tissue paper. The ring was verified to be clean by measuring the surface tension of deionized water (75 dynes/cm). Each surfactant concentration was measured in triplicate. The measured surface tension values were plotted against the logarithm of surfactant concentration (% v/v). The CMC was determined to be the concentration beyond which there was no further significant change in surface tension.



a. Distention of surface film during surface tension measurement.



b. Condition of surface film at breaking point.

Figure 6. Measurement of surface tension.
(Modification of figures taken from CSC DuNOUY Tensiometer Owners Manual, page 9. CSC Scientific Company, Inc.).

BioTreat was provided as the sole carbon source to determine if it provided any nutrient effect to G4. G4 was cultured on agar plates for 7 days, scraped, and resuspended in sterile minimal salts media (MSM, Little et al., 1988) to an OD = 1.5 at 600 nm. An aqueous BioTreat solution was prepared from the sterile 10% stock solution in deionized water and added to a final concentration of 0.05% v/v in sterile MSM in a 250 ml Erlenmeyer flask. The flask was inoculated with 0.3% inoculum of the G4 suspension and incubated at ambient temperature on a rotary shaker (250 rpm). The control flask contained only G4 and MSM with no BioTreat (no carbon source). The purity of the G4 suspension and the sterility of the surfactant solution were verified by streaking on nutrient agar plates and observing after several days for growth. No growth was seen from the surfactant solution. The G4 suspension revealed a pure culture as observed phenotypically. The OD in the flasks was monitored daily for 16 days by withdrawing 1 ml aliquots and recording the absorbance at 600 nm with a spectrophotometer. A blank consisting of the appropriate concentration of BioTreat in water was used daily to zero the spectrophotometer. Additional 0.05% v/v BioTreat was spiked to the flask at days 8 and 15. Surface tension readings of the cultures were taken daily to confirm the degradation of surfactant. An increase in surface tension is indicative of surfactant biodegradation. The experiment was discontinued at day 16 because it was no longer possible to determine if the turbidity of the solution was due to bacterial growth or the presence of BioTreat in solution. The sterility of the surfactant solution and the purity of the culture throughout the duration of the experiment was verified by streaking on nutrient agar plates.

The effect of BioTreat as a secondary carbon source on the growth and expression of the enzyme activity of G4 was determined by culturing G4 with glucose and BioTreat. Single colonies of G4 were selected from agar plates and inoculated into 10 ml BSM + 20 mM glucose in sterile 15 ml centrifuge tubes. BioTreat was added

from the sterile 10% stock to final concentrations of 0.01, 0.05, and 0.1% v/v in solution to represent concentrations below, at, and above the CMC. The tubes were incubated at ambient temperature on a rotary shaker for 3 days. On the third day, 1 ml was withdrawn from each tube and the OD at 600 nm was measured. The remaining 9 ml of each culture was transferred to 41 ml of BSM + 20 mM glucose and adjusted to the appropriate concentration of BioTreat. The OD was monitored daily until the OD $\geq$ 2 on the fifth day. On day 5, the TFMP oxidation assay was performed on each culture and the specific activity of the enzyme calculated.

3.3 TCE Solubilization Experimental Design

The effect of various concentrations of BioTreat on increasing the apparent aqueous solubility of TCE was determined through a series of experiments. All experimental treatments were performed in triplicate in 16.3 ml clear glass vials (EPA vials) with screw top lids and Teflon lined septa. All glassware was ashed as described earlier. All lids and septa were autoclaved at 120°C and 16 psi for 30 minutes. Surfactant solutions (0.001, 0.01, 0.05, 0.5, 1, and 5% v/v) were prepared in a sterile phosphate buffered solution (PBS, 1.2 g Na₂HPO₄, 2.2 g NaH₂PO₄, 8.7 g NaCl per liter distilled water). A stock solution of TCE saturated water was prepared by adding approximately 2 ml of pure TCE (trichloroethylene, stabilized, J.T. Baker Inc., Phillipsburg, NJ) to 10 ml sterile deionized water in a 16 ml sterile glass vial and allowing to equilibrate on a rotary shaker for 1 - 2 hr. TCE from this stock solution was added to the vials by injection via glass syringe (Hamilton Co., Reno, NV) before capping to obtain a total concentration of either 0.1 ppmv (parts per million by volume) or 1.0 ppmv. All TCE concentrations added to sample vials in these and the following experiments were calculated as if all the TCE was in the aqueous phase. Each vial contained a volume of 5 ml of liquid and 11.3 ml headspace. The vials were inverted

and equilibrated overnight at ambient temperature ($25^{\circ}\text{C} \pm 1^{\circ}\text{C}$) on a rotary shaker at 250 rpm.

TCE headspace measurements were performed on a Hewlett Packard 5890 Series II Plus gas chromatograph (GC) equipped with an electron capture detector (ECD) and a DB624 megabore capillary column (30 m, 0.53 mm inner diameter, Alltech, Deerfield, IL). Argon-methane (Liquid Air Corp., Walnut Creek, CA) was the carrier gas (flow = 7.0 ml / minute). The injector temperature was 100°C . The oven temperature began at 70°C and increased to 110°C at a rate of $10^{\circ}\text{C} / \text{minute}$. The detector temperature was 300°C . A headspace volume from each treatment vial of 30 μl was injected. Following headspace measurements, the samples were extracted by the addition of 3 ml of hexane (99+% capillary GC grade, Sigma Chemical Co., St. Louis, MO) injected through the septa, and allowed to extract while shaking on a rotary shaker overnight. Approximately 1 ml of the organic phase was withdrawn by Pasteur pipette and placed in an autosampler vial. A volume of 1 μl was analyzed on the GC ECD using the autosampler. The same column was used for headspace and hexane extraction analysis. The GC conditions were the same as for the headspace method with the exception of the following: the oven temperature started at 40°C and increased at a rate of 10°C per minute to 120°C , and the injector temperature was 200°C .

3.4 TCE Sorption Experimental Design

The sorption of TCE to G4 cells was measured by exposing G4 to 1, 5, and 10 ppmv TCE. Sorption experiments were performed in sterile 16.3 ml glass vials in triplicate. The vials were capped with autoclaved Teflon lined septa and screw type lids. G4 cultures were killed by autoclaving at 120°C and 16 psi for 30 minutes to eliminate degradation potential. The TFMP oxidation assay was performed to verify that no enzyme activity was present. Each vial contained 0.5 ml of an autoclaved G4

culture at OD = 2, 4.5 ml sterile PBS, and the appropriate TCE concentration. TCE was added to the vials by injection via glass syringe before capping to obtain a total concentration of 1, 5, or 10 ppmv. The vials were inverted and incubated on a rotary shaker at 250 rpm at ambient temperature. Headspace measurements were performed at 0, 24, and 48 hr.

3.5 TCE Degradation Experimental Design

The effect of BioTreat on enhancing the rates of TCE biodegradation was investigated using various BioTreat concentrations and 1, 5, and 10 ppmv TCE. Degradation experiments were carried out in sterile 16.3 ml glass vials in triplicate. The vials were capped with autoclaved Teflon lined septa and screw type lids. BioTreat solutions were prepared from a filter sterilized 10% v/v stock into autoclaved PBS. G4 was cultured as described in section 3.1 and the enzyme activity was tested before the addition of 0.5 ml bacteria to 4.5 ml of surfactant solution. TCE was added via syringe before capping to obtain the appropriate total concentration. The vials were inverted and incubated at room temperature on a rotary shaker. Surfactant solution, bacteria, and TCE were added in this order with no equilibration time between additions.

The first experiment measured the effects of a range of BioTreat concentrations above and below the CMC on the degradation of 1.0 ppmv TCE. G4 was incubated with BioTreat (0.01, 0.05, 0.5, 1, and 5%) and 1.0 ppmv TCE for 48 hr. Headspace analysis were performed at 4, 24 and 48 hr. After 48 hr, the residual TCE was extracted with 3 ml hexane and 1 μ l of the extractant was analyzed on the GC ECD. All headspace and hexane extraction GC methods were the same as those used in the solubilization experiments.

Since it appeared that TCE was degraded within 24 hr in the first experiment, degradation between 0 and 24 hr was investigated in the second experiment. G4 was

exposed to BioTreat at the CMC and 1.0 ppmv TCE for 24 hr. Headspace measurements were performed at 4 and 24 hr. Prior to the addition of hexane, 100 μ l of liquid was withdrawn by syringe through the septa to assess microbial numbers. The samples were hexane extracted and analyzed at the end of the 24 hr degradation period.

In order to obtain the number of viable colony forming units (CFUs) in solution, serial dilutions and spread plating were performed. Liquid samples from treatment vials were spread plated in triplicate on R2A agar plates (Difco Laboratories, Detroit, MI). R2A is a complex growth media and was used because it is non-selective. The bacteria were enumerated after 5 - 7 days, or after easily visible colonies were observed.

In the third experiment, the TCE concentration was increased to 5 ppmv. Since no solubilization was observed below the CMC for 1.0 ppmv, BioTreat was added at concentrations equal to the CMC, 10, and 100 times higher than the CMC (0.05, 0.5, and 5%). Analysis of the headspace TCE concentrations were performed at 0, 24, and 48 hr. The samples were hexane extracted at the end of the 48 hr degradation period. Cell numbers were measured in triplicate via serial dilutions and spread plating on R2A agar.

The last experiment focused on 0.05% BioTreat and 10 ppmv TCE. For this experiment, no headspace measurements or CFU data were collected. Only hexane extraction data at 48 hr were collected.

Data for solubilization and degradation experiments were statistically analyzed with the student's T-test available in Microsoft Excel version 5.0. The statistics are provided in Appendix III. For a 95% confidence level, P-values greater than 0.05 were used to determine that there was no statistical difference between sample means. P-

values less than 0.05 were used to determine that there was a statistical difference between sample means.

Chapter 4

Results and Discussion

4.1 Nutrient-Surfactant Characterization of BioTreat

BioTreat is a commercial product manufactured by Rem-Tec Group, Inc. (Winston-Salem, NC) marketed for the clean-up of industrial contaminants, grease and food solids, and for use in wastewater treatment systems. Its structure and composition are listed as proprietary information (MSDS in Appendix I). Its nutrient and surfactant properties were investigated for its effect on the bioremediation of TCE by *Burkholderia cepacia* G4 PR1₃₀₁.

Bacteria grown in liquid batch cultures can be described by 4 major phases of growth (Monod, 1949). Lag phase is characterized by little or no growth because the bacteria are adapting to the new environment and synthesizing needed cellular components, such as enzymes, for growth. During the exponential growth or log phase, the bacteria are growing and multiplying at their maximum rate. Exponential growth ends when nutrients become limiting to the bacteria, or when excessive toxic metabolites accumulate. Cell density is constant in stationary phase where growth rates are balanced by death rates. During the last phase, known as endogenous respiration, cell numbers begin to decline due to an increasing death rate from substrate depletion. Cellular components from lysed cells can be used by living cells as substrate during this phase, but there is still a net decrease in cell density.

The nutrient supplying properties of BioTreat were determined by culturing G4 with BioTreat as the sole carbon source (Figure 7). Three phases of bacterial growth can be used to describe Figure 7. There was a rapid increase in growth between days 1

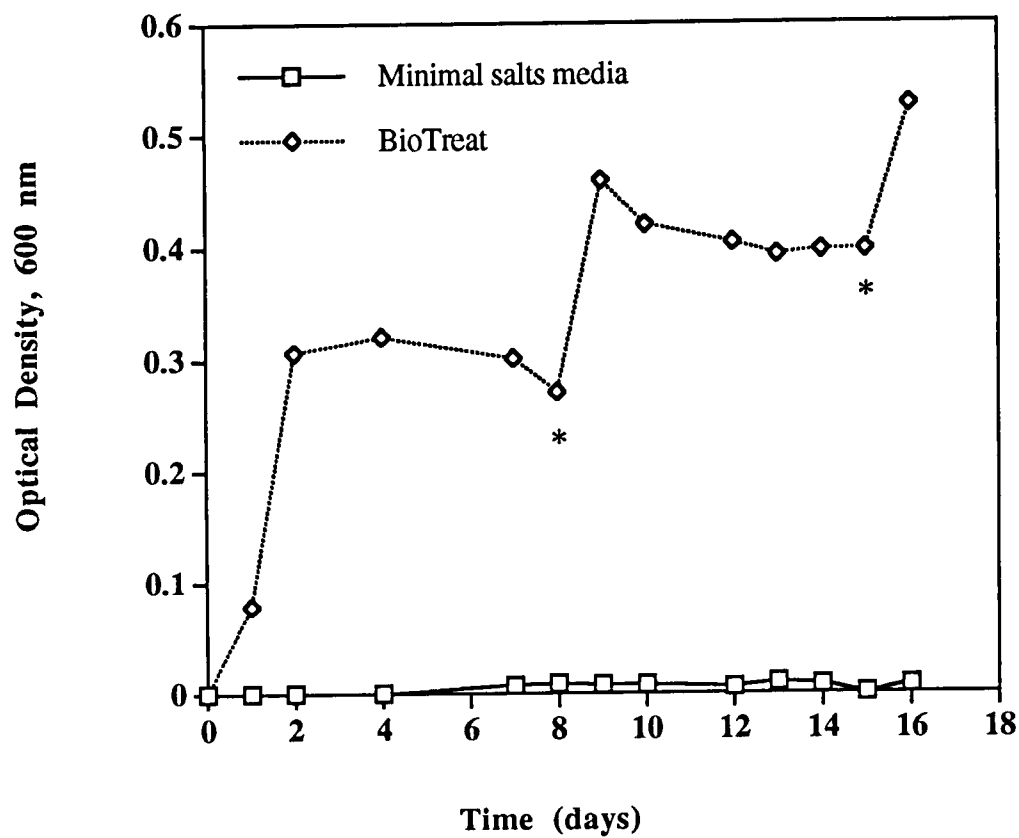


Figure 7. Growth of G4 with BioTreat as sole carbon source. Initial BioTreat concentration at 0.05%. Addition of BioTreat as indicated by * to a total concentration of 0.1% (day 8) and 0.15% (day 15).

and 2 (increasing to OD = 0.3). This period of rapid growth is most likely the exponential growth phase. Stationary growth phase was observed from days 2 - 8, as shown by the constant optical density. The beginning of endogenous growth phase is observed on day 8 with a slight decrease in OD ($\Delta OD = - 0.03$). The addition of BioTreat to a total concentration of 0.1% on day 8 resulted in a return to the exponential growth phase. After a 1 day incubation, the OD increased to 0.48. Stationary phase is again observed from days 10 - 15, with an OD = 0.42. At day 15, a further addition of BioTreat resulted in an increase in OD to 0.54 after an overnight incubation. Daily surface tension measurements confirmed that BioTreat was partially biodegraded. Control samples without BioTreat exhibited a constant surface tension of 76 dynes/cm. The surface tension of water was also measured at 76 dynes/cm. The treatment flask showed a corresponding increase in surface tension with increasing OD. The initial surface tension of the treatment flask was 36.7 dynes/cm, owing to the surfactant properties of BioTreat. The surface tension increased to 37.5 dynes/cm by day 1 and to 40.0 dynes/cm by day 2, presumably due to degradation of some components of the BioTreat mixture. After the addition of 0.05% BioTreat on day 8, the surface tension decreased to 37 dynes/cm, with a subsequent increase to 39.4 dynes/cm after a 1 day incubation. It appears that the surfactant components of BioTreat are degraded during the exponential growth phase. The nonsurfactant components may provide additional nutrients to sustain cell populations between days 3 and 7. After day 7, the carbon and energy sources in BioTreat that could be utilized by G4 were probably exhausted. The experiment was discontinued at day 16 because it became impossible to distinguish cell density from the cloudiness provided by BioTreat at 0.15% (total surfactant added up to day 16) in the samples used to blank the spectrophotometer. This data shows that BioTreat can provide the necessary requirements for the growth of G4 and that the surfactant components of BioTreat are partially biodegradable by this organism.

However, the growth of G4 on BioTreat is much slower when compared to a traditional growth media, such as glucose. It was shown in Chapter 3 that a 10% inoculum of G4 grew to an OD of 2.0 within 2 days when grown on 2 mM glucose. Figure 8 depicts a growth curve of G4 over a 24 hr period from a 10% inoculum.

The surfactant properties of BioTreat were determined by measuring its ability to lower the surface tension of water. Surface tensions were measured by the DuNUOY ring method. The energy per unit surface area required to pull the ring free from suspension in the solution is measured in dynes/cm (Figure 6a and 6b, Chapter 2). Surfactant monomers are amphiphilic by nature and tend to concentrate at the air-liquid interface, thus lowering the surface tension of the solution. Above a certain concentration, identified as the critical micelle concentration (CMC), surfactant monomers will form clusters called micelles. A micelle can be pictured as an aggregation of surfactant monomers with the hydrophobic tails oriented toward the inside of the cluster and the hydrophilic heads toward the outside (West and Harwell, 1992). This aggregation creates a hydrophobic core into which contaminants can partition. A plot of surface tension versus the log of surfactant concentration is used to determine the CMC. At concentrations above the CMC, there is little or no decrease in surface tension with increasing surfactant concentration.

An average CMC value for BioTreat was determined to be 0.05% from surface tension measurements in water (Figure 9); the corresponding surface tension is 37 dynes/cm. The surface tension of deionized water was measured at 75 dynes/cm. When compared to CMC curves for other single compound surfactants, such as Tergitol NP-10 (C₉PE_{10.5}, Figure 10), it may appear that BioTreat does not have a distinct CMC value. Heterogeneous surfactant mixtures typically do not have sharply defined CMC values (Kile and Chiou, 1989; Jafvert et al., 1994). These mixtures may consist of surfactants with hydrophobic tails of different lengths.

