A Microcosm Study of the Biodegradability of Adsorbed Toluene by Acclimated Bacteria in Soils

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(ABSTRACT)

Groundwater contamination by man-made chemicals is increasingly being reported in the United States. The potential for detrimental health effects is substantial and has been addressed by the environmental engineering profession. Typically, contaminated groundwater is pumped to the surface and treated in a variety of methods including air stripping, carbon adsorption, and biodegradation. In situ biodegradation is increasingly being considered as an alternative to pump-and-treat technology.

The primary goal of this research was to determine the fate of an organic chemical adsorbed to a subsurface soil when exposed to acclimated bacteria. Toluene was chosen as a representative compound
because it is a major constituent of groundwater contaminated by gasoline. In addition, toluene is known to be both biodegradable and adsorbable. Sybron Biochemical, Inc. supplied the aerobic bacteria Pseudomonas putida known to readily transform toluene.

Soil microcosms were established in test-tubes and conditions simulated those of a saturated, aerobic aquifer. Gas chromatography was used to quantify changes in toluene concentration due to adsorption and biodegradation. The addition of an aqueous toluene solution to sterile microcosms resulted in the rapid and extensive adsorption of toluene to the soil. Subsequent analysis revealed the slow adsorption of an additional small fraction of toluene.

Biodegradation studies entailed the addition of acclimated bacteria to sterile soil microcosms in which substantial toluene adsorption had occurred. Addition of small doses of hydrogen peroxide effectively maintained aerobic conditions for biodegradation. As a result, P. putida was able to transform all measurable toluene in the microcosms.

Additional desorption studies revealed that a "resistant" component of toluene remained adsorbed to the soil during biodegradation. This component was neither acted upon by bacteria nor readily extractable by methylene chloride. However, slow desorption of toluene was shown to occur at a rate comparable to slow adsorption. To achieve complete removal, groundwater treatment methods must address the rate-controlled desorption of the resistant toluene component.
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**INTRODUCTION**

Groundwater resources are currently being threatened by excessive demand and deterioration of quality. Studies have revealed contamination of aquifers (Westrick et al., 1984) from numerous sources including septic fields, surface water recharge (Zeyer et al., 1986), and landfill leachate (Reinhard et al., 1984). Widespread use of underground storage tanks for chemical products, and their associated spillage and leakage, has produced yet another avenue for introduction of man-made compounds to the subsurface (Garrett, 1987). Only through wise management can mankind hope to maintain this valuable resource.

Remediation of known and future contamination sites is of utmost concern and is presently being undertaken through EPA's Superfund program. However, knowledgeable decisions need to be made to affect the greatest improvement. Thus, the need arises for an increased understanding of the interactions that occur in the subsurface between man-made chemicals, groundwater, and soils.

During the past 25 years, investigators have amassed a wealth of knowledge on subsurface phenomena (McCarty et al., 1981). Microbial biodegradation of chemical compounds in the subsurface has been widely proven and readily reproduced in the laboratory (Kobayashi and Rittman, 1982). In addition, dispersion and sorption have been investigated and mathematically modelled so that the movement and concentration of subsurface pollutants can be predicted (Voice and Weber, 1983).
Before one can correctly apply treatment technology for alleviating groundwater pollution, the fate of chemical compounds in the subsurface needs to be ascertained. Researchers have investigated both aerobic and anaerobic microbial processes involved in the degradation of numerous chemical compounds. Others have studied the sorption of substrates to soils, sediments and suspended materials. However, little is known about the complex interactions between these two processes in the subsurface.

The goal for this research was to address this relationship by performing a model study of the availability of an adsorbed organic compound for microbial biodegradation. Toluene was selected as a suitable compound for such a study because it is a major constituent of gasoline, a well-known groundwater pollutant, and a potential health hazard. In addition, toluene has been shown to be biodegradable under aerobic conditions, and a source of acclimated bacteria was readily available from a local company to facilitate the research.

Several specific objectives were proposed for the planned experiments: (1) to ascertain the extent and rate of adsorption of toluene to a selected soil and specifically address the approach to adsorption equilibrium, (2) to establish the potential for the use of hydrogen peroxide as a source of oxygen for aerobic biodegradation, and (3) to determine if and at what rate microbial degradation of adsorbed toluene occurs.
LITERATURE REVIEW

When organic compounds are introduced into subsurface soils, numerous physical and chemical processes, including adsorption, desorption, dispersion, advection, biodegradation, volatilization, and reduction/oxidation, can act upon them. The focus of this study was the adsorption of toluene to soil particles and the resultant effect on its biodegradation by bacteria in the saturated zone.

Adsorption

In search of a better understanding of the fate of chemical pollutants in subsurface soils, numerous researchers have conducted sorption studies to develop models to be used for predicting the capacity of soils to adsorb organic compounds. The goal has been to calculate a soil's sorptive capacity based on easily measured and/or known properties such as soil organic carbon content, solids concentration, octanol-water partition coefficient, and water solubility. Chemical properties of many organics are widely known and commonly tabulated in reference manuals. In addition, many soil properties can be readily determined by established laboratory techniques (i.e. porosity, grain size, organic carbon content). Thus, given a soil sample and a reliable predictor, one could determine the fate of a chemical in the subsurface and enjoy great savings in time and
money by eliminating the need for elaborate field or laboratory studies.

Several adsorption equations that describe the adsorption of a solute to a solid have been developed over the years. Generally, these equations describe the relationship between the amount of solute adsorbed to a known mass of adsorbent and the equilibrium aqueous concentration. Details of equilibrium equations such as Langmuir, Freundlich, BET, and Gibbs have been presented by several authors (Montgomery, 1985, Voice and Weber, 1983, Weber, 1972) and will not be evaluated here.

Because the solutions to each equation sometimes provide different predictions of adsorption, the suitability of a given model depends in part on the properties of the adsorbate and adsorbent. For dilute concentrations, commonly found in the subsurface, several of the equations reduce to a linear proportionality given by \( q = K \cdot C \), where \( q \) is the amount of solute adsorbed per unit weight of adsorbent, \( C \) is the equilibrium aqueous concentration, and \( K \) is the distribution, or partition, coefficient. This linear "partitioning" has been widely used by researchers to predict the sorptive behavior of soils, sediments, and suspended solids.