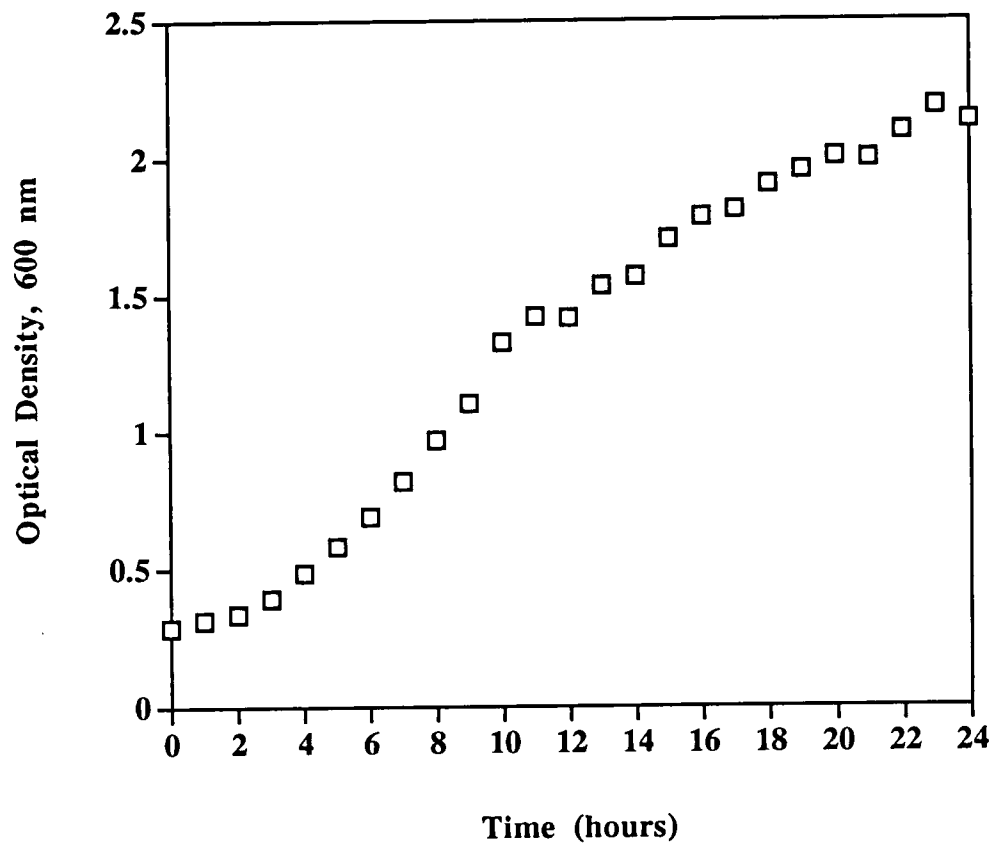


Figure 8. Growth curve for G4. Carbon source is 2 mM glucose, 10% inoculum.

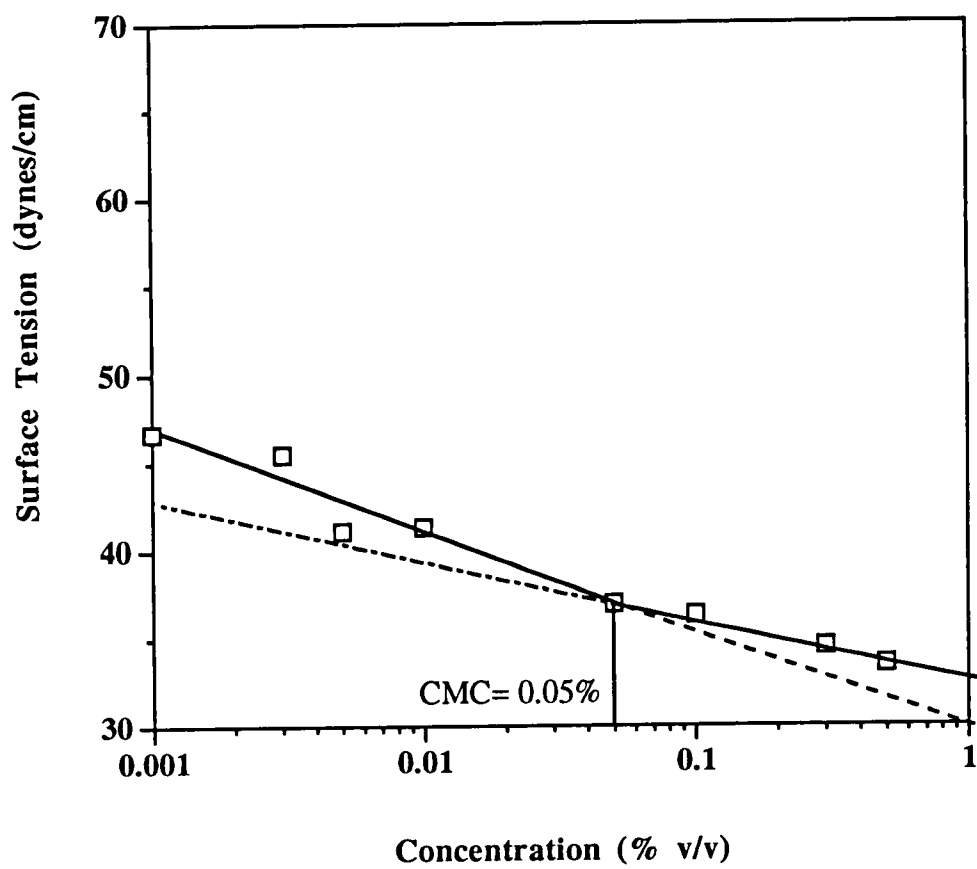


Figure 9. Determination of critical micelle concentration of BioTreat in water.

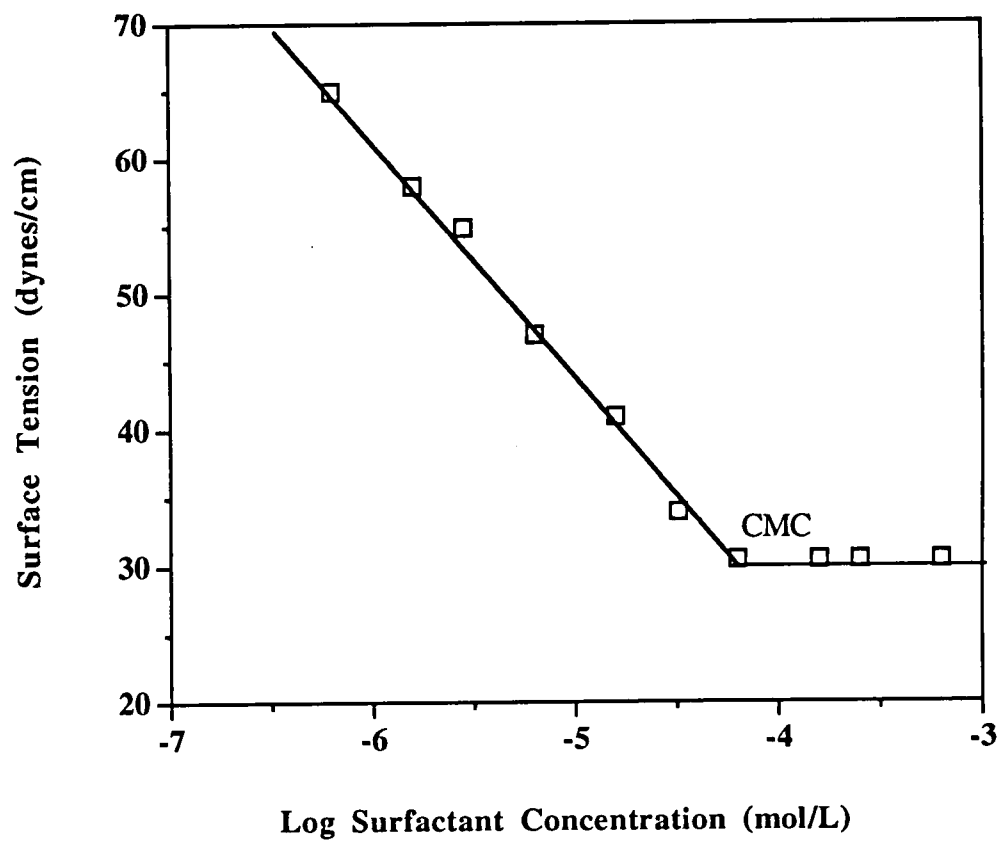


Figure 10. CMC Curve for Tergitol NP-10(C9PE10.5). (Source: Edwards et al., 1991).

Surfactant monomers with longer, more hydrophobic tails tend to form micelles at lower concentrations than monomers with shorter tails. Individual heterogeneous monomers in solution may have sequential CMC values because of their differing solubilities in water. BioTreat is a mixture of nonionic biodegradable surfactants (surfactants are EPA listed) with a pH of 7 - 8 (personal communication, T. Smith, Rem-Tec Group, Inc., Winston-Salem, NC). This may explain the lack of a sharply distinct CMC value for BioTreat. The presence of even very low concentrations of BioTreat can lower the surface tension of water (surface tension of 47 dynes/cm at 0.001% BioTreat), indicating that there may be micelles in solution at low BioTreat concentrations. The highest surfactant concentration tested was 0.5% with a surface tension of 33.5 dynes/cm.

Contaminant mobilization by surfactants can enhance remediation efforts. Typically surfactants used for the mobilization of contaminants are added at high concentrations to achieve ultra-low interfacial tensions at the water-NAPL (nonaqueous phase liquid) interface. The downward migration of dense nonaqueous phase liquids (DNAPLs) may be impeded by capillary forces. Ultra-low surface tensions induced by surfactant addition can cause downward movement of the contaminant, possibly leading to the contamination of previously clean areas (Fountain et al., 1991; Jackson, 1993/94; Pennel et al., 1994; West and Harwell, 1992). Recently, researchers have found that ultra-low surface tensions are not necessary to obtain enhanced remediation rates of contaminants in soil, thus minimizing the risk of downward contaminant migration (Aronstein and Alexander, 1992; Fountain et al., 1991; Jafvert et al., 1995; Pennel et al., 1994; Yeom et al., 1996). Remediation in these cases is enhanced not by mobilization of contaminant, but by enhanced solubilization. The apparent aqueous solubility is increased by the partitioning of the hydrophobic contaminant into the

hydrophobic cores of micelles. Although soil remediation is beyond the scope of this research, the mobilization of contaminants by BioTreat is conceivable.

4.2 Effect of BioTreat on Enzyme Activity

Experiments were conducted to determine whether BioTreat decreases the activity of the enzyme responsible for TCE degradation. Enzyme activity was monitored when G4 was cultured with 2 mM glucose as the primary growth substrate in the presence of 0.01, 0.05, and 0.1% BioTreat. These concentrations were chosen based on the average CMC (0.05%) and represent values 5 times lower than the average CMC (0.01%) and 2 times greater (0.1%) than the average CMC. Each culture was started by scraping bacteria from an agar plate, inoculating into 10 ml BSM-20 mM glucose and the appropriate concentration of BioTreat, and increasing the volume to 100 ml after a 2 day incubation period as described earlier in section 3.1. The optical density and enzyme activity were assayed for 5 consecutive days. All cultures (controls and treatments) reached OD = 2 on the fifth day of incubation. Enzyme activity was below the detection limits of the assay in all cultures until the OD had increased to 2 on day 5. Enzyme activity may be present at lower cell densities, but is not detectable by the colorimetric TFMP oxidation assay (personal communication, Dr. M. Shields). The data is summarized in Table 3.

Table 3. Effect of BioTreat on Growth and Enzyme Activity of G4

Treatment	Optical Density at day 5	Enzyme Activity nmol/min/mg protein/ml
No BioTreat	2.03	4.23 $\pm$ 0.11
0.01% BioTreat	2.01	4.70 $\pm$ 0.09
0.05% BioTreat	2.05	3.45 $\pm$ 0.16
0.1% BioTreat	2.04	2.60 $\pm$ 0.16

Control samples containing no BioTreat increased to OD = 2.03 with an enzyme activity of 4.23 $\pm$ 0.11 nmol/min/mg protein/ml. Samples containing 0.01% BioTreat reached an activity of 4.70 $\pm$ 0.09 nmol/min/mg protein/ml. A statistical analysis provided evidence that the difference between the means was significant ($P < 0.05$). The reason for the slight increase in enzyme activity over that of the control with no BioTreat is unclear. It is possible that the additional nutrients provided by BioTreat were utilized by the cells and increased the growth and reproduction and thus the amount of enzyme produced in that treatment sample. Enzyme activity is depressed when BioTreat is added at the average CMC and at twice the average CMC (0.05% and 0.1%) as compared to the control. At these concentrations, there is more surfactant in solution to possibly interact with the cells. Other researchers have proposed toxicity effects of surfactants on bacterial cells. Laha and Luthy (1992) propose a surfactant-cell membrane binding that disrupts membrane structures. Cell membranes, which are composed of a phospholipid bilayer, could be disrupted by interactions with the hydrophobic tails of surfactants. This disruption of the membrane may interfere with

the passage of substrate (TFMP) into the cell, thereby lowering the enzyme activity measured in the TFMP oxidation assay.

4.3 TCE Solubilization Studies

Solubilization is a widely recognized mechanism by which surfactants can enhance remediation rates (Aronstein et al., 1992; Chaiko et al., 1984; Edwards et al., 1990; Jackson, 1993/94; Jafvert et al., 1994; Kile and Chiou, 1989; Park and Jaffe, 1993; Pennell et al., 1994). Surfactants increase the apparent aqueous solubility of contaminants by the partitioning of hydrophobic compounds from the soil, aqueous, or gas phase into the hydrophobic core of the micelles. Park and Jaffe (1993) found that as the concentration of surfactant increased, the mass of contaminant sorbed decreased because more contaminant is solubilized into the micelles. In general, the more hydrophobic the contaminant, the greater the partitioning effect (Kile and Chiou, 1989; Shiao et al., 1994). For example, the micellar-aqueous partition coefficient, $\log K_m$, (defined as the ratio of the mole fraction of TCE in the micellar pseudophase to the mole fraction of TCE in the aqueous pseudophase), was reported to be 4.50 for PCE, 3.27 for TCE, and 2.76 for 1,2-DCE by Shiao et al. (1994). Larger K_m values indicate greater solubilization into micelles.

Experiments were performed to determine the combined effects of BioTreat and TCE concentration on enhancing TCE solubilization. BioTreat concentrations of 0.001, 0.01, and 0.05% did not have a significant effect ($P > 0.30$) on the partitioning of 0.1 ppmv or 1.0 ppmv TCE (reported as if all TCE was in the aqueous phase) (Figures 11 and 12) as measured by headspace gas chromatography. BioTreat does not enhance TCE solubility when added below the CMC, nor does it measurably enhance TCE solubility when added at the CMC.

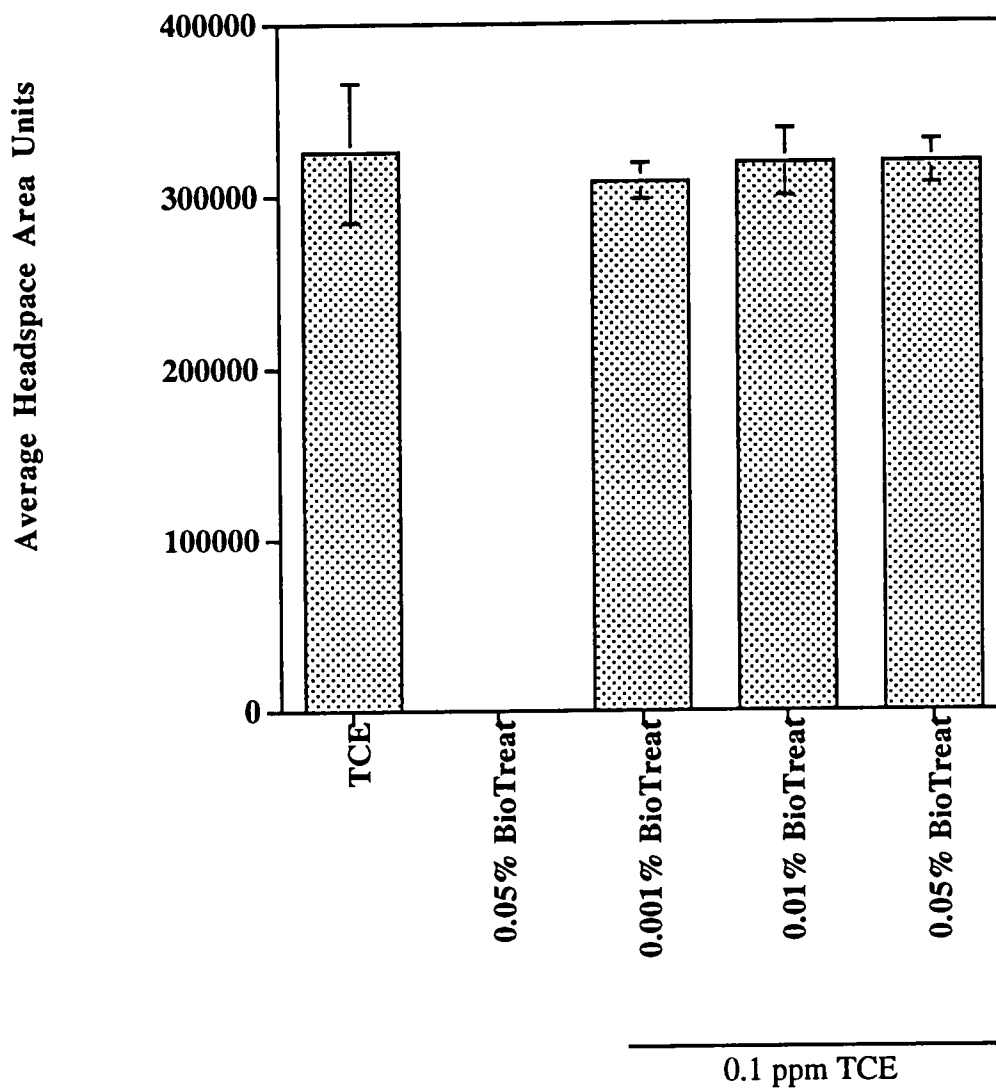


Figure 11. Abiotic solubilization of 0.1 ppm TCE by BioTreat at low concentrations. Controls are TCE only and 0.05% BioTreat only. $n=3$, stdev within error bars.

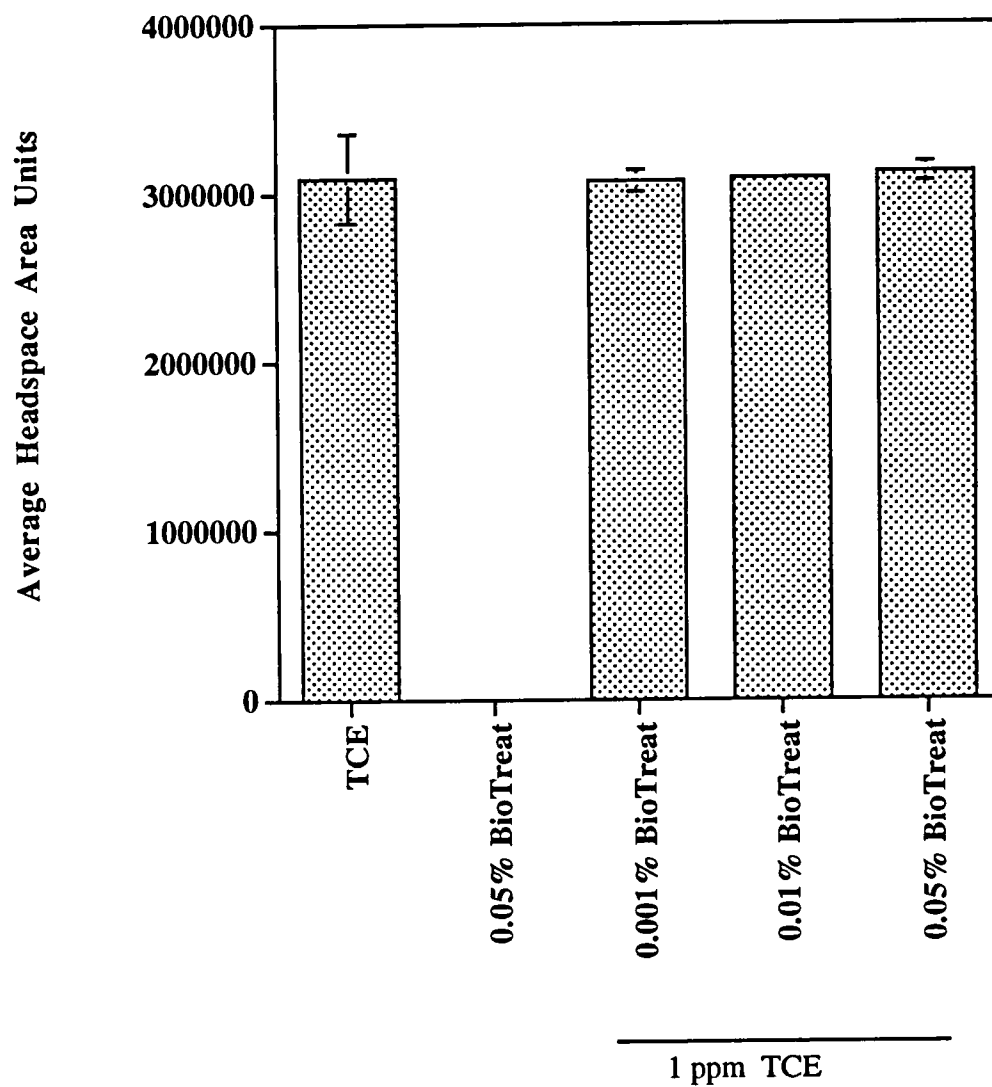


Figure 12. Abiotic solubilization of 1 ppm TCE by BioTreat at low concentrations. Controls are TCE only and 0.05% BioTreat only. $n=3$, stdev within error bars.

At 0.05% BioTreat, there may be micelles in solution; however, the solubility of TCE is not significantly enhanced because insufficient micelles are present to significantly increase partitioning of TCE into the aqueous phase. For treatments that do not contain BioTreat, the concentration of TCE in the gas phase is described by:

$$C_{\text{gas}} = \frac{M_T}{V_{\text{gas}} + \frac{V_{\text{aq}}}{H}}$$

where K_{m2} = concentration of TCE in the gas phase

V_{aq} = volume of aqueous phase

V_{gas} = volume of the gas phase

M_T = total mass of TCE added

H = Henry's Law constant for TCE (0.476).

A similar relationship is used to describe the TCE concentration in the gas phase for treatments containing BioTreat.

$$C_{\text{gas}} = \frac{M_T}{V_{\text{gas}} + \frac{K_{m2} \cdot \text{mass surfactant}_{\text{micelles}} + V_{\text{aq}}}{H}}$$

where K_{m2} = partitioning coefficient for TCE on a mass basis (see equation A-5).

A comparison of the gas phase TCE concentrations in treatments with and without 0.05% BioTreat reveals that 0.05% BioTreat did not significantly enhance the apparent aqueous solubility of TCE. For samples containing no BioTreat and 1 ppmv TCE, 0.25 µg/ml TCE was calculated to be in the headspace. A slight change (4.4%) in TCE headspace concentrations were calculated for treatments containing 0.05% BioTreat. At this BioTreat concentration, there are few micelles available for partitioning, thus no solubility enhancement is observed.

Similar results for other nonionic surfactants are reported by West (1992) and Kile and Chiou (1989) for chlorinated organics. West observed no detectable enhancement of PCE, TCE, or DCE solubility with four nonionic surfactants at concentrations below the CMC. Kile and Chiou (1989) reported no enhanced solubility of trichlorobenzene with four nonionic surfactants at sub-CMC concentrations. At these surfactant concentrations, there are few micelles available for contaminant partitioning. However, Kile and Chiou (1989) reported that solubilization of a highly hydrophobic compound, DDT, was enhanced with a nonionic surfactant at concentrations below the CMC. They reported that the potential to enhance solubility increases with nonpolar to polar group ratios. A partition-like effect was described in which the tail of a surfactant monomer interacts with the hydrophobic contaminant. However, since TCE is not considered a highly hydrophobic compound (solubility = 1100 mg/L), solubilization enhancement by surfactant monomers is expected to be insignificant. Jafvert et al., (1994) determined that the hydrophobicity of the solubilized chemical is more significant in determining K_m than the specific surfactant used.

Hexane extractions were performed to show that there were no significant abiotic TCE losses ($P > 0.30$) and that the interference of BioTreat with the extraction efficiency of hexane for TCE was insignificant ($P > 0.20$) (Figures 13 and 14).

In contrast to low BioTreat concentrations, solutions containing 0.5, 1, and 5% BioTreat enhanced the solubilization of TCE (Figure 15). These concentrations represent 10 times the average CMC (0.5%), twenty times the average CMC (1%) and 100 times the average CMC (5%). A decrease in headspace concentration indicates an increase in solubilization of TCE as compared to buffer-only controls. Samples containing 0.05% BioTreat had 30% less TCE present in the headspace as compared to controls with TCE and buffer only. Samples containing 1% BioTreat had 50% less

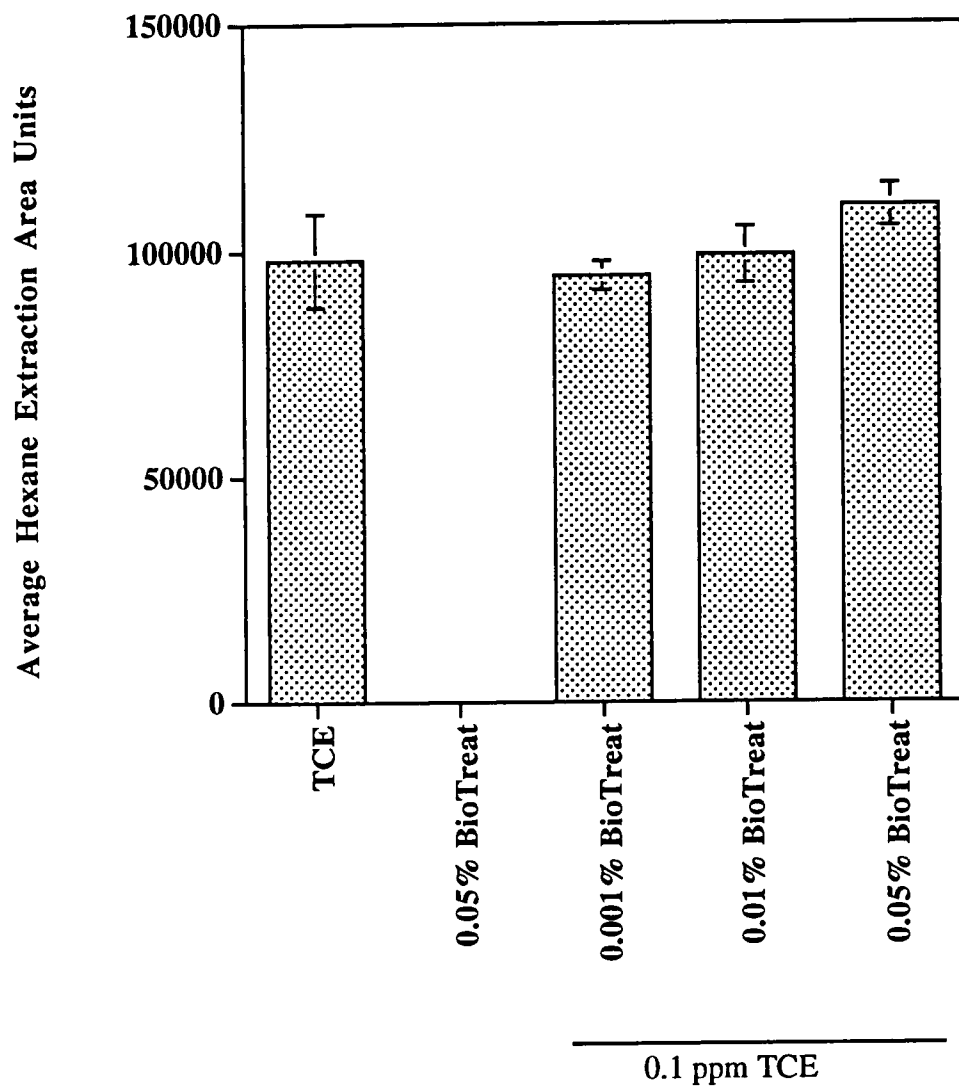


Figure 13. Hexane extraction efficiency for 0.1 ppm TCE with low BioTreat concentrations. Controls are TCE only and 0.05% BioTreat only. $n=3$, stdev within error bars.

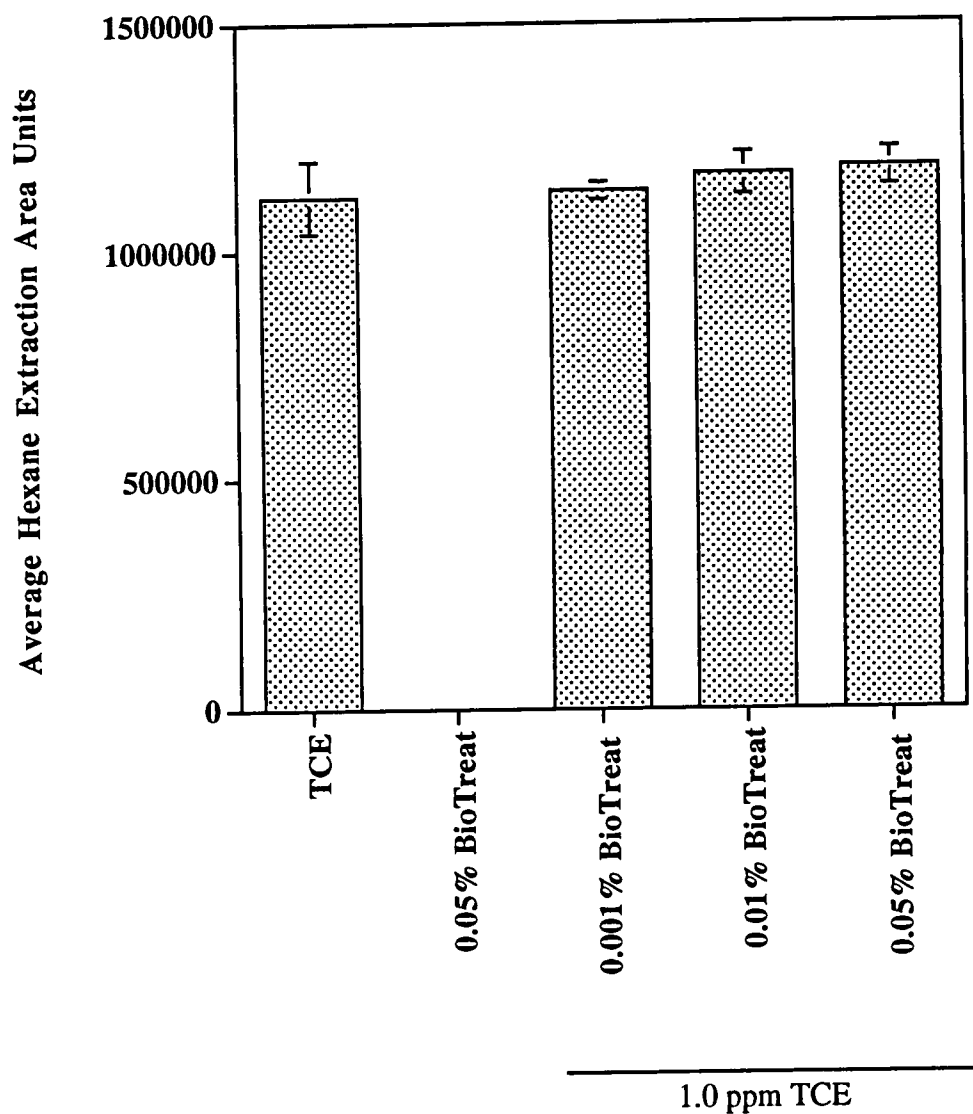


Figure 14. Hexane extraction efficiency for 1.0 ppm TCE with low BioTreat concentrations. Controls are TCE only and 0.05% BioTreat only. n=3, stdev within error bars.

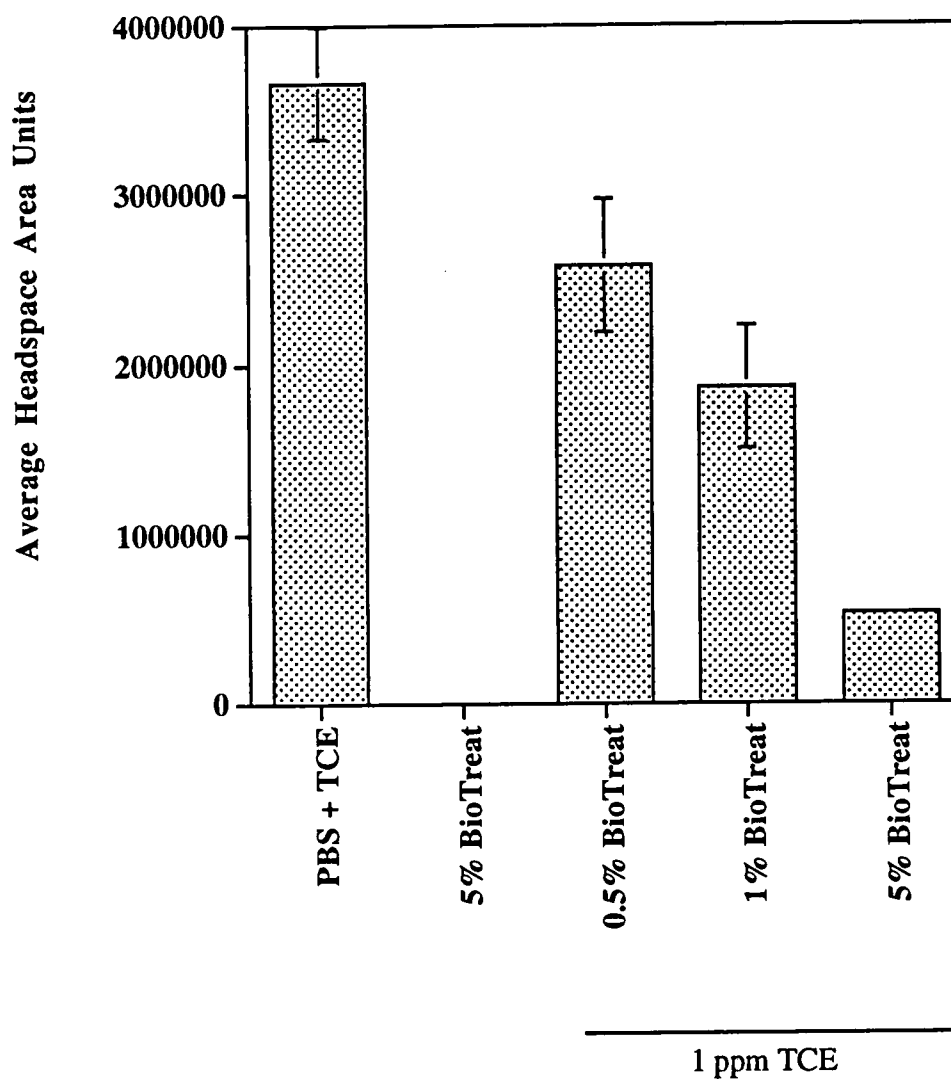


Figure 15. Abiotic solubilization of 1 ppm TCE by BioTreat at high concentrations. Controls are TCE only and 5% BioTreat. $n=3$, stdev within error bars.

TCE in the headspace than in controls. An increase in TCE solubility with 5% BioTreat is measured by a decrease of 70% less TCE in the headspace than in control samples. At these concentrations, more micelles are present; therefore more TCE can be solubilized into the hydrophobic cores because there are more hydrophobic cores available. The partitioning of organic contaminants into the hydrophobic cores of micelles increases the apparent aqueous solubility (Kile and Chiou, 1989; West and Harwell, 1992; Shiau et al., 1994). These results are supported by Parke and Jaffe (1993) who have shown that the partitioning of three nonionic organic compounds, carbon tetrachloride, naphthalene, and phenanthrene, increases as the concentration of surfactant in solution above the CMC increases. Jafvert et al. (1994) observed a linear increase in hexachlorobenzene solubility with increasing surfactant concentration above the CMC. Edwards et al. (1990) reported similar results for the solubilization of polycyclic aromatic hydrocarbons (PAH) by 4 nonionic surfactants. Again, at surfactant concentrations greater than the CMC, the solubility of PAH increases due to higher micelle volume available for solubilization.

A hexane extraction was performed to show that abiotic TCE losses were insignificant ($P > 0.05$) and that BioTreat did not significantly affect the hexane extraction efficiency ($P > 0.05$) (Figure 16).