Caution must be used when applying linear sorption equations as stated by Mingelgrin and Gerstl (1983). In general, adsorption isotherms are linear only when the solute concentration is low. However, the specific concentration range over which linear adsorption occurs is subject to debate. Most often, the applicable solution
concentration range is compared to the chemical's solubility limit. Karickhoff and Morris (1985) mentioned one-half of water solubility as the upper concentration limit that one can apply linear adsorption. The fact that numerous instances of linear adsorption have been documented reveals the potential for greatly simplifying the prediction of adsorption (Choiu et al., 1979, Karickhoff and Morris, 1985).

Building upon the evidence of linear adsorption for low concentrations, Chiou et al. (1979a, 1979b) claimed that adsorption of nonionic organic compounds by soils is actually a partitioning process analogous to the partitioning of a solute between two solvents (e.g. octanol and water). Their research entailed correlating the equilibrium aqueous concentration to the amount bound to the soil for concentrations approaching a compound's solubility limit. Evidence of linear adsorption for several compounds, even up to 95 percent of solubility, was suggested to be proof of strict partitioning. Mingelgrin and Gerstl (1983) took issue with these results and presented evidence of other nonionic compounds that exhibited nonlinear adsorption. They stated that adsorption is also dependent on the availability of surface sites and may exhibit linearity in cases of low surface loading, even for concentrations approaching solubility. The importance of solids concentration has been revealed in studies that showed that partition coefficients varied by more than an order of magnitude over several orders of adsorbent concentrations (DiToro and Horzempa, 1982, O'Connor and Connolly, 1980).
Some researchers have considered the cases of linearity as evidence that adsorption is a strict partitioning process and not an adsorption phenomenon (Chiou et al., 1979a). While adsorption is a surface/interface phenomenon, partitioning is an interaction that occurs between two phases. When applying partitioning to a two solvent system, i.e. octanol-water, one can easily envision a solute distributing itself between the two solvents to varying degrees. Applying the same concept to a solid/liquid system, one has difficulty describing the state of the solute in the solid phase. It appears that to define the partitioning process, one would have to rely on the adsorption theory that is capable of explaining the surface phenomena, such as molecular dipole attractions and hydrogen bonding. To date, the proponents of strict partitioning have addressed neither what the state of the solute in the solid phase is nor the many cases of non-linear partitioning. Thus, the partitioning concept seems to have limited usefulness and might even confuse the understanding of adsorption.

Karickhoff and Morris (1985) were most pointed in stating, "The high degree of variability and complexity in sediment composition and potential sorptive interactions seems to preclude the possibility of developing a simple, systematic procedure for predicting sorption parameters." Despite the complexity of the problem, Karickhoff maintained hope that an estimation of sorption behavior could be obtained through the use of a "limited set" of indicators. Among the sorption parameters considered to have the greatest potential are soil
organic carbon content, solids concentration, chemical solubility, and octanol-water distribution coefficient.

By far, the most attention has been paid to the organic carbon fraction of soils. As far back as 1965, Lambert (1965) related the adsorption of nonionic organic pesticides to the "effective organic matter" of a soil. Others have elaborated on this research to verify the validity of organic carbon as a major factor in determining sorption behavior (Carter and Suffet, 1982, Garbarini and Lion, 1986, Karickhoff and Morris, 1985, Lambert et al., 1965, Swanson and Dutt, 1973).

Regardless of the experimental method used by these scientists, they always showed that a soil's organic carbon content was well correlated with the adsorption distribution coefficient when the carbon content was greater than one percent (Chiou et al., 1979b, McCarty et al., 1981). When comparing all results, one can see that predictions of adsorption within an order of magnitude and often closer are possible for most organic compounds. However, some researchers have taken issue with the degree of correlation between organic carbon and adsorption and have examined other soil components, such as oxygen and clay content, in order to improve predictions of adsorption (Garbarini and Lion, 1986, Mingelgrin and Gerstl, 1983). In some instances, the inorganic fraction of soil controls sorption, usually for soils with low organic content. With these limitations in mind, organic carbon content has been shown to be a useful predictor of adsorption.
The octanol-water partition coefficient, $K_{ow}$, has also received much attention as an adsorption indicator. For a given soil, $K_{ow}$ and $K$ (or $K_{ow} = K +$ organic carbon fraction) have been shown to be highly correlated, and numerous empirical equations that describe adsorption based on these coefficients have been derived (Chiou et al., 1979b, Karickhoff and Morris, 1985). However, different equations may lead to coefficients that differ by more than an order of magnitude for the same system. A variation of this magnitude is far from Karickhoff's goal of estimates within a factor of 2, but these predictions are still useful for estimation purposes. Mingelgrin and Gerstl's experimental data (1983) revealed that the degree of adsorption may vary by more than an order of magnitude for a single nonionic compound. These results reveal the difficulties in applying the empirical equations with the current understanding about sorption behavior.

Similarly, empirical relationships have shown solubility ($S$) to be inversely proportional to the distribution coefficient ($K$). Once again, the empirical equations yield values of $K$ that are only accurate to within an order of magnitude (Karickhoff and Morris, 1985, Mingelgrin and Gerstl, 1983). Depending on the application, such estimates may be sufficiently accurate to predict the adsorption capacity of soils.

In summary, the use of chemical and soil properties has resulted in many promising relationships for predicting sorption behavior. Relationships involving adsorption as a function of the soil organic carbon fraction, solubility and octanol-water partition coefficient
yield the most reliable predictions of sorption, although the predictions should be considered broad estimates. Caution must be used when applying these predictors to all types of soil or sediments because organic carbon is not always the dominant sorption factor. When applying these relationships, one should always specify the type of soil and the range of concentrations that are suitable for the adsorption prediction.

**Conceptual Models**

In the interest of understanding the sorption process better, some researchers have developed mathematical models to describe the physical interactions between the chemical molecules and the soil particles. Both Peel and McKay, among others, have used the concept of macropores and micropores to reflect the observation of fast and slow stages of adsorption (McKay et al., 1987, McKay, 1984, Peel et al., 1981). Others have recorded the existence of an initially rapid stage of adsorption, often achieved within minutes of exposure, and a subsequently slower stage that lasts days or even months (Karickhoff et al., 1979, Karickhoff, 1980, Miller and Weber, 1985, Sugiura et al., 1975).

Karickhoff (1979, 1980) has advanced the concept of a two-compartment system whereby the bulk phase, \( P \), of a pollutant in solution rapidly sorbs to a compartment of a soil particle, \( S_i \), and achieves
equilibrium. In the next stage, the sorbed pollutant slowly diffuses into a second soil compartment, $S_2$. Thus, movement of the pollutant can be depicted by the following schematic diagram:

\[
P \leftarrow k_1 \rightarrow S_1 \leftarrow k_2 \rightarrow S_2
\]

where rate constant $k_1 = \frac{X_t}{X}K$, $X_t$ = fraction of total sorptive capacity achieved rapidly, $K$ = equilibrium partition coefficient, and $k_2$ = diffusion rate constant.

McKay and others (Hand et al., 1983, McKay et al., 1987, McKay, 1984, Peel et al., 1981) have developed similar adsorption relationships based on the important role of internal pore diffusion. Sorption model improvements have led to the use of three major factors: (1) mass transfer through an external film, (2) diffusion into adsorbent macropores, and (3) diffusion into adsorbent micropores that contribute to adsorption. Use of the micropore diffusion term has yielded exceptional predictions for long-term adsorption that were not possible with simpler models.

Desorption

Desorption, like adsorption, has been shown to occur in both fast and slow stages. Several investigators have recorded a fast initial
release of a significant portion of sorbate followed by a much slower rate of approach to equilibrium (Karickhoff et al., 1979, Karickhoff, 1980, Miller and Weber, 1985, Sugiura et al., 1975). This phenomenon has been observed in experiments in which desorption was induced by both aqueous dilution and gaseous purge. Sorbent particles appear to release sorbate from surfaces and/or macropores and rapidly achieve equilibrium with the aqueous phase. Subsequently, the gradient within the particle generates the rate-controlled migration of substrate from the micropores to the macropores and bulk phase.

Research to date has also shown that adsorption of organic compounds to soils is not always a reversible process as was once believed (DiToro and Horzempa, 1982, DiToro et al., 1982, Swanson and Dutt, 1973, Uchrin and Katz, 1985, van Genuchten et al., 1977). Clearly, hysteretic traits have been revealed by the noticeable differences in adsorption and desorption isotherms generated by numerous studies. DiToro and his colleagues (1982) have proposed that the existence of a "resistant component", i.e. one that does not desorb, can explain the irreversibility of the process. The resistant component was described as being more permanently bound to the soil/sediment and, thus, would not readily desorb. Their model, dividing the sorbate into reversible and resistant components, described the fate of PCB's in sediments and soils and provided remarkable correlations to actual sorption data.
Another important finding of DiToro et al. (1982) related to the amount of time an adsorbent was exposed to the substrate. Increasing the contact time between sorbate and sorbent resulted in a marked increase in the partition coefficient for the resistant component and a similar decrease in the partition coefficient for the reversible component. Their finding corroborates the claim of Karickhoff et al. (1979) that the degree of release of a sorbate from the sorbent is dependent on the "incubation time" of the sorption system. Both studies pointed to an increasing difficulty in recovering sorbed pollutants with increased time of exposure of soil to the compound.

The fact that DiToro and Horzempa (1982) found no change in the extent of desorption for increased desorption recovery times is at odds with other researchers. Karickhoff et al. (1979) mentioned the nature of some sorbates to slowly "bleed" off of the sorbent for days or months before equilibrium was approached. In their study, all of the sorbate was eventually recovered by purging the system with an inert gas. More research is needed to verify the existence of a permanently bound sorbate component.

The concentration of adsorbent is another aspect of sorption that may influence the amount of adsorption. One study (Karickhoff and Morris, 1985) revealed that adsorption is not affected by solids concentrations. Other studies (Carter and Suffet, 1982, DiToro et al., 1982) have established an inverse relationship between adsorbent concentration and the distribution coefficient. These conflicting
results preclude one's ability to make any sound conclusions.

**Biodegradation**

Through biodegradation of chemical compounds, bacteria have exhibited tremendous potential for removing groundwater pollutants. Aerobic respiration utilizing organic compounds is a well-established fact and researchers have revealed the existence of such processes in a variety of subsurface environments (Garbarini and Lion, 1986, Marshall, 1971). Anaerobic respiration using alternative electron acceptors (nitrate, sulfate, CO$_2$) and fermentation processes have also exhibited measurable removal rates notably for many recalcitrant compounds. Experimental procedures using culture vessels, test-tube microcosms, and flow-thru columns have successfully simulated biodegradation by indigenous microorganisms in subsurface systems (Bouwer and McCarty, 1984, Kuhn et al., 1985, Major et al., 1988, Novak et al., 1985, Pfaender, 1987, Smith and Novak, 1987, White et al., 1986, J.T. Wilson et al., 1987, Zeyer et al., 1986). Novak and fellow researchers at Virginia Polytechnic Institute and State University (1985, Smith and Novak, 1987, White et al., 1986) have established kinetic rates for bacterial utilization of known groundwater pollutants such as methanol, TBA, phenol and chlorophenol. Other aromatic compounds, including toluene, are also readily degradable as shown in numerous studies.
Additional researchers have revealed the ability of denitrifying and methanogenic bacteria to degrade organic compounds under anoxic and anaerobic conditions (Bouwer and McCarty, 1984, Evans, 1977, Grbic-Galic and Vogel, 1987). Generally, these processes breakdown the complex aromatic hydrocarbons at a much slower rate than aerobic respiration.