Since the molecular weight of BioTreat is not known by this researcher, a K_m value could not be calculated. However, an alternative partitioning coefficient, K_{m2} , was determined from experimental data (see Figure A.1 in Appendix I). In contrast to K_m , which is the molar partitioning coefficient that describes the ratio of the mole fraction of TCE in the micellar pseudophase to the mole fraction of TCE in the aqueous pseudophase, K_{m2} was calculated in a similar manner using mass relationships. K_{m2} can be defined as:

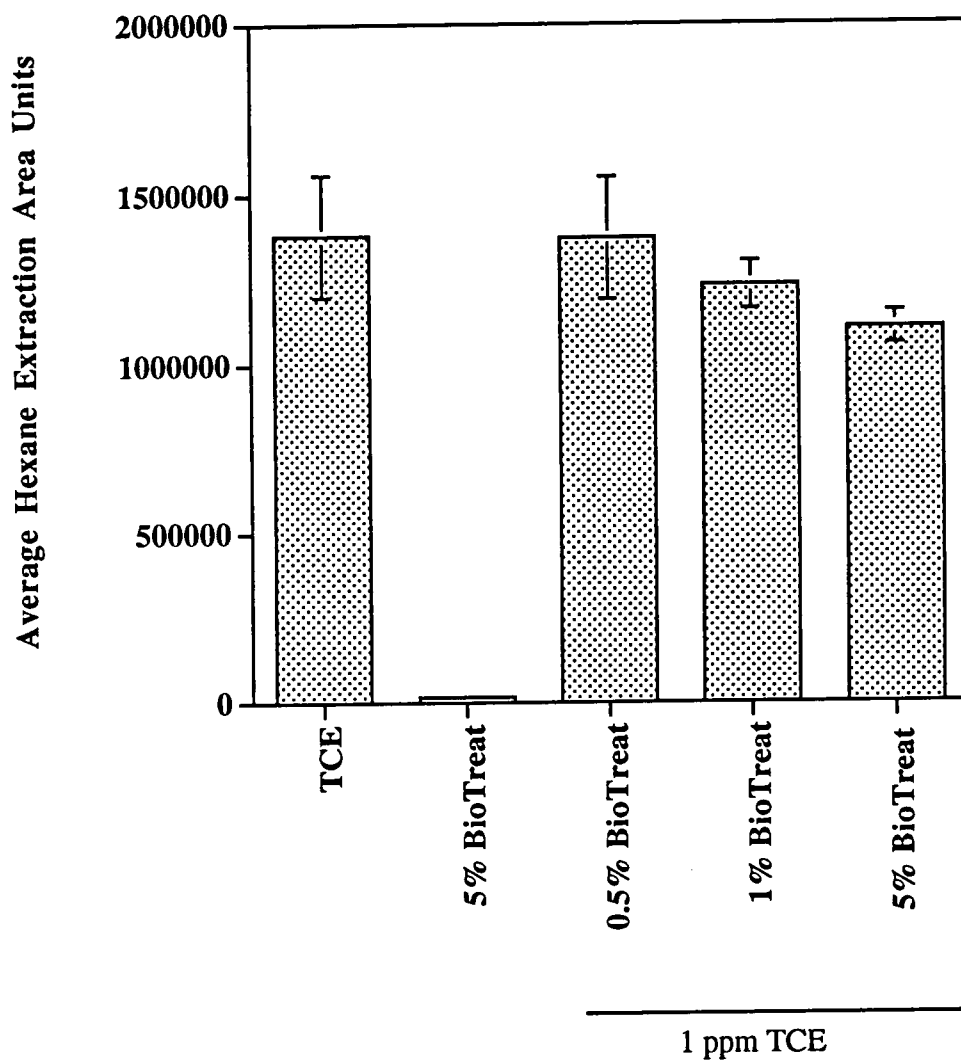


Figure 16. Hexane extraction efficiency for 1.0 ppm TCE with high BioTreat concentrations. Controls are TCE only and 5% BioTreat. $n=3$, stdev within error bars.

$$K_{m2} = \frac{\text{mass TCE solubilized per g surfactant}_{\text{micelles}}}{\text{aqueous phase TCE concentration}}$$

K_{m2} can be extracted from published K_m values with the following relationship:

$$K_{m2} = \frac{K_m}{\text{mol. wt. surfactant} \cdot 0.0555}$$

A comparison of K_{m2} values for various surfactants is presented in Table 4. For a complete development of the equations for calculating K_{m2} , refer to Appendix I.

Table 4. K_m and K_{m2} values for several surfactants.

Surfactant	K_{m2}	Log K_m
Aerosol OT*	127	3.50
BioTreat	214	N/A
Inipol EAP 22*	75	N/A
SDS*	152	3.38
Triton X100*	125	3.63
Tween 80*	140	4.00

*Measured values obtained from Dr. Frederic Gadelle, Oak Ridge National Laboratory Environmental Sciences Division, unpublished data.

By comparison of K_{m2} values for the surfactants presented in Table 4, it can be concluded that BioTreat is effective at increasing the apparent aqueous solubility of TCE. The calculated K_{m2} value for BioTreat is larger than values for the other nonionic surfactants (Triton X100, and Tween 80), the anionic surfactants (Aerosol OT, and SDS), and for the oleophilic fertilizer, Inipol EAP22. This indicates that of the

surfactants presented in Table 4, BioTreat is the most effective on a mass basis at increasing the solubilization of TCE.

From these solubility studies, it is evident that BioTreat concentrations greater than the average CMC can enhance the apparent aqueous solubility of TCE.

4.4 TCE Sorption Studies

The sorption of TCE to G4 cells was measured as a function of TCE headspace concentration. From an addition of 0.05 ml of G4 culture at OD = 2 to 4.5 ml of buffer, there are approximately $1 - 3 \times 10^8$ cells/ml (personal communication, Dr. F. Gadelle). Headspace measurements at 0 and 24 hr showed that no TCE is sorbed to G4 cells inactivated by autoclaving when added at concentrations of 1, 5, and 10 ppmv ($P > 0.10$) (Figure 17). The 10 ppm controls at 0 hr had not reached equilibrium, hence the low headspace readings at that time period. This data is supported by Chang and Alvarez-Cohen (1995) who also showed that TCE sorption onto bacterial cells (mixed cultures of methane, propane, toluene, and phenol oxidizers) is insignificant.

4.5 TCE Degradation Studies

The effects of BioTreat on the TCE degradation rates of TCE by G4 were investigated. Initial experiments showed that BioTreat can support cell growth and enhance the apparent aqueous solubility of TCE at concentrations in excess of the CMC, however above the CMC, BioTreat adversely affected enzyme activity. The combined influence of all these factors were tested in degradation experiments. Enhanced solubility could lead to increased rates of TCE degradation by the bacteria, provided that the enzyme activity was not significantly decreased and that micellized TCE is bioavailable. Viable cell counts would show if BioTreat's nutrient

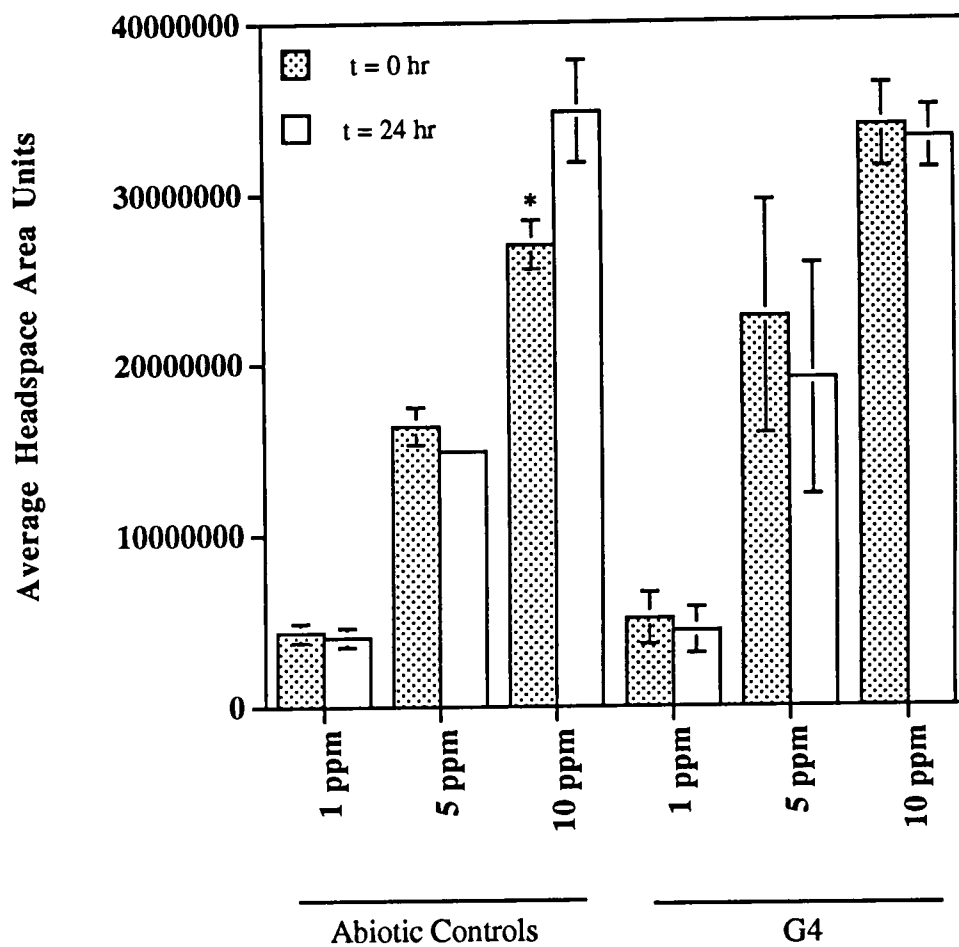


Figure 17. Sorption of TCE to autoclaved G4 cells. *Indicates sample not at equilibrium. n=3 stdev within error bars.

characteristics lead to any increase in degradation rates from an increase in cell numbers.

G4 was exposed to 1 ppm TCE and 0, 0.01, 0.05, 0.5, 1, and 5% BioTreat (Figure 18). TCE only and BioTreat only controls were included. At $t = 4$ hr, the decrease in headspace TCE concentrations in samples containing G4 and G4 + 0.01% BioTreat is attributed to degradation. At 0.01% BioTreat, no enhanced solubilization is observed (see Figure 12). No abiotic losses of TCE at this time point are expected because there were no punctures in the septa until measurements were taken at $t = 4$ hr. A decrease of TCE measured in the headspace over time in the TCE only controls indicate abiotic losses. Samples containing 0.05, 0.5, 1, and 5% BioTreat show a statistical difference in the TCE concentrations when compared to the control with G4 and no surfactant ($P < 0.05$). An increase in degradation at these surfactant concentrations may be due to the additional nutrients provided by BioTreat. An increase in solubilization may also contribute to the decrease in TCE headspace concentration with 0.5, 1, and 5% BioTreat.

After 24 and 48 hr incubations, no TCE was measured in the headspace except for treatments containing 5% BioTreat. A hexane extraction to quantify residual TCE (Figure 19) confirmed that all of the TCE was degraded in samples containing 0.01, 0.05, 0.5, and 1% BioTreat. Approximately 11.5% TCE remained in the 5% BioTreat samples as compared to TCE only controls after the 48 hour degradation period. Biodegradation appears to be inhibited at this high concentration of BioTreat.

There are four major explanations for inhibited biodegradation rates at high surfactant concentrations: surfactant toxicity, lower bioavailability of the contaminant, surfactant interactions with the cell membranes (Laha and Luthy, 1992), and oxygen limitations. At high substrate concentrations, the bacteria may quickly consume the

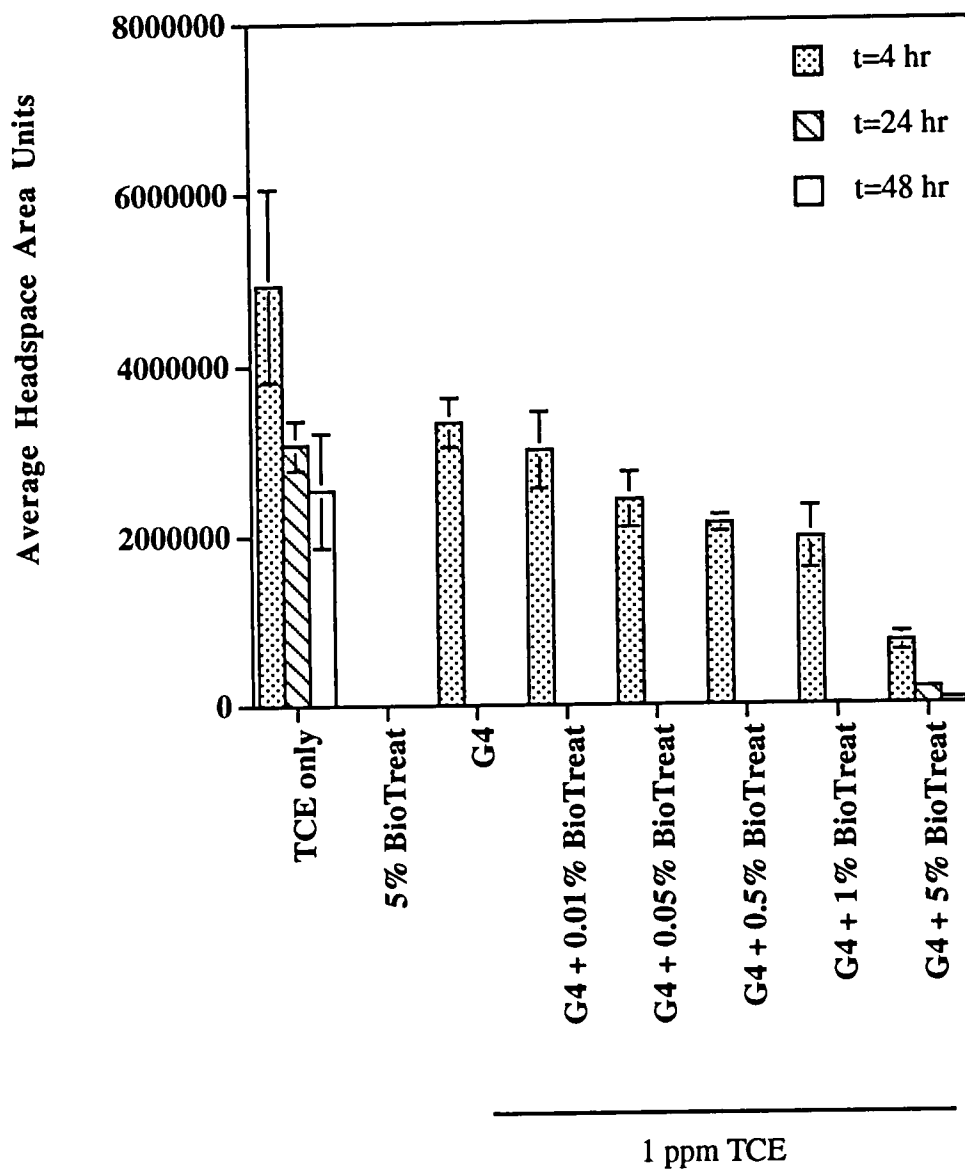


Figure 18. Effect of BioTreat concentration on TCE disappearance with the use of G4. n=3, stdev within error bars.

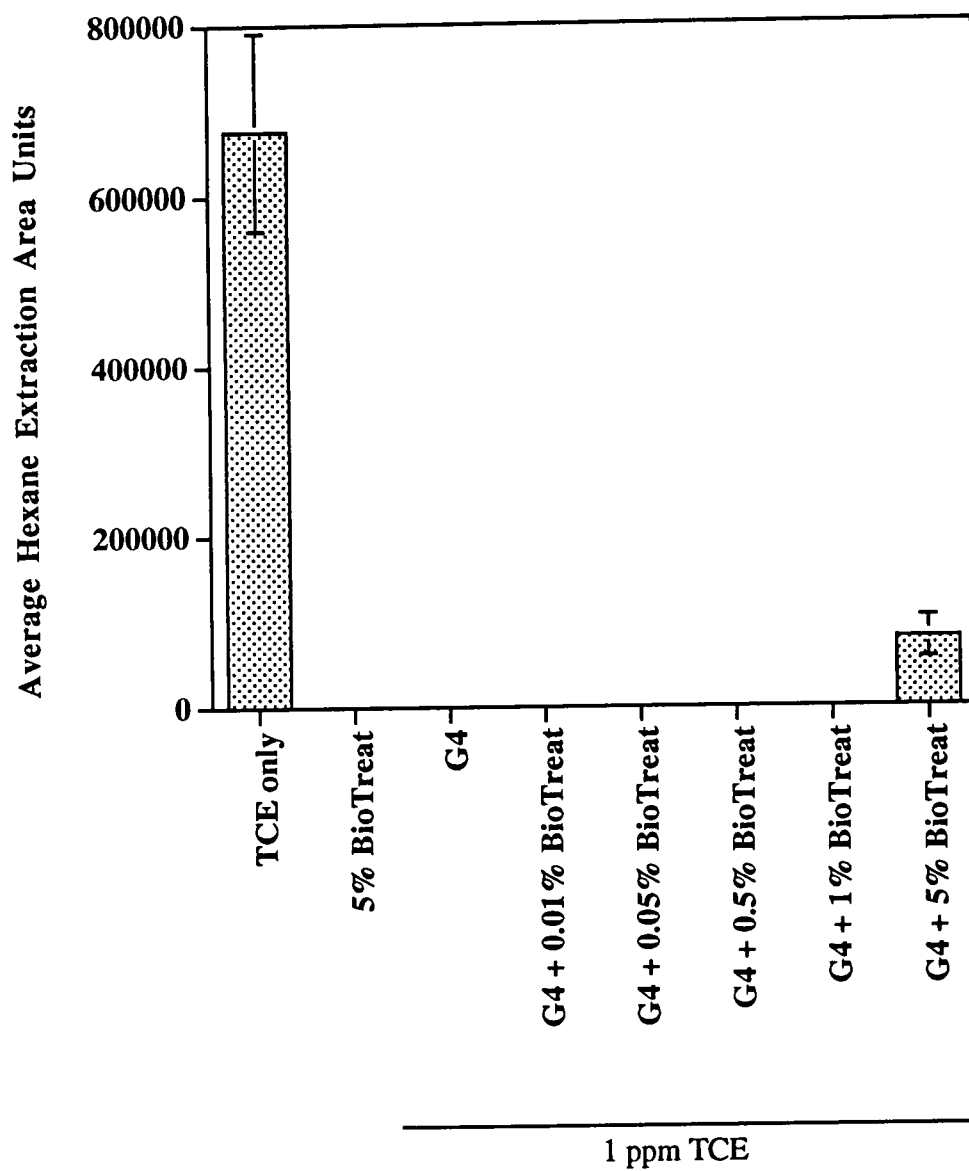


Figure 19. Residual TCE after 48 hr degradation period for 1 ppm TCE and low BioTreat concentrations. n=3, stdev within error bars.

dissolved oxygen available while metabolizing the carbon and energy source. Low dissolved oxygen concentrations may limit growth and reproduction, resulting in fewer cells and less overall degradation. An analysis of oxygen peak sizes from chromatograms showed a 53% decrease in oxygen. Under these conditions, degradation may not be limited. Several researchers have reported on surfactant toxicity effects on bacterial cells. Ankley and Burkhard (1992) proposed that the toxicity of the effluent from a publicly owned treatment works that received no secondary treatment was due to the presence of surfactants, not to the residual chlorine concentrations. Aronstein et al. (1991) reported that a nonionic surfactant at twice the CMC was toxic to phenanthrene degraders. Larson and Maki (1982) reported that microbial exposure to an anionic surfactant depressed rates of glucose metabolism in a microbial ecosystem. Glucose metabolism is an indirect measure of the chemical's toxicity to the microorganisms. Another mechanism by which surfactants can increase toxicity to the bacteria is by enhancing contaminant solubilization, leading to toxic levels of the contaminant in contact with the bacteria (Thibault et al., 1996).