Since toluene is one of the important organic compounds associated with groundwater polluted by gasoline, it has been examined by numerous researchers in the hopes of better understanding the efficacy of gasoline removal from the environment. In 1964, Claus and Walker published the results of microbiological studies revealing the ability of soil bacteria to degrade toluene (1964). Degradation pathways and intermediate compounds were investigated through experimentation with bacteria of the genus Pseudomonas. Other researchers have studied various bacteria and determined that metabolic pathways are very species dependent. Finette et al. (1984) presented initial degradation steps for three species. Concerning the aerobic biodegradation of toluene, P. putida has been identified as playing an important role (Finette et al., 1984, Gibson et al., 1968, Kuhn et al., 1988, B.H. Wilson et al., 1986).
Bioavailability

In addressing the relationship between sorption and biodegradation, several researchers have studied the effects of soil particles on microbial activity (Martin et al., 1978, McCarthy et al., 1985, Sorensen, 1975, Steen et al., 1980, Verma et al., 1975). As presented by Marshall (1971), three types of physical interactions between soil and microorganisms are possible in the subsurface: (1) bacterial cells adsorbed to larger soil particles, (2) smaller soil particles adsorbed to bacteria, and (3) aggregation of particles and cells of the same size.

Typical experiments have quantified the evolution of radiolabeled CO₂ transformed from labeled organic compounds and related the amount of CO₂ to microbial degradation. By adding soils and/or humic materials, any change in CO₂ evolution has been attributed to adsorption (Martin et al., 1978, Sorensen, 1975, Verma et al., 1975). Addition of humic materials was shown to inhibit degradation of proteins and some amino acids but not most readily degradable organics (Martin et al., 1978). On the other hand, some clays have been shown to stimulate bacterial activity (Marshall, 1971). Similarly, studies of humic sorption effects on enzyme activity have yielded both inhibitory and stimulatory results that were dependent on concentration levels (Ladd and Butler, 1975). Experiments by Sorensen (1975) revealed that the amount of silt plus clay in soil was proportional to stabilization of cellulose, apparently
due to adsorption. However, this soil fraction was not proportional to resistance to "stress treatment", including air drying-wetting, biodegradation stimulation by glucose addition, and exposure to chloroform vapor. Varied results revealed that a soil's silt and clay fraction was not able to irreversibly protect cellulose from such treatments. In conclusion, soil components, including humic materials, were found to be important in decreasing the biodegradation of only certain organic compounds. A wider range of organics, especially man-made compounds, needs to be examined before broader relationships can be derived.

Another study directly addressing bioavailability was performed by Speitel and DiGiano (1987). Using a flow-through column and applying radiolabeling techniques, they determined biodegradation rates of acclimated bacteria for substrates previously adsorbed to granulated activated carbon (GAC). Further experimentation on desorption kinetics and comparisons to diffusion mathematical models yielded a comprehensive study on the relationship between sorption and biodegradation. Desorption kinetics, specifically diffusive transport within the GAC particles, was considered to be the controlling factor for biodegradation of sorbed materials.
Summary

Research to date on the adsorption of organic compounds to soil has shown that the application of predictive models is a worthwhile endeavor. Particularly in the case of dilute aqueous solutions and for soils with an organic carbon content of at least one percent, the removal of a solute by an adsorbent can be described by a linear relationship. In addition, the distribution coefficient, the slope of such a line, has been shown to correlate fairly well with both an organic compound's octanol-water coefficient and its solubility. While predictions are valid only to within an order of magnitude, the use of such established parameters may preclude the need for expensive and time-consuming research. Organic carbon has been shown to play an important role in adsorption and biodegradation. For some soils, the extent of adsorption was revealed to be proportional to the amount of organic matter. In addition, some soil components, particularly the humic materials, have exhibited an ability to decrease biodegradation of organic compounds in soil.

Recent findings have also revealed the importance of desorption in soil systems. Hysteretic effects have proven that in some cases adsorption is not a simple reversible process. Current research has also suggested that desorption may be a major factor involved with availability of organic compounds for biodegradation.
Further research into these topics will result in a refinement of our knowledge of the actual processes occurring in the subsurface. Through an improved understanding of the fate of organic compounds in soil systems, better decisions can be made for treating groundwater pollution.
M E T H O D S & M A T E R I A L S

E x p e r i m e n t a l P l a n

Before experimentation began, a plan was developed in which three microcosm studies were to be used to accomplish the research objectives. These included: (1) the incubation of soil in a dilute aqueous toluene solution to determine adsorption characteristics, (2) the addition of acclimated bacteria after adsorption equilibrium had been achieved to reveal whether or not microbes are able to biodegrade adsorbed substrates, and (3) an investigation into the use of hydrogen peroxide as a source of oxygen to support aerobic biodegradation.

These experiments were undertaken at the beginning of the research with the understanding that subsequent experiments would build upon their results. Because the outcome of some of the experiments was uncertain, it was not possible to establish a complete and detailed plan at the beginning of the research. Therefore, the experimental methods evolved as the research progressed.

During the early stages of experimentation, toluene's high volatility proved to be a problem. Before useful experimental results could be obtained, all volatile losses had to be eliminated or quantified; therefore, several studies were carried out to establish an acceptable method for containing toluene in the test-tube microcosms. Once methods were developed for accounting for all the toluene, experiments could be conducted to accomplish the objectives.
As previously stated, the adsorption characteristics were to be determined first. The next step was to investigate the use of hydrogen peroxide as an oxygen source. From this experiment, the optimal dose of H₂O₂ was to be established for use in conjunction with determining the ability of bacteria to biodegrade adsorbed toluene. Based on the results of these trials, additional experiments on desorption were undertaken to gather additional information.

The following sections explain in detail the materials and methods used throughout the research.

**Soil Microcosms**

Toluene sorption and biodegradation experiments were conducted using batch soil microcosms that were established in screw-capped, glass test tubes (13mm x 100mm). The microcosms were sealed with Teflon-coated, silicone septa, and samples for analysis were withdrawn with a microliter syringe by piercing the septa, thus minimizing volatilization of toluene. To eliminate contamination by foreign microbes, all implements and glassware were autoclaved at 15 psi and 120°C for 30 minutes.

Microcosms were weighed on a Mettler balance (Model AC100) before and after additions of soil and solution to determine the respective masses in each test tube. Toluene stock solution (approximately 500
mg/L) was prepared by dissolving the pure compound in sterile distilled water, and because dissolution of toluene occurred very slowly, the solution was allowed to equilibrate over 12 hours. Appropriate dilutions of the stock solution provided the microcosm toluene solutions with concentrations ranging from 10 to 60 mg/L as determined by gas chromatography (GC). Initially, the microcosms were partially filled so that the toluene solution just covered the soil, agitated vigorously with a vortex mixer to remove entrapped air, and then completely filled with solution to eliminate gaseous headspace. Finally, all microcosms were agitated with a vortex mixer and stored in an inverted position at 20°C in the dark to simulate subsurface conditions.

In order to define the relationship between adsorption and biodegradation by acclimated bacteria, it was necessary to eliminate the effects of indigenous soil microbes. To accomplish this, the soil was sterilized by autoclaving at 15 psi and 120°C for 30 minutes on five occasions. The first four times, soil was distributed evenly in an enamel-coated tray and covered with aluminum foil. This soil was then placed in the sterile test-tube microcosms prior to the final autoclaving to kill any foreign microbes possibly introduced during the filling procedure.
Soil Samples

Subsurface soil was collected from an uncontaminated site at the Harwood's Mill Water Filtration Plant in Newport News, Virginia. This soil, composed primarily of sand and silt, was manually removed from a saturated zone from two to three feet below the surface. All collection implements and containers had been heat sterilized and any soil that contacted contaminated surfaces was removed with a sterile blade and discarded.

After removing the top two feet of soil, all the soil between two and three feet was aseptically collected and placed in a sterile cooler. Individual samples were then removed from different areas and depths within the cooler and randomly placed in previously autoclaved, quart "Mason" jars. The storage jars, filled with composite samples, were capped with sterile Teflon lids and stored in the dark at 10°C.

The Newport News soil's organic carbon content was measured by combusting soil samples in a muffle furnace at 550°C as per Physical Examination 209D in the 16th edition of "Standard Methods for the Examination of Water and Wastewater". This procedure yields a measurement of the volatile solids that is considered to be an approximation of the amount of organic matter in the drying sample. This soil's organic carbon content was estimated to be 2.9 percent by weight.
Acclimated Bacteria

Pseudomonas putida (biotype B) was selected as the microorganism to be used for the biodegradation experiments because it is known to utilize toluene as a sole carbon and energy source. Sybron Biochemical (Salem, Virginia) supplied the acclimated bacteria that had originally been isolated from contaminated soil and maintained on agar slants. A sufficient population of microbes was grown in rubber-stoppered, 250 mL Erlenmeyer flasks over 5 days following inoculation of a solution of toluene (500 mg/L) and minimal-salts medium.

Minimal-salts medium (pH 7.2) was prepared, as recommended by Doug Goldsmith of Sybron Biochemical, by adding 1 mL of salts solution, consisting of 4.0g MgSO₄, 0.2g NaCl, 0.2g FeSO₄ * 7 H₂O, 0.2g MnSO₄, and 0.2g CaCl₂ dissolved in 100 mL distilled water, to 1 liter of Sorenson's Buffer, 3.8g KH₂PO₄, 12.5g K₂HPO₄, and 1.0g (NH₄)₂PO₄ dissolved in 1 L of distilled water. Both solutions were autoclaved prior to mixing.

Toluene was removed from the culture solution before adding the bacteria to the microcosms by washing the microbes with minimal-salts medium three times. This process entailed centrifuging the bacterial suspension at 5000 rpm for 15 minutes, discarding the supernatant, and refilling the centrifuge tube with minimal-salts media before repeating the process. This dilution procedure effectively removed any measurable trace of toluene from the bacterial solution as verified by GC analysis.
Given that one bacterial cell weighs roughly $10^{-12}$g, as per Doug Goldsmith, the concentration of microorganisms in the culture was estimated from the solids concentration for a known volume of media. Solids concentration was determined by following Physical Examination 209C in Standard Methods, "Total Suspended Solids Dried at 105°C". Typically, five to ten mL of bacterial suspension was filtered through a 0.20 µm glass-fiber filter disk to remove a measurable number of cells. The filter disks were dried in aluminum planchets to a constant weight and the difference from the initial value was attributed to cell mass.

Microcosms were inoculated with enough bacterial suspension, typically 0.5-1.0 mL, to achieve a final concentration of 50 mg bacteria per liter. To make room for the inoculum, a second syringe was inserted through the septum to remove an equal volume of aqueous solution from the microcosms as the microorganism slurry was added. The syringe needles were inserted to different depths and mixing was minimized to avoid removal of the inoculum in the second syringe. Inoculation was completed by replacing the punctured septa and mixing the contents vigorously.

**Hydrogen Peroxide**

The use of aerobic bacteria for the biodegradation experiments required that oxygen levels be maintained in the microcosms. An aqueous...
hydrogen peroxide solution (H$_2$O$_2$; 35 percent by weight) was selected as an oxygen source because it is readily soluble in water and liquid doses can be easily measured. Experiments were designed to determine the effect of varying the dose of H$_2$O$_2$ on biodegradation. Small doses (one to five μL) of hydrogen peroxide were to be directly injected into the microcosms with a microliter syringe. From these data an optimal dose was to be determined to accomplish complete toluene biodegradation. Well-known toxicity effects were to be avoided by maintaining concentrations below 0.05 percent (by volume) as determined by Britton (1985).

**Analytical Methods**

To quantify the extent of adsorption and biodegradation, microcosms were "sacrificed", i.e. measured one time only, after certain periods of time. Changes in aqueous toluene concentrations were considered to accurately reflect removal by adsorption and biodegradation. Prior to sampling, microcosms were centrifuged at 2000 rpm for 15 minutes to shift the soil to the bottom of the test tube and settle suspended particles. Next, aqueous concentrations were determined by direct injection of 2 μL of supernatant into a Hewlett Packard Model 5880A gas chromatograph (GC).
GC conditions were as follows: nitrogen carrier gas flowed at 30 mL/min through a 6 foot by 1/8 inch stainless steel column packed with 0.2 percent Carbowax 1500 on 80/100 mesh Carbopak-C. Toluene was measured isothermally at 150°C with the temperature of the injector port at 150°C and that of the flame ionization detector (FID) at 225°C. The detection limit was established to be 0.1 mg/L.