Volkering et al. (1995) concluded that PAH's inside micelles are not readily available to microorganisms. At high surfactant concentrations, many micelles are present which decrease the aqueous phase concentration of contaminant for biodegradation, thus decreasing bioavailability. They proposed that the PAH sequestered in micelles acts as a reservoir that replenishes PAH aqueous-phase concentrations as they are degraded. However, the exit rates of the contaminant may be slower than the degradation rates, and therefore may limit degradation (Laha and Luthy, 1992). At 5% BioTreat, more micelles are present than at the lower concentrations (0.01, 0.05, 0.5, 1% BioTreat); therefore, more TCE can be sequestered into the micelles. In a study by Grimberg et al. (1996), micelles were treated as a separate phase in which micellarized phenanthrene was not directly available for biodegradation.

Surfactant-cell interactions can also occur which inhibit contaminant degradation. The hydrophobic tail of surfactant monomers can penetrate the phospholipid bilayer that composes cell membranes and interfere with transport of substrate or contaminant into the cell for metabolism. However, experiments by Laha and Luthy (1992) showed these supra-CMC micellar surfactant-bacteria interferences were reversible upon decreasing the surfactant concentration below the CMC and do not permanently damage cells.

Since 1 ppm TCE was degraded within 48 hours in most of the treatments, an experiment was designed to observe the effect of 0.05% BioTreat on the degradation of 1 ppm TCE within a 24 incubation. Headspace measurements were performed at 4 and 24 hours. No enhanced TCE solubilization is expected at this BioTreat concentration based on the results of previous experiments (see Figures 11 and 12). However, data in Figure 20 shows enhanced degradation rates in the presence of 0.05% BioTreat ($P < 0.01$). Since no enhanced solubilization is expected at this BioTreat concentration, the increase in degradation rates could be attributed to increased cell numbers due to the nutrients supplied by BioTreat. The experimental design does not include a carbon source other than BioTreat and TCE. G4 does not gain energy for cell growth from TCE metabolism; thus, any increase in cell numbers would be due to BioTreat. The carbon in BioTreat could serve as an electron donor (carbon source) for cell growth. Increased cell growth corresponds to an increase in cell numbers, which could in turn correspond to an increase in degradation rates. Previous experiments showed that 1 ppm TCE was not detected in the headspace after 24 hr (see Figure 18). This was not found to be true in this experiment, and can only be explained by differences in the bacterial cultures used in these two discrete experiments. Previous experiments showed a decrease in enzyme specific activity with a 0.05% BioTreat concentration (Table 3). However, in those experiments, BioTreat was present throughout the

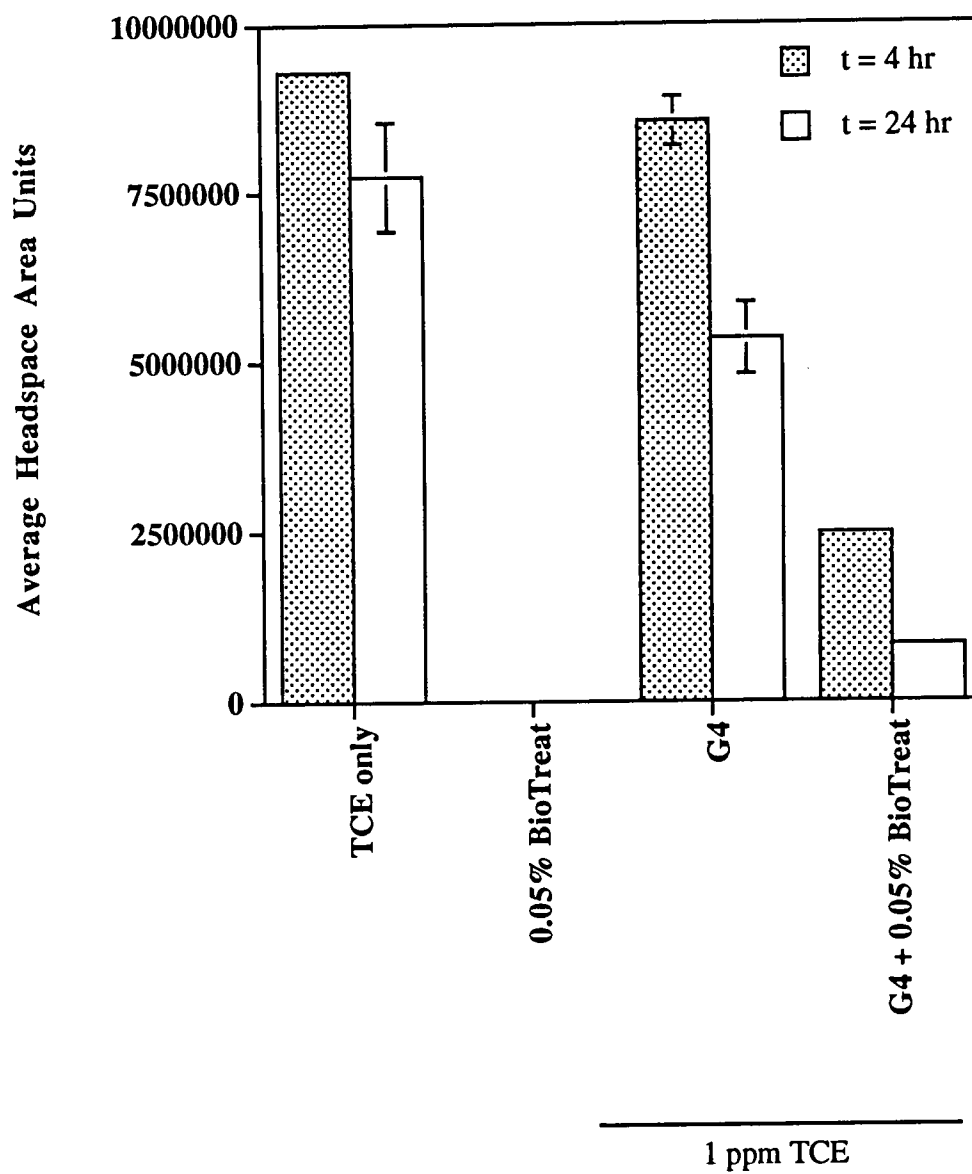


Figure 20. Effect of 0.05% BioTreat on TCE degradation by G4.
n=3, stdev within error bars.

growth cycle. In this degradation experiment, G4 was added after it reached late stationary phase (grown on glucose) and the enzyme was already being expressed. These differences could explain the lack of inhibition in this experiment.

A hexane extraction showed that treatments containing 0.05% BioTreat had approximately 82% less TCE than the sterile control ($P < 0.01$) (Figure 21). Again, enhanced solubilization by BioTreat does not occur at this concentration. Increased degradation as compared to treatments with bacteria and TCE only were most likely due to increased cell numbers from the additional nutrient source available to the bacteria from the presence of BioTreat. Serial dilutions and spread plating were done to enumerate the bacteria present in each treatment. Viable cell counts revealed $121 \times 10^6 \pm 27.0$ cells/ml for treatments containing bacteria and TCE only. An increase of 37% ($192 \times 10^6 \pm 11.8$ cells/ml, $P < 0.01$) over the control samples with no BioTreat was observed for treatments containing G4, TCE and 0.05% BioTreat. Therefore the enhanced degradation rates were most likely due to increased cell numbers in samples containing BioTreat.

The effect of BioTreat on the degradation rate of 5 ppm TCE is shown in Figure 22. Three BioTreat concentrations were used to represent the average CMC (0.05%), ten times the average CMC (0.5%), and 100 times the average CMC (5%). Headspace measurements were performed at 0, 24, and 48 hr. At $t = 0$ hr, there is no significant difference between 0.05% BioTreat and the control ($P = 0.166$). This is expected since there is no enhanced solubilization at this concentration. At 0.5% and 5% BioTreat, a decrease in TCE headspace concentration is observed. Again, this is expected because of the increase in TCE solubilization observable at these concentrations. No measurable degradation was measured in samples containing no or 0.05% BioTreat at this sampling time.

After a 24 hr degradation period, only samples containing 0.5% BioTreat

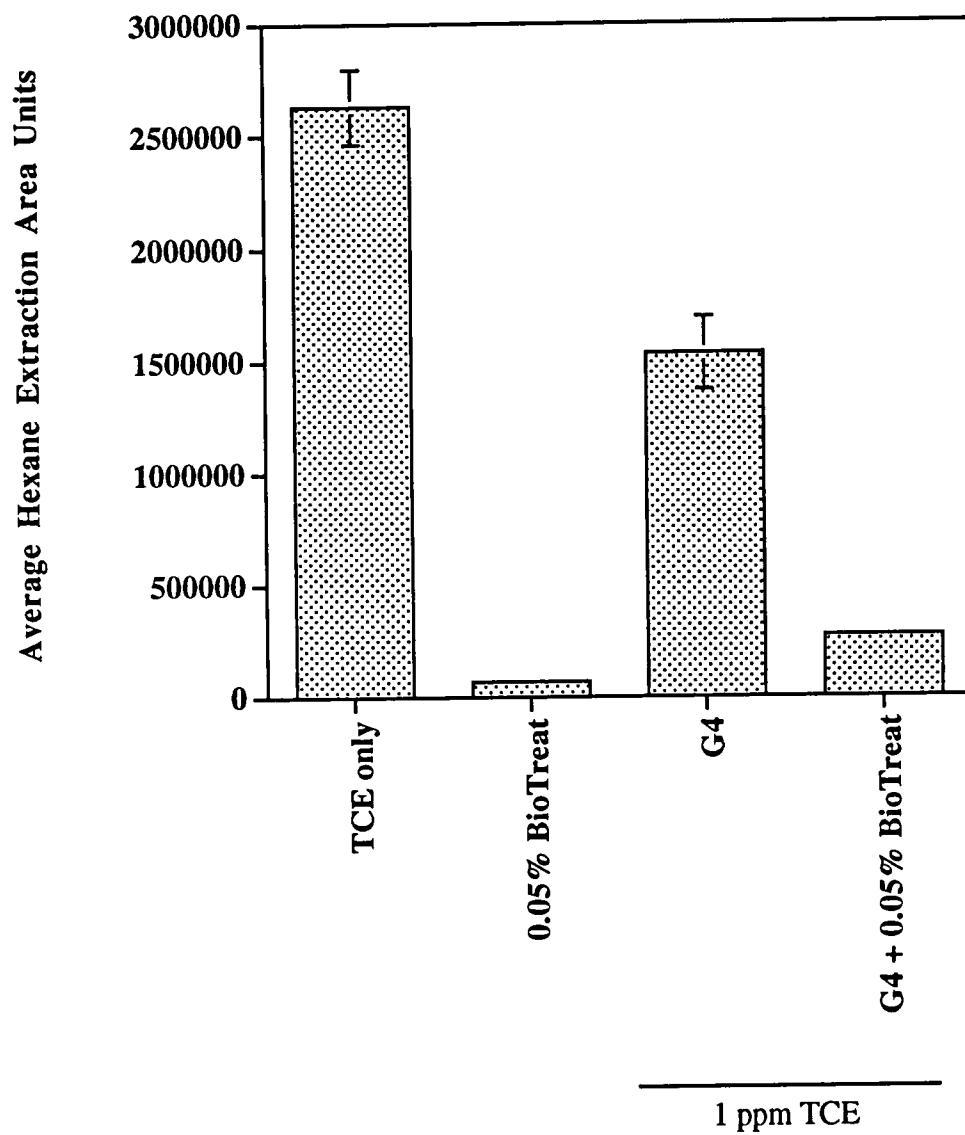


Figure 21. Residual TCE after 24 hr degradation period for 1 ppm TCE with 0.05% BioTreat. n=3, stdev within error bars.

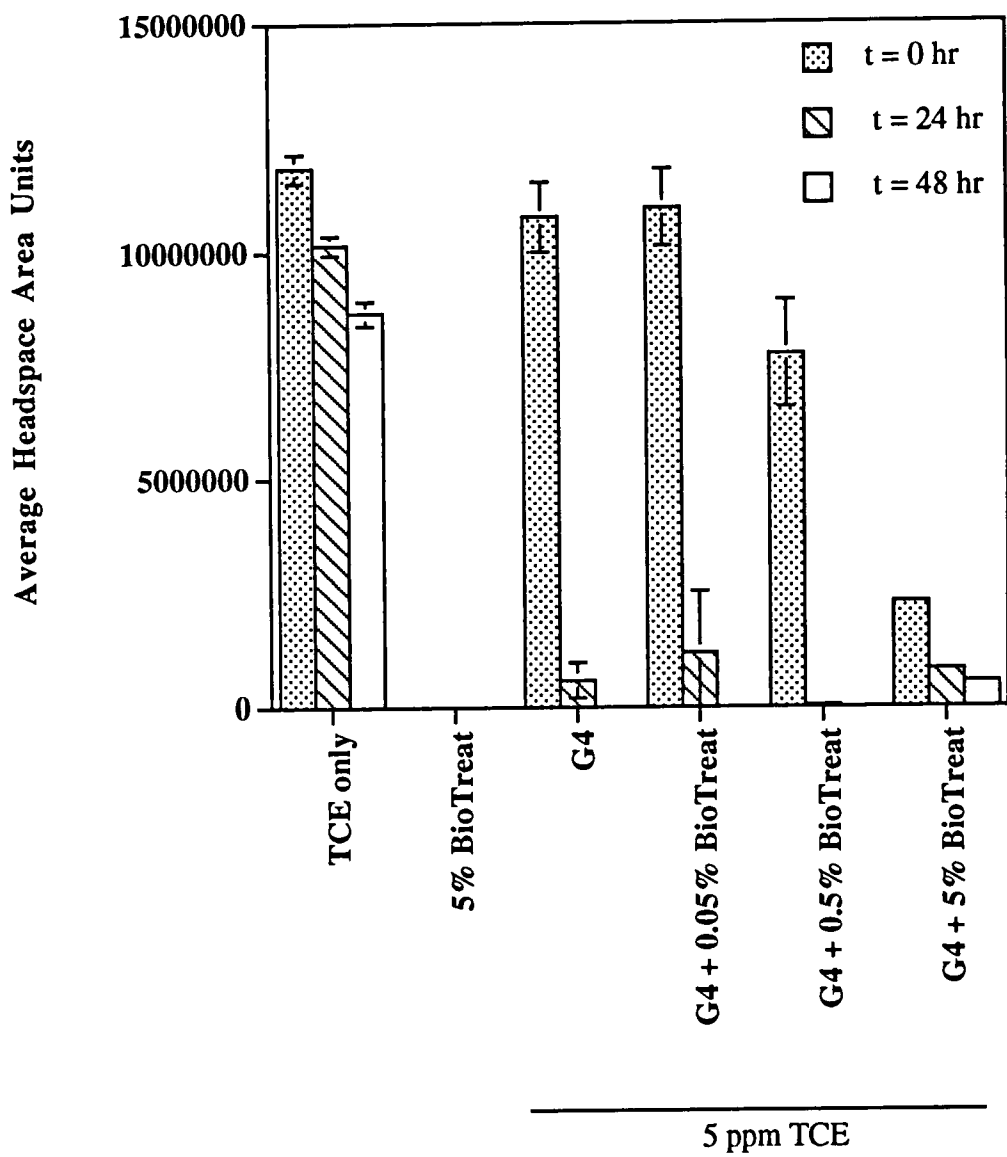


Figure 22. Effect of BioTreat concentration on disappearance of 5 ppm TCE with G4. n=3, stdev within error bars.

showed no measurable amounts of TCE in the headspace. Samples containing 0.05% and 5% BioTreat had approximately 7 and 5% TCE remaining, respectively. As the incubation time increased to 48 hr, all samples showed complete degradation, except 5% BioTreat. A hexane extraction after 48 hours provided verification that 5 ppm TCE was degraded in samples with 0.05%, 0.5% and no BioTreat (Figure 23). Samples containing 5% BioTreat had approximately 69% less TCE than the sterile controls. Again, this may be due to several reasons such as potential surfactant toxicity to the bacteria, lower bioavailability of the contaminant due to partitioning into the micelles, surfactant interactions with the cell membranes, and oxygen limiting conditions. Serial dilutions and plate counts were performed to measure the changes in cell numbers in samples containing BioTreat. Samples with 0.05% BioTreat had 107% more cells than the control with no BioTreat (control = $107 \times 10^6 \pm 19.8$ CFU/ml; 0.05% BioTreat = $222 \times 10^6 \pm 12.7$ CFU/ml, $P < 0.01$). Treatments containing 0.5% BioTreat had the greatest number of colony forming units, with an increase of 150% over the control ($267 \times 10^6 \pm 20.0$ CFU/ml, $P < 0.001$). However, samples with 5% BioTreat showed a decrease in colony forming units by 72.8% as compared to controls ($29 \times 10^6 \pm 4.0$ CFU/ml, $P < 0.001$)).

The last experiment in this study focused on the effects of BioTreat on the degradation of 10 ppm TCE (Figure 24). For this experiment, residual TCE after a 48 hr incubation was quantified by a hexane extraction only. A control sample with BioTreat and TCE was added to show BioTreat does not interfere with the extraction efficiency of hexane ($P > 0.04$). In samples containing G4 with no BioTreat, 87.4% less TCE than in the sterile controls was measured after 48 hours. Samples with 0.05% BioTreat and bacteria had 8% less TCE than samples with G4 and TCE only. At this concentration (0.05% BioTreat), no enhanced solubilization affects are visible.

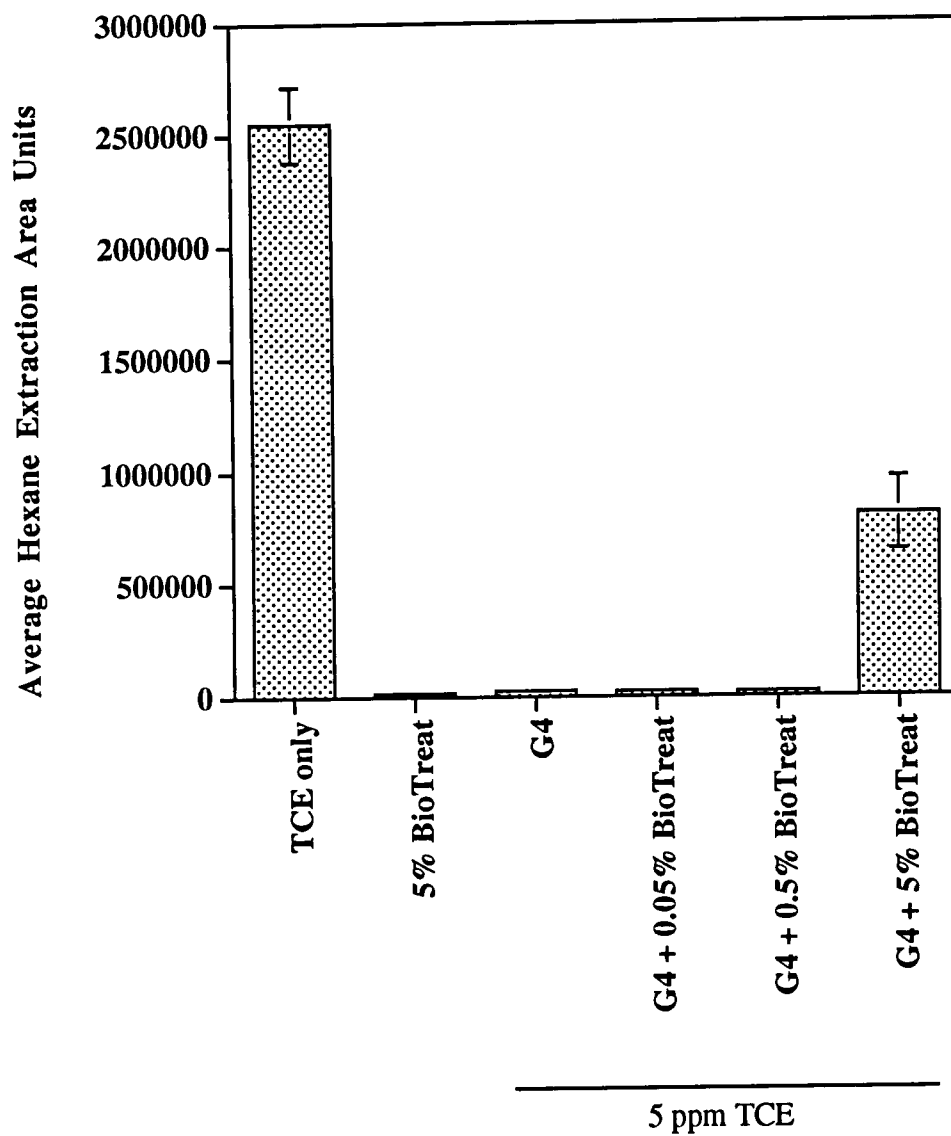


Figure 23. Residual TCE after 48 hr degradation period for 5 ppm TCE with various BioTreat concentrations. n=3, stdev within error bars.

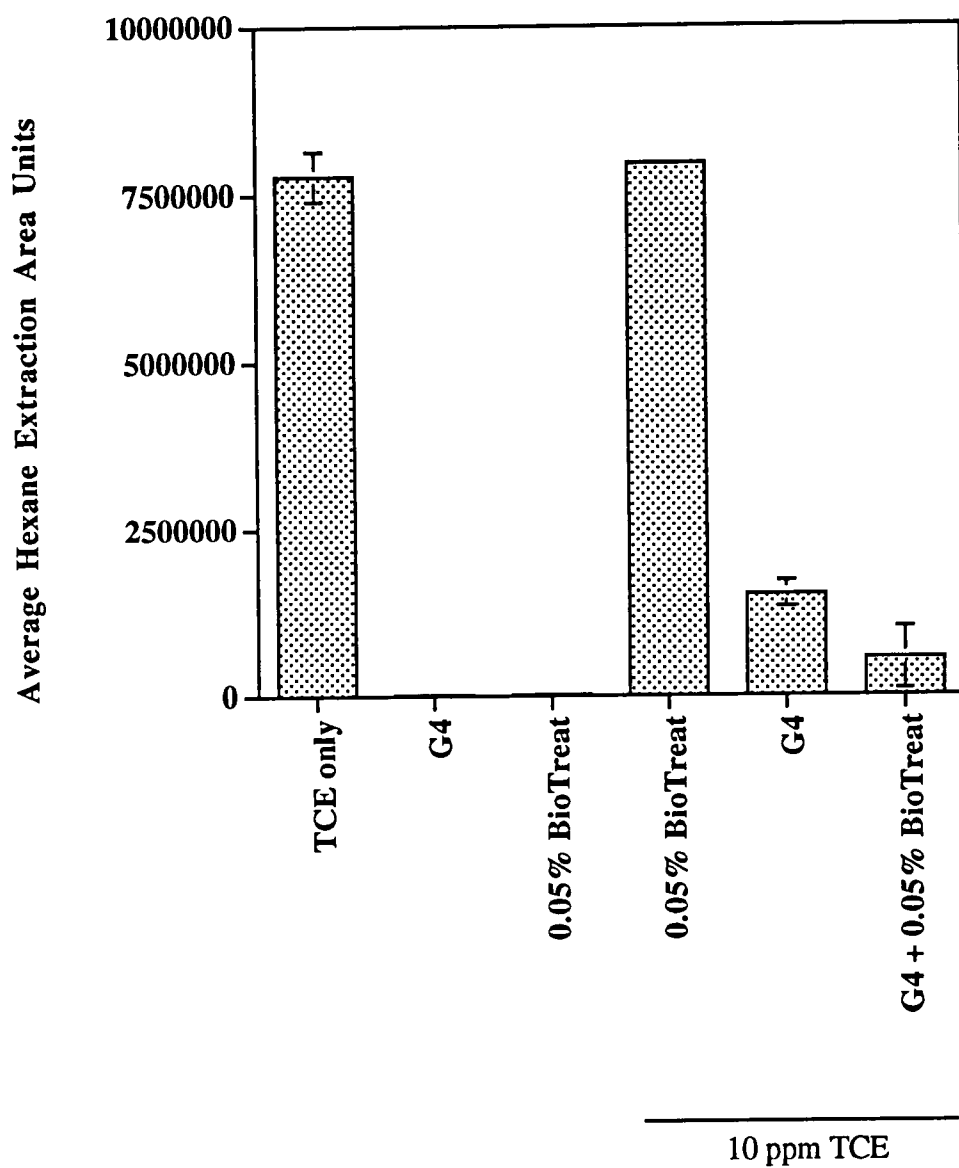


Figure 24. Residual TCE after 48 hr degradation period for 10 ppm TCE with 0.05% BioTreat. $n=3$, stdev within error bars.

From previous evidence of increased cell numbers in treatments containing low concentrations of BioTreat, the increase in TCE degraded over controls can be attributed to increased cell numbers due to the additional nutrients provided by BioTreat.

Chapter 5

Conclusions

The effects of BioTreat, a nutrient-surfactant compound, on *Burkholderia cepacia* G4 PR1₃₀₁ and the degradation of TCE by this bacteria were investigated in this research. Experiments were performed to determine if BioTreat is biodegradable by G4 and can support cell growth, thus confirming that BioTreat is a nutrient mixture useful in bioremediation activities. The effects of BioTreat on G4's enzyme activity were studied to determine if BioTreat had any adverse effects on the ability of G4 to degrade TCE. The enhancement of the apparent aqueous solubility of TCE by BioTreat concentrations ranging from two times below the average CMC to 100 times greater than the average CMC were determined. Finally, the effects of BioTreat on the degradation of G4 on various TCE concentrations were examined.

From these experiments, the following conclusions can be drawn.

1. BioTreat does have surfactant properties, as evidenced by its ability to lower the surface tension of water. The average CMC value of BioTreat is 0.05%.
2. BioTreat is partially biodegradable by G4 and can support cell growth of G4. Cell growth with BioTreat as the sole carbon and energy source is slow.
3. BioTreat, at concentrations above the average CMC, inhibits enzyme activity. The mechanism for this unknown. It is possible that there are

surfactant-cell membrane interactions which inhibit degradation. Reduced enzyme activity is first seen at the CMC concentration.

4. BioTreat does not enhance TCE aqueous solubility at or below the average CMC. Solubilization occurs at concentrations greater than the average CMC.
5. BioTreat can enhance degradation rates. At or below the average CMC, degradation could be enhanced due to increased cell numbers made possible by the increased nutrients available from BioTreat.
6. BioTreat at very high concentrations may inhibit cell growth , resulting in fewer cells. This is likely due to toxicity effects of BioTreat on the bacteria or oxygen limiting conditions.

G4 is a good candidate for remediation of TCE contaminated sites because it does not require an inducing compound and can be grown with nonhazardous substrates as opposed to strains which require induction by phenol, toluene, or other chemicals. G4 should not be used in sites that are heavily contaminated with TCE due to the toxicity effect at high concentrations.

BioTreat has two effects that may enhance remediation: enhanced solubility and bionutrients. Enhanced solubilization of TCE in aqueous only systems by BioTreat can be achieved at concentrations greater than the CMC. However, enhanced solubility of TCE by partitioning into micelles does not necessarily translate into enhanced biodegradation rates. Micellarized TCE may decrease bioavailability and therefore decrease the amount of TCE that can be degraded. The use of BioTreat concentrations

above the CMC must also be balanced with the fact that at these concentrations, a decrease in enzyme activity is observed. BioTreat at low concentrations could be used to provide an additional source of nutrients for the bacteria, but it should not be used as the primary growth substrate since the growth of G4 on BioTreat is slow under these conditions. The effects of BioTreat-soil interactions should be investigated before use at actual sites.

This research was performed in support of a field demonstration of TCE remediation at the Department of Energy Kansas City Plant in Kansas City, Missouri.

References

- Agency for Toxic Substances and Disease Registry, U.S. Public Health Service. 1989. Toxicological profile for trichloroethylene. ATSDR/TP-88/24. p. 134.
- Ankley, G. T., and L. P. Burkhard. 1992. Identification of surfactants as toxicants in a primary effluent. *Env. Tox. and Chem.* 11:1235-1248.
- Arciero, J. A., and J. S. Suk. 1986. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Biochem. Biophys. Res. Comm.* 159:240.
- Aronstein, B. N., and M. Alexander. 1992. Surfactants at low concentrations stimulate biodegradation of sorbed hydrocarbons in samples of aquifer sands and soil slurries. *Environ. Tox. and Chem.* 11:1227-1233.
- Aronstein, B. N., Calvillo, Y. M., and M. Alexander. 1991. Effects of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soil. *Environ. Sci. Technol.* 25:1728-1731.
- Attwood, D., and A. T. Florence. 1983. *Surfactant Systems: Their Chemistry, Pharmacy, and Biology*. Chapman and Hall: New York, 1983.
- Basseres, A. and A. Ladousse. 1993. Bioremediation with oleophilic fertilizer. *ACS Preprints, Bioremediation and BioProcessing.* 38:246-253.
- Beller, H. R., Grbic-Galic, D., and M. Reinhard. 1992. Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. *Appl. and Environ. Microbiol.* 58:786-793.
- Brockman, F. J., Payne, W., Workman, D. J., Soong, A., Manley, S., and T. C. Hazen. 1995. Effect of gaseous nitrogen and phosphorus injection on in situ bioremediation of a trichloroethylene-contaminated site. *Journ. of. Haz. Mater.* 41:287-298.
- Chaiko, M. A., R. Nagarajan, and E. Ruckenstein. 1984. Solubilization of single-component and binary mixtures of hydrocarbons in aqueous micellar solutions. *Journ. of Coll. and Inter. Sci.* 99:168-182.
- Chang, H. and L. Alvarez-Cohen. 1995. Transformation capacities of chlorinated organics by mixed cultures enriched on methane, propane, toluene, or phenol. *Biotech. and Bioeng.* 45:440-449.
- Chu, K. H., and L. Alvarez-Cohen. 1996. Trichloroethylene degradation by methane-oxidizing cultures grown with various nitrogen sources. *Water Environ. Res.* 68:76-82.
- Churchill, S.A., Griffin, R. A., Jones, L. P., and P. F. Churchill. 1995. Biodegradation and Bioremediation. Biodegradation rate enhancement of hydrocarbons by an oleophilic fertilizer and a rhamnolipid biosurfactant. *Journ. Environ. Qual.* 24:19-28.

- Cohen, A. K., and N. Johnson. 1989. Thermal destruction of military waste by incineration and thermal stripping. A&WMA Annual Meeting Proceedings, 82nd A&WMA Annual Meeting. Air and Waste Manag. Assoc, Pittsburg, PA. p. 11
- Criddle, C. S., DeWitt, J. T., Grbic-Galic, D., and P. L. McCarty. 1990. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. Appl. and Environ. Microbiol. 56:3240-3246.
- Deziel, E., Paquette, G., Villemur, R., Lepine, F., and J. Bisailon. 1996. Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. and Environ. Microbiol. 62:1908-1912.
- Dzombak, D. A., and R. G. Luthy. 1984. Estimation of adsorption of polycyclic aromatic hydrocarbons on soils. Soil Sci. 137:292-308.
- Edwards, D. A., Luthy, R. G., and Z. Liu. 1991. Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. Environ. Sci. Technol. 25:127-133.
- Ely, R. L., Hyman, M. R., Arp, D. Guenther, R. B., and K. J. Williamson. 1995. A cometabolic kinetics model incorporating enzyme inhibition, inactivation, and recovery: II. Trichloroethylene degradation experiments. Biotech. and Bioeng. 46:232-245.
- EPA Working Group. 1981. EPA-440/4-85-019. An exposure and risk assessment for trichloroethylene.
- Fan, S., and K. M. Scow. 1993. Biodegradation of trichloroethylene and toluene by indigenous microbial populations in soil. Appl. and Environ. Microbiol. 59:1911-1918.
- Fliermans, C. B., Phelps, T. J., Ringelberg, D., Mikell, A. T., and D. C. White. 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. Appl. and Environ. Microbiol. 54:1709-1714.
- Folsom, B. R., Chapman, P. J., and P. H. Pritchard. 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. and Environ. Microbiol. 56:1279-1285.
- Fountain, J. C., Klimek, A., Beikirch, M. G., and T. M. Middleton. 1991. The use of surfactants for in situ extraction of organic pollutants from a contaminated aquifer. Journ. of Haz. Mater. 28:295-311.
- Fox, Brian, G., Borneman, J. G., Wackett, L. P., and L. D. Lipscomb. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. 29:6419-6427.
- Gandi, P., Erickson, L. E., and L. T. Fan. 1995. A simple method to study the effectiveness of bioremediation aided, pump-and-treat technology for aquifers contaminated by non-aqueous phase liquids. II. Multicomponent systems. Journ. of Haz. Mater. 41:185-204.

- Gantzer, C. J. and L. P. Wackett. 1991. Reductive dechlorination catalyzed by bacteria transition-metal coenzymes. *Environ. Sci. Technol.* 25:715-722.
- Gillespie, M. T., and J. M. Strong-Gunderson. 1997. Effects of a nutrient-surfactant compound on solubilization rates of TCE. *Appl. Biochem. and Biotechnol.* 63-65:835-843.
- Graham, D. W., Smith, V. H., and K. P. Law. 1995. Application of variable nutrient supplies to optimize hydrocarbon biodegradation. In *Bioremediation of Recalcitrant Organics*. Hinchee, R. E., Hoeppe, R. E., and D. B. Anderson, eds. Battelle Press, Columbus, OH. p. 331-340.
- Grimberg, S. J., Stringfellow, W. T., and M. D. Aitken, 1996. Quantifying the biodegradation of phenanthrene by *Pseudomonas stutzeri* P16 in the presence of a nonionic surfactant. *Appl. and Environ. Microbiol.* 62:2387-2392.
- Haley, J. L., Hanson, B., Enfield, C., and J. Glass. 1991. Evaluating the effectiveness of groundwater extraction systems. *GWMR*. Winter, 119-124.
- Harms, H., and A. J. B. Zehnder. 1995. Bioavailability of sorbed 3-chlorodibenzofuran. *Appl. and Environ. Microbiol.* 61:27-33.
- Hopkins, G. D., and P. L. McCarty. 1995. Field evaluation of in situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. *Environ. Sci. Technol.* 29:1628-1637.
- Hyman, M. R., Russel, S. A., Ely, R. L., Williamson, K. J., and D. J. Arp. 1995. Inhibition, inactivation, and recovery of ammonia-oxidizing activity in cometabolism of trichloroethylene by *Nitrosomonas europaea*.. *Appl. and Environ. Microbiol.* 61:1480-1487.
- IARC. 1995. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. 63:75-158.
- Jackson, R. E. 1993/94. Surfactant-enhanced remediation of DNAPL zones in granular aquifer systems. *Remediation*. Winter: 77-91.
- Jafvert, C. T., Van Hoof, P. L., and J. K. Heath. 1994. Solubilization of non-polar compounds by non-ionic surfactant micelles. *Wat. Res.* 28:1009-1017.
- Jafvert, C. T., Van Hoof, P. L., and W. Chu. 1995. The phase distribution of polychlorobiphenyl congeners in surfactant-amended sediment slurries. *Wat. Res.* 29:2387-2397.
- Kile, D. E., and C. T. Chiou. 1989. Water solubility enhancements of DDT and trichlorobenzene by some surfactants below and above the critical micelle concentration. *Environ. Sci. Technol.* 23:832-838.
- Kuneida, H., Nakano, A., and M. Akirmaru. 1995. The effect of mixing of surfactants on solubilization in a microemulsion system. *Journ. of Coll. and Inter. Sci.* 170:78-84.