Before measurements were made of the toluene concentrations in the microcosms, the GC was recalibrated by analysis of a 50 mg/L toluene standard solution. New standards were made for each day of sampling by diluting a stock solution of toluene dissolved in methanol (1000 mg/L) with distilled water. Two test tubes were filled and analyzed repeatedly until results for each were within 5 percent. These results were then entered into the GC integrator's recalibration program. A preliminary calibration study verified the linearity of detector response for the concentration range used in this research (See Appendix A, Figure A-1).

**Soil Extraction**

In subsequent experiments, the amount of toluene adsorbed to the soil was to be quantified. To accomplish this, the soil in a microcosm was extracted with methylene chloride, and the solvent was analyzed on the GC. The method proceeded as follows: after the aqueous toluene
concentration in a microcosm was determined, the supernatant was
decanted, its volume measured, and the microcosm was refilled with
methylene chloride. The contents were then mixed vigorously with a
vortex-mixer for four minutes during which the microcosm was inverted
every 30 seconds. The soil and liquid phases were then separated by
centrifuging the microcosm for six minutes at 2000 rpm. These steps
were typically repeated two or three times to maximize toluene removal.
Direct injection of 2 μl of the methylene chloride into the GC
quantified the amount of toluene that was extractable by this procedure.
The extractable toluene concentration included both the toluene
dissolved in the pore water and the toluene extracted from the soil.
Pore water that was displaced by the solvent during extraction was
measured by GC analysis and it was revealed that the aqueous toluene
completely partitioned out of the water and into the methylene chloride.
Thus, the extraction procedure was effective at recovering the total
amount of toluene in the pore water.

Due to the need to establish the effectiveness of the extraction
method, some preliminary results will be presented that define the
limitations of its usefulness. Unfortunately, preliminary extraction
efficiency studies showed that the procedure was not able to extract the
total amount of toluene adsorbed to the soil. A matrix of microcosms
was filled with soil and toluene solutions with initial concentrations
of 10, 50, and 100 mg/L. After allowing for adsorption to occur,
microcosms were extracted with methylene chloride and the mass of
recoverable toluene calculated. Knowing the initial mass of toluene added, it was possible to determine the percent recovery of the adsorbed fraction given that the aqueous toluene was completely recovered.

Incomplete recovery (less than 100 percent efficiency) was not considered to be a problem as long as consistent removal could be accomplished. Given a consistent extraction efficiency and the amount of toluene extracted, all of the adsorbed toluene would be accountable. However, some variation in extraction efficiencies for the microcosm matrix was encountered. Mean efficiencies ranged from 60 to 70 percent recovery and the variation from the mean ranged from 9 to 19 percent (See Appendix B, Table B-1). While the extraction procedure effectively recovered a majority of adsorbed toluene, the observed variation in efficiencies precluded precise quantification. Thus, with these limitations, solvent extraction of soil was utilized throughout the microcosm experiments in determining the fate of adsorbed toluene.
RESULTS & DISCUSSION

Adsorption Equilibrium

To study the relationship between biodegradation and adsorption, it was necessary to first characterize the adsorption process in the absence of biological activity. Initially, experiments were undertaken to determine the adsorption characteristics of toluene onto soil from the Newport News site. A group of sacrificial microcosms was sampled over time to quantify the change in aqueous toluene concentration. Since any toluene adsorbed to soil would not be measurable as part of the aqueous solution, the change in supernatant concentration was considered to be an accurate reflection of the adsorption process.

As shown in Figure 1, toluene adsorbed rapidly to the soil at three different initial concentrations. Within the first two days, the concentrations of toluene decreased to approximately half of their initial values demonstrating rapid and extensive sorption. However, after this rapid stage of adsorption, the solution concentrations remained relatively constant indicating that the removal of toluene from solution had slowed considerably and perhaps even ceased. Continued monitoring over forty four days showed that the aqueous concentrations had stabilized and achievement of adsorption equilibrium was considered complete. From the measurements taken, it was determined that the toluene concentrations were essentially unchanged from their values measured two weeks earlier.
Figure 1. Adsorption of toluene in sterile soil microcosms with different initial aqueous concentrations.
However, additional analyses performed on this same group of microcosms five months later revealed further decreases in the aqueous concentrations. This decrease was apparent for the data at 215 days that fall below the curves that were fit by linear regression. Expressed as a fraction of the initial concentration, Figure 2 reveals that after seven months the residual concentration had decreased by ten percent or greater. All the normalized data followed the same trend indicating that adsorption was linear.

It was postulated that this drop in the aqueous toluene concentration was due to further adsorption of the solute onto the soil. Additional "slow" adsorption sites could have attributes such that physical access to them is rate-limited by transport processes, i.e. film or pore diffusion. Another explanation for slow adsorption could be the weak bonding of the solute to sites that have a low affinity for the compound.

There are many explanations for the observance of continued slow adsorption but this research was not designed to directly investigate such phenomena. Furthermore, at this stage in the research, volatilization and/or oxidation of solute could not be completely ruled out as the cause of the decline in solution toluene over the seven month period. However, based on these results, adsorption of toluene appeared to be a two-stage process, with an initially fast period followed by a slower adsorption of an additional small fraction of the solute.
Figure 2. Two-stage adsorption of toluene in sterile soil microcosms. Initial stage was rapid and extensive; long-term stage entailed slow adsorption of a small fraction of toluene. Data normalized to initial concentrations ($C_0$) follow the same curve indicating linear adsorption. (Dashed line is a continuation of the linear regression fit to the data up to 45 days.)
Additional information about the adsorption of toluene to soil can be derived from Figure 3 where the data in Figure 2 has been plotted in the form of adsorption isotherms. The mass of adsorbate was calculated by subtracting the final aqueous mass (final concentration * solution volume) of toluene from the initial mass added to each microcosm. Dividing the mass of adsorbate by the mass of soil in each microcosm provided the ordinate values (X/M) to be plotted versus the equilibrium aqueous concentrations (C). Replicate data for given time periods were averaged and shown as single data points on the plot. Statistical analysis by linear regression provided the values of the correlation coefficients, $r^2 = 0.99$, (Table 1) that are reflective of an excellent fit to linearity for the isotherms.

The linear isotherms presented are in good agreement with previous research that has suggested a linear adsorption relationship for dilute aqueous solutions and for soils with an organic carbon content above one percent. In this case, concentrations were well below half the solubility limit for toluene ($515 + 2 = 258$ mg/L) and the organic carbon content was estimated to be 2.9 percent.

The importance of this finding was that, for the concentration range studied, the adsorption process can be greatly simplified by applying the observed linear relationship. The slope of the line is defined as the distribution coefficient, $K$, and is a measure of the ratio of the adsorbed solute to the solution concentration. Based on
Figure 3. Linear toluene adsorption isotherms. The change in slope with time was indicative of long-term, slow adsorption as the microcosms approached equilibrium.
Table 1. Toluene distribution coefficients (K) derived from the linear adsorption isotherms.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Slope (=K)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>0.94</td>
<td>0.999</td>
</tr>
<tr>
<td>2-6 weeks</td>
<td>1.21</td>
<td>0.990</td>
</tr>
<tr>
<td>7 months</td>
<td>1.42</td>
<td>0.998</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: See Figure 3.

\textsuperscript{b}: $r^2$ = correlation coefficient, see Appendix C for statistical analyses.
the final adsorption isotherm (7 months), the distribution coefficient for toluene adsorbed to this particular soil was calculated to be 1.42 (mL/g). Thus, given a value for aqueous concentration, the mass of adsorbed toluene could be determined by multiplying the value by both 1.42 and the mass of soil in the microcosm. Calculations such as this are valuable in predicting the fate of organic compounds in the subsurface. For instance, compounds that are not adsorbed to any great extent will travel with the groundwater and have the potential to pollute drinking water supplies.

Other researchers have shown that the organic carbon fraction \((f_o)\) of a soil plays an important role in the adsorption of organic compounds (Garbarini and Lion, 1986, Karickhoff and Morris, 1985, Lambert et al., 1965, McCarty et al., 1981). Commonly, K values have been normalized to the organic carbon content yielding the organic carbon distribution coefficient, \(K_{oc} (K_{oc}=K+f_o)\). The usefulness of this value is apparent when comparing soils that have different compositions. \(K_{oc}\) values for specific compounds have been shown to be fairly consistent, within an order of magnitude, for a wide range of soils. Thus, given a \(K_{oc}\) value for an organic compound, one could calculate the distribution coefficient and the extent of adsorption after simply measuring the organic carbon content of the soil of interest \((K=f_o*K_{oc})\). Normalizing the distribution coefficient from this experiment for organic carbon resulted in a \(K_{oc}\) value of 49.0 mL/g.
Because adsorption studies have not been carried out on many organic chemicals, researchers have attempted to equate $K_{oc}$ to other known properties such as solubility, $S$, and octanol-water partition coefficient, $K_{ow}$. In this way, by deriving an empirical relationship between $K_{oc}$ and $S$, for example, one could predict the extent of adsorption by knowing only the compound's aqueous solubility and the organic carbon content of the soil. Predictions such as these could potentially save many research dollars and hours.

Empirical equations using solubility have commonly been considered to have good potential for estimating values of $K_{oc}$. As seen in Table 2, the $K_{oc}$ calculated by Mingelgrin and Gerstl's equation was only 1.4 times larger than the experimental value obtained in this research. Being able to predict within a factor of two should be considered an excellent result and would be very valuable in actual applications. The worst prediction, using Karickhoff's equation (Table 2), was still within an order of magnitude of the actual $K_{oc}$. Even this correlation should be considered acceptable given the complexities of the adsorption process. In conclusion, these results reaffirm the usefulness of the empirical equations in predicting adsorption.

By grouping the isotherm data temporally, the change in the rate of adsorption can be seen (Figure 3). The isotherm for the first week reflects the extensive adsorption that occurred upon addition of toluene to the microcosms. After another one to five weeks, additional solute had adsorbed to the soil as shown by the increase in slope of the
Table 2. Comparison of organic carbon distribution coefficient derived from present study ($K_{oc}=49.0$ mL/g) to predicted and measured values.

<table>
<thead>
<tr>
<th>Investigator</th>
<th>$K_{oc}$ (mL/g)</th>
<th>predicted or measured value $\div 49.0$ mL/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mingelgrin &amp; Gerstl\textsuperscript{a}</td>
<td>69.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Means et al\textsuperscript{b}</td>
<td>95.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Garbarini &amp; Lion\textsuperscript{c}</td>
<td>151</td>
<td>3.1</td>
</tr>
<tr>
<td>Karickhoff et al\textsuperscript{d}</td>
<td>396.4</td>
<td>8.1</td>
</tr>
</tbody>
</table>

a: $\log K_{oc} = 4 - 0.576 \log S$ (1983)
b: $\log K_{oc} = 0.686 \log S + 4.723$ (Mingelgrin & Gerstl, 1983)
c: measured value from soil adsorption experiment (1986)
d: $\log K_{oc} = -0.54 \log S + 0.44$ (1979)

$S$ = aqueous solubility
isotherm. Finally, after seven months, the slope increased again, indicating additional adsorption. Because this increase occurred over a much longer period of time, the rate of adsorption must have slowed down. Thus, after the initial rapid adsorption stage, it appears that adsorption continued to occur at a rate that decreased with time. The attainment of true adsorption equilibrium appears to require a substantial period of time. Toluene may take up to a year or more to approach the equilibrium state where no net exchange occurs between the relative components.

However, in the interests of obtaining useful data within a reasonable amount of time, it was decided that a minimum of two weeks would be a sufficient microcosm incubation period. Since the majority of the aqueous toluene was adsorbed to the soil within two days, two weeks was thought to provide enough time to establish a substantial adsorbed component. Even though true adsorption equilibrium would not be achieved, it was felt that applicable results on the effect of adsorption on biodegradation would still be obtainable. Throughout the remainder of Results & Discussion, "adsorption equilibrium" will refer to the time period after the "fast" adsorption stage when a majority of the toluene had already adsorbed to the soil.
Volatile Losses

Throughout the preliminary experiments with toluene, volatilization proved to be a problem. The consequences of volatile losses are profound upon quantification of adsorption and biodegradation, especially when performing mass balance calculations. Therefore, in order to quantify the losses, a study was undertaken to monitor the toluene concentration in microcosms filled with an aqueous toluene solution. Soil was not added to eliminate the effects of adsorption in the mass balance calculations.