- Laha, S., and R. G. Luthy. 1991. Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. *Environ. Sci. Technol.* 25:1920-1930.
- Laha, S., and R. G. Luthy. 1992. Effects of nonionic surfactants on the solubilization and mineralization of phenanthrene in soil-water systems. *Biotechnol. and Bioeng.* 40:1367-1380.
- Landa, A. S., Sipkema, E. J., Weijma, J., Beenackers, A. A. C. M., Dolfin, J., and D. B. Janssen. 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. *Appl. and Environ. Microbiol.* 60:3368-3374.
- Larson, R. J., and A. W. Maki. 1982. Effect of linear alkylbenzene sulfonate on the structure and function of microbial communities in model ecosystems. *Aquatic Toxicology and Hazard Assessment: Fifth Conference, ASTM STP 766*. Pearson, J. G., Foster, R. B., and W. E. Bishop, eds. American Society for Testing and Materials. p. 120-136.
- Leahy, J. G., Byrne, A. and R. H. Olsen. 1996. Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. *Appl. and Environ. Microbiol.* 62:825-833.
- Leavitt, M. E., and K. L. Brown. 1994. Biostimulation versus bioaugmentation-three case studies. In *Hydrocarbon Bioremediation*. Hinchee, R. E., Alleman, B. C., Hoepfel, R. E., and R. N. Miller. Lewis Publishers, Boca Raton, FL. p. 72-79.
- Lee, M. D., Thomas, J. M., Borden, R. C., Bedient, P. B., Ward, C. H. and J. T. Wilson. 1988. Bioremediation of aquifers contaminated with organic compounds. *CRC Critical Reviews in Environmental Control.* 18:29-89.
- Leuser, R. M., Velazquez, L. A., Cohen, A., and J. Janssen. 1989. Remediation of PCB soil contamination by on-site incineration. *Journ. of Haz. Mat.* 197th National Meeting on Characterization and Cleanup of Chemical Waste Sites. 375-383.
- Lindstrom, J. E., Prince, R. C., Clark, J. C., Grossman, M. J., Yeager, T. R., Braddock, J. F., and E. J. Brown. 1991. Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the T/V Exxon Valdez oil spill. *Appl. and Environ. Microbiol.* 57:2514-2522.
- Little, C. D., Palumbo, A. V., Herbes, S. E., Lidstrom, M. E., Tyndall, R. L., and P. J. Gilmer. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. *Appl. and Environ. Microbiol.* 54:951-956.
- Maymo-Gatell, X., Tanyol, V., Gossett, J. M., and S. H. Zinder. 1995. Characterization of an H₂-utilizing enrichment culture that reductively dechlorinates tetrachloroethylene to vinyl chloride and ethene in the absence of methanogenesis and acetogenesis. *Appl. and Environ. Microbiol.* 61:3928-3933.
- McGrath, R. 1995. Managing sediment remediation costs. *Poll. Eng.* July:34-35.

Merck Index, 1976. Ninth edition. Windholz, M., Budavari, S., Stroumstos, L. Y., and M. N. Fertig, eds. Merck & Co., Inc., Rahway, NJ. p. 1238.

Monod, J. 1949. The growth of bacterial cultures. *Ann. Rev. of Microbiol.* 3:371.

Munakata-Marr, J., McCarty, P. L., Shields, M. S., Reagin, M., and S. C. Francesconi. 1996. Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered *Burkholderia* (*Pseudomonas*) *cepacia* G4 and PR1. *Environ. Sci. Technol.* 30:2045-2052.

Nelson, M. J. K., Montgomery, S. O., Mahaffey, W. R., and P. H. Pritchard. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. *Appl. and Environ. Microbiol.* 52:949-954.

Nelson, M. J. K., Montgomery, S. O., O'Neill, E. J., and P. H. Pritchard. 1986. Aerobic cometabolism of trichloroethylene by a bacterial isolate. *Appl. and Environ. Microbiol.* 52:383-384.

Oldenhuis, R., Oedzes, J. Y., van der Waarde, J. J., and D. B. Jansenn. 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Appl. and Environ. Microbiol.* 57:7-14.

Parke, J. and P. R. Jaffe. 1993. Partitioning of three nonionic organic compounds between adsorbed surfactants, micelles, and water. *Envir. Sci. Technol.* 27:2559-2565.

Pennel, K. D., Jin, M., Abriola, L. M., and G. A. Pope. 1994. Surfactant enhanced remediation of soil columns contaminated by residual tetrachloroethylene. *Journ. of Contam. Hydrol.* 16:35-53.

Phelps, T. J., Niedzielski, J. J., Schram, R. M., Herbes, S. E., and D. C. White. 1990. Biodegradation of trichloroethylene in continuous-recycle expanded-bed bioreactors. *Appl. and Environ. Microbiol.* 56:1702-1709.

Raymond, R. L. 1974. Reclamation of hydrocarbon contaminated ground water. U. S. Patent 3,846,290.

Reij, M. W., Kieboom, J., de Bont, J. A. M., and S. Hartmans. 1995. Continuous degradation of trichloroethylene by *Xanthobacter* sp. strain Py2 during growth on propene. *Appl. and Environ. Microbiol.* 61:2936-2942.

Schwartz, A., and R. Bar. 1995. Cyclodextrin-enhanced degradation of toluene and *p*-toluic acid by *Pseudomonas putida*. *Appl. and Environ. Microbiol.* 61:2727-2731.

Shiau, B., Sabatini, D. A., and J. H. Harwell. 1994. Solubilization and microemulsification of chlorinated solvents using direct food additive (edible) surfactants. *Ground Water.* 32:561-569.

Shields, M. S., and M. J. Reagin. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. *Appl. and Environ. Microbiol.* 58:3977-3983.

Shields, M. S., Montgomery, S. O., Chapman, P. J., Cuskey, S. M., and P. H. Pritchard. 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. *Appl. and Environ. Microbiol.* 55:1624-1629.

Shields, M. S., Montgomery, S. O., Cuskey, S. M., Chapman, P. J., and P. H. Pritchard. 1991. Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. *Appl. and Environ. Microbiol.* 57:1935-1941.

Shields, M. S., Reagin, M. J., Gerger, R. R., Campbell, R., and C. Somerville. 1995. TOM, a new aromatic degradative plasmid from *Burkholderia (Pseudomonas) cepacia* G4. *Appl. and Environ. Microbiol.* 61:1352-1356.

Shields, M.S. 1995. A bacterial system for the remediation of trichloroethylene. AL/EQ-TR-1995-0024.

Smith, G. A., Christian, S. D., Tucker, E. E. and J. F. Scamehorn. 1989. Solubilization of hydrocarbons by surfactant micelles and mixed micelles. *Journ. Colloid Interface Sci.*, 130:254.

Speitel, G. E., and J. M. Leonard. 1992. A sequencing biofilm reactor for the treatment of chlorinated solvents using methanotrophs. *Wat. Environ. Res.* 64:712-719.

Stanley, S. H., Prior, S. D., Leak, D. J., and H. Dalton. 1983. Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms; studies in batch and continuous cultures. *Biotechnol. Lett.* 5:487-492.

Strong-Gunderson, J. M., and A. V. Palumbo. 1995. Surfactant and surfactant-like compounds enhance the bioavailability of contaminants and natural organic matter. In *Microbial Processes for Bioremediation*. Hinchee, R. E., Brockman, F. J., and C. M. Vogel, eds. Battelle Press, Columbus, OH. pp. 33-40.

Strong-Gunderson, J. M., Applegate, B., Sayler, G. S., and A. V. Palumbo. 1995. Biosurfactants and increased bioavailability of sorbed organic contaminants: measurements using a biosensor. In *Gas, Oil, and Environ. Biotech. VI*. Srivastava, V., Smith, J., and T. D. Hayes, eds. Institute of Gas Technology, Des Plaines, IL. pp. 17-26.

Sturman, P. J., Stewart, P. S., Cunningham, A. B., Bouwer, E. J., and J. H. Wolfram. 1995. Engineering scale-up of in situ bioremediation processes: a review. *Journ. of Contam. Hydrol.* 19:171-203.

Sveum, P., Fakness, L. G., and S. Ramstad. 1994. Bioremediation of oil-contaminated shorelines: the role of carbon in fertilizers. In *Hydrocarbon Bioremediation*. Hinchee, R. E., Alleman, B. C., Hoeppel, R. E., and R. N. Miller. Lewis Publishers, Boca Raton, FL. p. 163-174.

- Tartara, G. M., Dybas, M. J., and Criddle, C. S. 1993. Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. Strain KC. Appl. and Environ. Microbiol. 59:2126-2131.
- Thibault, S. L., Anderson, M., and W. T. Frankenberger, Jr. 1996. Influence of surfactants on pyrene desorption and degradation in soils. Appl. and Environ. Microbiol. 62:283-287.
- Tompson, A. F. B., Knapp, R. B., Hanna, M. L., and R. T. Taylor. 1994. Simulation of TCE migration and biodegradation in a porous medium under conditions of finite degradation capacity. Advan. in Water Resour. 17:241-249.
- Tsien, H. C., Brusseau, G. A., Hanson, R. S., and L. P. Wackett. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. and Environ. Microbiol. 55:3155-3161.
- Uchiyama, H., Oguri, K., Nishibayashi, M., Kokufuta, E., and O. Yagi. 1995. Trichloroethylene degradation by cells of a methane-utilizing bacterium, *Methylocytis* sp. M, immobilized in calcium alginate. Journ. of Ferm. and Bioeng. 79:608-613.
- Venosa, A. D., Haines, J. R., Nisamanepong, W., Govind, R., Pradhan, S. and B. Siddique. 1992. Efficacy of commercial products in enhancing oil biodegradation in closed laboratory reactors. Journ. of Ind. Microbiol. 10:13-23.
- Volkerling, F., Breure, A. M., van Andel, J. G., and W. H. Rulkens. 1995. Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl. and Environ. Microbiol. 61:1699-1705.
- Wackett, L. P., and D. T. Gibson. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. and Environ. Microbiol. 54:1703-1708.
- West, C. C. 1992. Surfactant-enhanced solubilization of tetrachloroethylene and degradation products in pump and treat remediation. In Transport and Remediation of Subsurface Contaminants: Colloidal, Interfacial, and Surfactant Phenomena, Sabatini, D. A., and R. C. Knox, eds. American Chemical Society, 159-168.
- West, C. C., and J. H. Harwell. 1992. Surfactants and subsurface remediation. Environ. Sci. Technol. 26:2324-2330.
- Yeom, I. T., Ghosh, M. M., and C. D. Cox. 1996. Kinetic aspects of solubilization of soil-bound polycyclic aromatic hydrocarbons. Env. Sci. Technol. 30:1589-1595.
- Yeom, I. T., Ghosh, M. M., Cox, C. D., and K. G. Robinson. 1995. Micellar solubilization of polynuclear aromatic hydrocarbons in coal tar-contaminated soils. Environ. Sci. Technol. 29:3015-3021.
- Zhang, Y., and R. M. Miller. 1995. Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of *n*-alkanes. Appl. and Environ. Microbiol. 61:2247-2251.

Appendices

Appendix I. Calculation of K_{m2} partitioning coefficient.

A mass balance for the standards containing water and TCE yields an expression for the total TCE in the system as distributed between the aqueous and the gas phase.

$$M_T = M_{gas} + M_{aq} \quad (A-1)$$

where M_T = total mass of TCE in the system

M_{gas} = mass of TCE in the gas phase

M_{aq} = mass of TCE in the aqueous phase.

Henry's law can be applied to estimate the amount of TCE in the headspace of standards given the known concentration of TCE added to each vial.

$$C_{gas} = C_{aq} H \quad (A-2)$$

where C_{aq} = TCE concentration in aqueous phase (mol/L)

C_{gas} = TCE concentration in gas phase (mol/L)

H = Henry's Law constant for TCE (0.476).

A combination of equations A-1 and A-2 solving for the mass of TCE in the gas phase yields:

$$M_{gas} = \frac{M_T}{\frac{V_{aq}}{V_{gas} H} + 1} \quad (A-3)$$

where V_{gas} = volume of the gas phase

V_{aq} = volume of the aqueous phase.

A calibration curve can be constructed given that the electron capture detector response of the gas chromatograph is directly proportional to C_{gas} (personal communication, Dr. F. Gadelle). This calibration curve is used to relate the amount of TCE in the gas phase in samples to the GC peak area.

A similar method is used to estimate the amount of TCE in the micellar pseudophase. A mass balance of a system containing water, TCE and surfactant yields an expression for the total TCE in the system as distributed between the liquid (water pseudophase + micellar pseudophase) and the gas phase.

$$M_T = M_{\text{gas}} + M_{\text{aq}} + M_{\text{micelle}} = C_{\text{gas}} V_{\text{gas}} + C_{\text{aq}} V_{\text{aq}} + C_{\text{mic}} V_{\text{aq}} \quad (\text{A-4})$$

where M_{micelle} = mass of TCE in the micellar pseudophase

C_{mic} = concentration of TCE in the micelles.

The concentration of TCE in the micelles is a function of the partitioning of TCE into the micelles, the concentration of TCE in the aqueous phase, and the concentration of micelles in solution (personal communication, Dr. C. Cox).

$$C_{\text{mic}} = K_{m2} C_{\text{aq}} Z_{\text{mic}} \quad (\text{A-5})$$

where $K_{m2} = \frac{\text{mass TCE solubilized per g surfactant}_{\text{micelles}}}{\text{aqueous phase TCE concentration}}$

and the concentration of micelles in solution is:

$$Z_{mic} = \frac{M_{T_{surf}} - CMC V_{aq}}{V_{aq}} \quad (A-6)$$

where $M_{T_{surf}}$ = total mass of surfactant added.

An linear relationship between the inverse concentration of TCE in the gas phase and the concentration of micelles in solution can be obtained by combining equations A-2, A-4, and A-5 (Figure A-1).

$$\frac{1}{C_{gas}} = \frac{K_{m2} V_{aq}}{H M_T} Z_{mic} + \frac{V_{gas} + V_{aq}}{M_T (1 + H)} \quad (A-7)$$

A plot of $1/C_{gas}$ versus Z_{mic} yields a line with slope:

$$\frac{K_{m2} V_{aq}}{H M_T} \quad (A-8)$$

Since V_{aq} , H , and M_T are known, K_{m2} can be calculated from the slope of the line.

K_{m2} can be compared to K_m by extracting K_{m2} from K_m (personal communication, Dr. F. Gadelle).

$$K_m = \frac{X_m}{X_a} \quad (2-2)$$

By assuming that the moles of TCE in the micelles $\ll$ moles of surfactant in the micelle, X_m simplifies to:

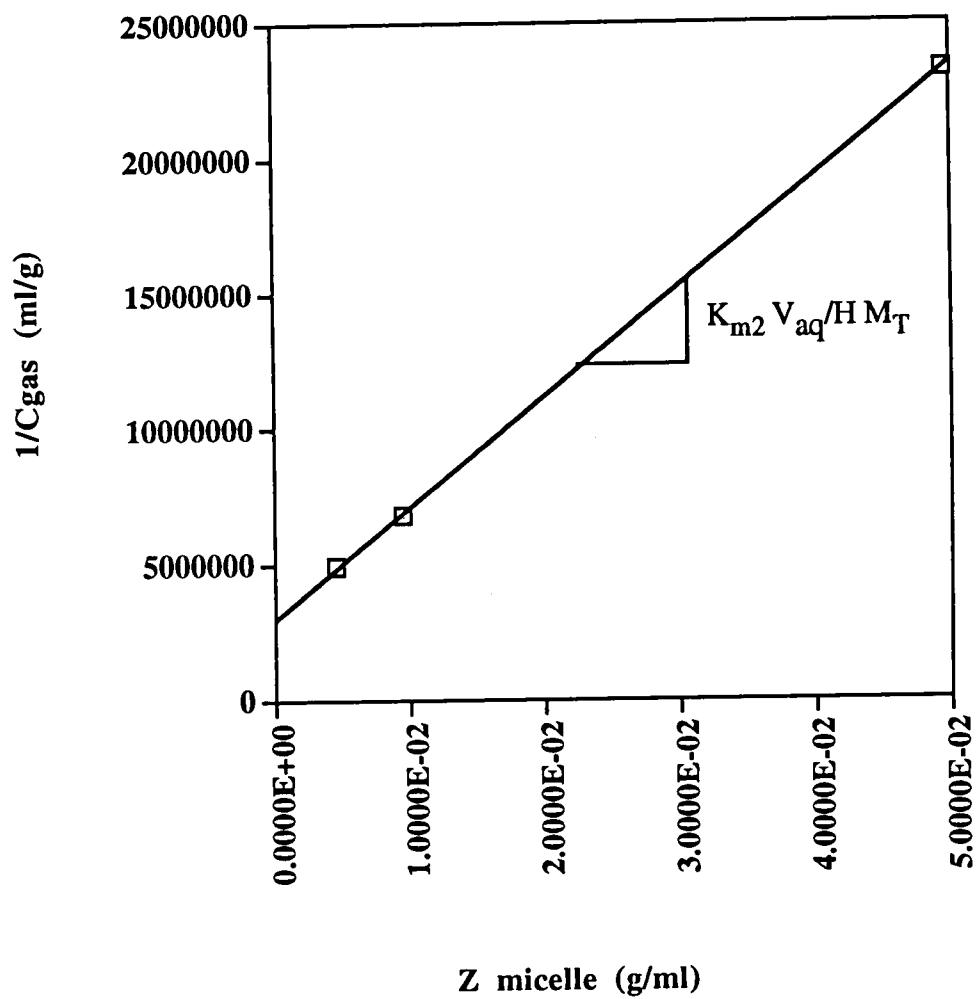


Figure A-1. Solubilization of TCE by BioTreat.

$$X_m = \frac{\text{moles TCE}_{\text{micelles}}}{\text{moles surfactant}_{\text{micelles}}} \quad (\text{A-9})$$

Since moles TCE $\ll$ moles water and by assuming an aqueous phase volume of 1 ml:

$$X_a = \frac{\frac{\text{moles TCE}}{1 \text{ g}}}{18 \text{ g - mol water}} = \frac{C_{\text{aq}}}{0.0555} \quad (\text{A-10})$$

where C_{aq} = concentration of TCE in the aqueous phase, mol/cm³.

Substituting equations 2-14 and 2-15 into 2-2 and further simplifying yields the following expression for K_m :

$$K_m = \frac{\text{moles TCE}_{\text{micelle}}}{\text{moles surfactant}_{\text{micelle}}} \cdot \frac{0.0555}{C_{\text{aq}}} \quad (\text{A-11})$$

$$K_m = \frac{\frac{\text{mass TCE}_{\text{micelles}} (0.0555) (\text{mol. wt. surfactant})}{\text{mass TCE}_{\text{aq}}}}{V_{\text{aq}}} \cdot \text{mass surfactant}_{\text{micelles}} \quad (\text{A-12})$$

The partitioning of TCE between the aqueous pseudophase and the micellar pseudophase in terms of mass can be written as such:

$$K_{m2} = \frac{\frac{\text{mass TCE}_{\text{micelles}}}{\text{mass surfactant}_{\text{micelles}}}}{\frac{\text{mass TCE}_{\text{aq}}}{V_{\text{aq}}}} = \frac{\text{mass TCE}_{\text{micelles}}}{\text{mass surfactant}_{\text{micelles}}} \cdot \frac{V_{\text{aq}}}{\text{mass TCE}_{\text{aq}}} \quad (\text{A-13})$$

where V_{aq} = ml.

By substitution of equation A-13 into A-12, K_{m2} can be calculated from published values of K_m when K_{m2} is expressed in units of ml/g.

$$K_m = K_{m2} \bullet \text{mol. wt. surfactant} \bullet 0.0555 \quad (\text{A-14})$$

Appendix II. Material Safety Data Sheet for BioTreat.

See following pages.

MATERIAL SAFETY DATA SHEET

REM-TEC , INC.
208 MORAVIAN CAMP RD.
LAUREL SPRINGS, NC 28644
TELEPHONE 910-359-2650

SECTION 1 PRODUCT IDENTIFICATION

PRODUCT NAME: BIO-TREAT 2
CHEMICAL SYNONYMS: -
FORMULA: Proprietary
MSDS NUMBER: 92-233A

SECTION 2 HAZARDOUS INGREDIENTS

INGREDIENTS	CAS NAME	CAS#	HAZARD	TLV / PEL
Proprietary			Irritant	No TLV established

Specific chemical name(s) and CAS number(s) will be a trade secret as allowed by 29 CFR 1910.1200.

SECTION 3 REGULATORY DATA

TITLE III - SARA

The following data is being supplied in compliance with Title III Superfund Amendments and Reauthorization Act (SARA) Part 313 and 40 CFR 372.

This product does not contain any chemicals
found on the list in 40 CFR 372.

TRANSPORTATION

Containers of less than 110 gal. (drums)	Containers of more than 110 gal. (bulk)
NOT REGULATED BY DOT	NOT REGULATED BY DOT

CALIFORNIA PROPOSITION 65

Contains no materials known to be on the California Proposition 65 list.

SECTION 4 HMIS AND NFPA RATINGS

<u>NCPA HMIS DERIVED HAZARD RATING</u>		
HMIS HEALTH RATING	1	0=LEAST
HMIS FIRE RATING	0	1=SLIGHT
HMIS REACTIVITY RATING	0	2=MODERATE
HMIS PERSONAL PROTECTION	X	3=SEVERE
		4=EXTREME

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NFPA 704 DERIVED HAZARD RATING

NFPA HEALTH RATING	1
NFPA HEALTH RATING	0
NFPA REACTIVITY RATING	0

SECTION 5 NOTES

None

SECTION 6 PHYSICAL DATA

APPEARANCE AND ODOR: Clear amber liquid-mild odor.

SOLUBILITY : Disperses in water

BOILING POINT: N.A.

% VOLATILES: -

VAPOR PRESSURE: N.A.

SPECIFIC GRAVITY: -

VAPOR DENSITY: N.A.

SECTION 7 REACTIVITY DATA

STABILITY:

STABLE

CONDITIONS TO AVOID:

NONE KNOWN

INCOMPATIBLE MATERIALS:

NONE KNOWN

HAZARDOUS POLYMERIZATION:

WILL NOT OCCUR

CONDITIONS TO AVOID:

NONE KNOWN

HAZARDOUS DECOMPOSITION PRODUCTS:

Not established.

SECTION 8 FIRE AND EXPLOSION HAZARDS

FLASH POINT: N.A.

FLAMMABLE LIMITS:LEL: N.A.

UEL: N.A.

EXTINGUISHING METHODS: Use water spray, carbon dioxide, dry chemical or foam.

SPECIAL FIRE FIGHTING PROCEDURES: None known.

UNUSUAL FIRE AND EXPLOSION HAZARDS: None known.

SECTION 9 HEALTH HAZARD INFORMATION

EFFECTS OF OVEREXPOSURE- ACUTE

INGESTION: May cause gastrointestinal irritation.

EYES: May cause irritation.

INHALATION: None currently known.

DERMAL: Prolonged or repeated contact may cause irritation.

EMERGENCY AND FIRST AID PROCEDURES

INGESTION: If material is swallowed, get medical attention.
EYES: Flush eyes with water for at least 15 minutes holding eyelids open occasionally. Call a physician.
INHALATION: If affected, remove individual to fresh air.
DERMAL: Flush exposed area with water. Remove contaminated clothing. Launder clothing before reuse. If irritation occurs, consult a physician.

SECTION 10 OVEREXPOSURE AND TOXICITY DATA

No data available for chronic overexposure.

SECTION 11 SPECIAL PROCEDURES INFORMATION

RESPIRATORY PROTECTION: None normally needed.
EYE PROTECTION: Flush with water for at least 15 minutes. If irritation occurs and persists, consult a physician.
PROTECTIVE GLOVES: None known.
VENTILATION: Sufficient mechanical (general) and/or local exhaust to remove any vapors formed.
OTHER PROTECTIVE EQUIPMENT: Impervious apron or clothing to minimize or prevent skin contact.

SECTION 12 SPECIAL PRECAUTIONS

PRECAUTIONS TO BE TAKEN IN: Avoid contact with eyes, skin, clothing.
HANDLING AND STORAGE: Wash thoroughly after handling.
OTHER PRECAUTIONS: Since emptied containers retain product residue, follow all label warning (if any) even after container is empty.

SECTION 13 SPILL OR LEAK PROCEDURES

SPILL AND LEAK PROCEDURES:

Small spill: Absorb onto dry sand, paper, vermiculite, etc. and sweep or scoop into containers.

Large spill: Dike to minimize contaminated area. Pump liquid to salvage tank. Absorb remaining liquid onto sand, clay, earth, etc. Scoop into containers.

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DISPOSAL METHOD: Incineration or landfill burial in accordance with federal, state or local regulations.

SECTION 14 NOTES AND OTHER INFORMATION

N.A. = Not Applicable

N.D. = Not Determined

The information given herein is believed to be accurate but is not warranted to be whether originating with Rem-Tec, Inc. or not. The information is offered solely for your consideration. Recipients are advised to confirm in advance of need that the information is current, applicable and suitable for their needs.

THIS MATERIAL SAFETY DATA SHEET REPLACES ALL PREVIOUS MSDS FOR: BIO-TREAT

REM-TEC, INC.
208 MORAVIAN CAMP RD.
LAUREL SPRINGS, N.C. 28644
TELEPHONE 910-359-2650

DATE: OCTOBER 7, 1992
BY: TRAVIS SMITH
TITLE: REGULATION COORDINATOR

MSDS NO. 92-233A2

Appendix III. Statistical Analysis.

Statistical analysis for Figure 11. Abiotic solubilization of 0.1 ppm TCE by BioTreat at low concentrations.

Treatments compared	P-value	Conclusion
TCE only & 0.05% BioTreat only	0.05	Different
TCE only & TCE + 0.001% BioTreat	0.35	Same
TCE only & TCE 0.01% BioTreat	0.36	Same
TCE only & 0.05% BioTreat	0.36	Same
TCE + 0.001% BioTreat & TCE + 0.01% BioTreat	0.45	Same
TCE + 0.01% BioTreat & TCE + 0.05% BioTreat	0.99	Same

Statistical analysis for Figure 12. Abiotic solubilization of 1.0 ppm TCE by BioTreat at low concentrations.

Treatments compared	P-value	Conclusion
TCE only & 0.05% BioTreat only	1.9×10^{-5}	Different
TCE only & TCE + 0.001% BioTreat	0.54	Same
TCE only & TCE 0.01% BioTreat	0.32	Same
TCE only & 0.05% BioTreat	0.38	Same
TCE + 0.001% BioTreat & TCE + 0.01% BioTreat	0.26	Same
TCE + 0.01% BioTreat & TCE + 0.05% BioTreat	0.76	Same

Statistical analysis for Figure 13. Hexane extraction efficiency for 0.1 ppm TCE with low BioTreat concentrations.

Treatments compared	P-value	Conclusion
TCE only & 0.05% BioTreat only	0.04	Different
TCE only & TCE + 0.001% BioTreat	0.35	Same
TCE only & TCE 0.01% BioTreat	0.38	Same
TCE only & 0.05% BioTreat	0.45	Same
TCE + 0.001% BioTreat & TCE + 0.01% BioTreat	0.33	Same
TCE + 0.01% BioTreat & TCE + 0.05% BioTreat	0.07	Same

Statistical analysis for Figure 14. Hexane extraction efficiency for 1.0 ppm TCE with low BioTreat concentrations.

Treatments compared	P-value	Conclusion
TCE only & 0.05% BioTreat only	1.7×10^{-5}	Different
TCE only & TCE + 0.001% BioTreat	0.79	Same
TCE only & TCE 0.01% BioTreat	0.40	Same
TCE only & 0.05% BioTreat	0.27	Same
TCE + 0.001% BioTreat & TCE + 0.01% BioTreat	0.12	Same
TCE + 0.01% BioTreat & TCE + 0.05% BioTreat	0.70	Same

Statistical analysis for Figure 15. Abiotic solubilization of 1 ppm TCE by BioTreat at high concentrations.

Treatments compared	P-value	Conclusion
TCE only & 5% BioTreat only	4.4×10^{-5}	Different
TCE only & TCE + 0.05% BioTreat	0.02	Different
TCE only & TCE + 1% BioTreat	0.003	Different
TCE only & TCE + 5% BioTreat	8.32×10^{-5}	Different
TCE + 0.05% BioTreat & TCE + 1% BioTreat	0.08	Different
TCE + 0.5% BioTreat & TCE + 5% BioTreat	0.008	Different
TCE + 1% BioTreat & TCE + 5% BioTreat	0.003	Different

Statistical analysis for Figure 16. Hexane extraction efficiency for 1.0 ppm TCE with high BioTreat concentrations.

Treatments compared	P-value	Conclusion
TCE only & 5% BioTreat only	0.0001	Different
TCE only & TCE + 0.05% BioTreat	0.96	Same
TCE only & TCE + 1% BioTreat	0.26	Same
TCE only & TCE + 5% BioTreat	0.06	Same
TCE + 0.05% BioTreat & TCE + 1% BioTreat	0.28	Same
TCE + 0.5% BioTreat & TCE + 5% BioTreat	0.07	Same
TCE + 1% BioTreat & TCE + 5% BioTreat	0.06	Same

Statistical analysis for Figure 17. Sorption of TCE to autoclaved G4 cells.

Treatments compared	P-value	Conclusion
1 ppm control 0-24 hr	0.02	Different
5 ppm control 0-24 hr	0.19	Same
10 ppm control 0-24 hr	0.01	Different
1 ppm trx 0-24 hr	0.17	Same
5 ppm trx 0-24 hr	0.82	Same
10 ppm trx 0-24 hr	0.68	Same
1 ppm control & trx @ t=0 hr	0.44	Same
5 ppm control & trx @ t=0 hr	0.18	Same
10 ppm control & trx @ t=0 hr	0.01	Different
1 ppm control & trx @ t=24 hr	0.47	Same
5 ppm control & trx @ t=24 hr	0.17	Same
10 ppm control & trx @ t=24 hr	0.47	Same

Statistical analysis for Figure 18. Effect of BioTreat concentration on TCE disappearance with the use of G4.

Treatments compared	P-value	Conclusion
TCE only & 5% BioTreat only @ t=4 hr	0.001	Different
TCE only & BioTreat only @ t=4 hr	0.07	Different
TCE only & G4 + 0.01% Biotreat @ t=4 hr	0.049	Different
TCE only & G4 + 0.05% Biotreat @ t=4 hr	0.01	Different
TCE only & G4 + 0.5% Biotreat @ t=4 hr	0.01	Different
TCE only & G4 + 1% Biotreat @ t=4 hr	0.01	Different
TCE only & G4 + 5% Biotreat @ t=4 hr	0.003	Different
G4 only & G4 + 0.01% BioTreat @ t=4 hr	0.35	Same
G4 + 0.01% BioTreat @ t=4 hr	0.02	Different
G4 + 0.01% BioTreat & G4 + 0.05% BioTreat @ t=4 hr	0.14	Same
G4 + 0.05% BioTreat & G4 + 0.5% BioTreat @ t=4 hr	0.22	Same
G4 + 0.05% BioTreat & G4 + 1% BioTreat @ t=4 hr	0.18	Same
G4 + 0.05% BioTreat & G4 + 5% BioTreat @ t=4 hr	0.001	Different

Statistical analysis for Figure 19. Residual TCE after 48 hr degradation period for 1 ppm TCE and low BioTreat concentrations.

Treatments compared	P-value	Conclusion
TCE only & 5% BioTreat only	0.0005	Different
TCE only & G4 + TCE only	0.0005	Different
TCE only & G4 + 0.01% BioTreat + TCE	0.0005	Different
TCE only & G4 + 0.05% BioTreat + TCE	0.0005	Different
TCE only & G4 + 0.5% BioTreat + TCE	0.0005	Different
TCE only & G4 + 1% BioTreat + TCE	0.0005	Different
TCE only & G4 + 5% BioTreat + TCE	0.0009	Different

Statistical analysis for Figure 20. Effect of 0.05% BioTreat on TCE degradation by G4.

Treatments compared	P-value	Conclusion
TCE only & 0.05% BioTreat only @ t=4 hr	0.005	Different
TCE only & G4 + TCE @ t=4 hr	0.36	Same
TCE only & G4 + 0.05% BioTreat + TCE @ t=4 hr	0.02	Different
G4 + TCE & G4 + 0.05% BioTreat + TCE @ t= 4 hr	0.006	Different
TCE only & 0.05% BioTreat only @ t=24 hr	0.004	Different
TCE only & G4 + TCE @ t=24 hr	0.90	Same
TCE only & G4 + 0.05% BioTreat + TCE @ t=24 hr	0.006	Different
G4 + TCE & G4 + 0.05% BioTreat + TCE @ t= 24 hr	0.008	Different

Statistical analysis for Figure 21. Residual TCE after 24 hr degradation period for 1 ppm TCE with 0.05% BioTreat.

Treatments compared	P-value	Conclusion
TCE only & 0.05% BioTreat only	0.003	Different
TCE only & G4 + TCE	0.59	Same
TCE only & G4 + 0.05% BioTreat + TCE	0.005	Different
G4 + TCE & G4 + 0.05% BioTreat + TCE	0.016	Different

Statistical analysis for Figure 22. Effect of BioTreat concentration on disappearance of 5 ppm TCE with G4.

Treatments compared	P-value	Conclusion
TCE only & 5% BioTreat only	3.4×10^{-7}	Different
TCE only & G4 +TCE @ t=0 hr	0.08	Same
TCE only & G4 + 0.05% BioTreat + TCE @ t=0 hr	0.17	Same
TCE only & G4 + 0.5% BioTreat + TCE @ t=0 hr	0.004	Different
TCE only & G4 + 5% BioTreat + TCE @ t=0 hr	1.1×10^{-6}	Different
G4 + TCE & G4 + 0.05% BioTreat + TCE @ t=0 hr	0.77	Same
G4 + TCE & G4 + 0.5% BioTreat + TCE @ t=0 hr	0.008	Different
G4 + TCE & G4 + 5% BioTreat + TCE @ t=0 hr	4.6×10^{-6}	Different
TCE only & G4 only @ t=24 hr	2.8×10^{-6}	Different
TCE only & G4 + 0.05% BioTreat + TCE @ t=24 hr	0.0003	Different
TCE only & G4 + 0.5% BioTreat + TCE @ t=24 hr	1.4×10^{-7}	Different
TCE only & G4 + 5% BioTreat + TCE @ t=24 hr	2.8×10^{-7}	Different

Statistical analysis for Figure 23. Residual TCE after 48 hr degradation period for 5 ppm TCE with various BioTreat concentrations.

Treatments compared	P-value	Conclusion
TCE only & 5% BioTreat only	1.3×10^{-5}	Different
TCE only & G4 only	1.3×10^{-5}	Different
TCE only & G4 + 0.05% BioTreat + TCE	1.3×10^{-5}	Different
TCE only & G4 + 0.5% BioTreat + TCE	1.3×10^{-5}	Different
TCE only & G4 + 5% BioTreat + TCE	0.0002	Different

Statistical analysis for Figure 24. Residual TCE after 48 hr degradation period for 10 ppm TCE with 0.05% BioTreat.

Treatments compared	P-value	Conclusion
TCE only & G4 only	3.2×10^{-6}	Different
TCE only & 0.05% BioTreat only	3.2×10^{-6}	Different
TCE only & 0.05% BioTreat + TCE	0.44	Same
TCE only & G4 + TCE	1.3×10^{-5}	Different
TCE only & G4 + 0.05% BioTreat + TCE	2.9×10^{-5}	Different
G4 + TCE & G4 + 0.05% BioTreat + TCE	0.029	Different

Vita

Marie T. Gillespie was born in Vung Tau, Vietnam on February 25, 1972. She attended private Catholic school until third grade, and finished her primary and secondary education in the public school system of Plainfield, Indiana. She graduated with high honors from Plainfield Junior Senior High School in June 1990. She entered Purdue University, West Lafayette, Indiana in August 1990 to study Interdisciplinary Engineering with a major in Bioengineering. She participated in a co-operative engineering education program with Purdue University and Martin Marietta Energy Systems throughout her college years. She graduated from Purdue University in December 1994 with a Bachelor of Science degree in Engineering and a certificate for completing the co-operative education program. She entered the Master's program in Environmental Engineering at the University of Tennessee, Knoxville, in September 1995 while continuing to work at Lockheed Martin Energy Research Corporation (formerly the X-10 plant managed by Martin Marietta Energy Systems) full time. The Master of Science degree was received in August 1997.