Repeated samplings of individual microcosms revealed a consistent decrease in the concentration of toluene due to volatilization (Figure 4). Losses occurred despite the use of Teflon/silicone septa that reportedly reseal themselves. Attempts to reseal the septa by capping with wax or silicone caulk proved ineffective. Fortunately, unpunctured septa were able to reliably contain the toluene as shown by the maintenance of concentration in previously unsampled ("new") microcosms. Thus, sacrificial sampling was determined to be the most reliable method of accounting for toluene. At this early stage in the research, it was not deemed necessary to carry out this storage loss experiment past thirty days since no volatilization losses in the new microcosms were apparent up to this point. In retrospect, given the long-term results from the adsorption equilibrium experiment, it would have been beneficial to have continued monitoring new microcosms for several months to determine whether any slow rate volatilization was occurring.
Figure 4. Storage losses from sterile toluene solutions after puncturing septa. Volatile losses were eliminated in unsampled test-tubes as indicated by maintenance of aqueous concentration.
At this point in the research, since volatile losses were not apparent within 30 days in previously unsampled microcosms, volatilization was considered inconsequential. The application of this assumption for subsequent experiments was justified by the fact that they were generally carried out within one month.

Knowing that additions of microbes and hydrogen peroxide were to be made to the microcosms in subsequent experiments which would require replacement of the septa, another study was undertaken to quantify toluene losses during removal of the screw cap. Repeated trials exhibited decreases of 2 percent when caps were removed for one minute and decreases of less than 1 percent for 30 second removals. Since septum replacement generally took less than 10 seconds, volatile losses during brief cap removal were considered to be insignificant.

**Hydrogen Peroxide**

The aerobic bacteria, *Pseudomonas putida*, that were added to degrade the toluene are strict aerobes. Therefore, small doses of hydrogen peroxide (H₂O₂; 35 percent by weight) were added to the microcosms in order to maintain oxygen levels. Because high concentrations of H₂O₂ are known to be toxic to microorganisms, one of the experimental objectives in this study was to determine the optimum
dose of hydrogen peroxide for microbial degradation. This study involved the addition of both acclimated bacteria and either 0, 1, 3, or 5 μL doses of H₂O₂ after an adequate adsorption period had transpired. The aqueous concentration was monitored to determine the mutual effects of hydrogen peroxide and microbes on both adsorption and biodegradation of toluene.

Since hydrogen peroxide is known to oxidize organic compounds, some microcosms were left unseeded to distinguish the oxidative effect of H₂O₂ on toluene. In addition, one subset of microcosms, called the "3 μL initial group", was dosed with 3 μL of H₂O₂ immediately after the addition of toluene, prior to adsorption, to determine longer term effects on toluene with respect to both biodegradation and adsorption.

In addition to the 3 μL initial group and a control group to which no H₂O₂ was added, there were three groups of test-tube microcosms that were dosed with either 1, 3, or 5 μL aliquots of hydrogen peroxide seventeen days after toluene was added. Bacteria were added several days after H₂O₂ addition to allow for the complete diffusion of the chemical within the microcosms and thus, eliminate the danger of toxicity.

In review, the microcosms were essentially divided into two subsets, those seeded with bacteria and those that were not. Within each subset, there were five groups to which different doses of H₂O₂ were added: 0, 1, 3, 3(initially), and 5 μL. With these groupings, it was possible to quantify the effect of the various hydrogen peroxide
doses on both the biodegradation and oxidation of toluene.

Oxidation Effects

The effect of \( \text{H}_2\text{O}_2 \) on unseeded microcosms will be examined first in Figure 5 where the aqueous toluene concentration was plotted versus time. As seen in the plot, even though data points for each group reveal rather erratic behavior, some general trends are discernible. Generally, of the groups dosed with \( \text{H}_2\text{O}_2 \) on day 17 (open symbols), the control group had the highest concentration and the 5 \( \mu \text{L} \) group had the lowest throughout the experiment. This was most likely due to toluene's high volatility and the considerable length of time (1 3/4 hours) required to fill all the microcosms with toluene solution. It appears that an initial concentration gradient was propagated during the filling procedure. Having measured a lower stock toluene solution concentration after filling all of the microcosms, it was not possible at the time to discern the effect on the microcosms. However, since the control group had been filled first, it probably had the largest concentration with the other groups decreasing in the following order: controls > 1 \( \mu \text{L} \) > 3 \( \mu \text{L} \) > 3 \( \mu \text{L initial} \) > 5 \( \mu \text{L} \). This relationship was generally observed throughout the experiment.

With this gradient factor in mind, it was still possible to draw some conclusions from the data. None of the subsets exhibited any
Figure 5. Effect of various doses of hydrogen peroxide (H$_2$O$_2$) on the adsorption of toluene in sterile soil microcosms. Possible oxidation of toluene in "3 μL initial group" dosed with H$_2$O$_2$ on day 0.
noticeable change in the aqueous toluene concentration after the addition of $H_2O_2$ on day 17. Thus, hydrogen peroxide did not appear to oxidize the toluene after a reasonable amount of adsorption had occurred. However, the group dosed initially with hydrogen peroxide (filled triangles) had the lowest toluene concentration. Thus, it appears that hydrogen peroxide may have oxidized some of the toluene. This oxidation appeared to amount to no more than 5 to 10 percent of the aqueous toluene. Thus, oxidation of toluene by hydrogen peroxide was not considered to be of major importance. However, because it was possible that some chemical oxidation occurred, in subsequent experiments, hydrogen peroxide was added just prior to the seeding of acclimated bacteria to minimize any possible effects of oxidation.

**Hydrogen Peroxide Dose**

The relationship between the dose of hydrogen peroxide and biodegradation can be derived from Figure 6 for the subset of seeded microcosms. This plot takes the concentration values on day 20 (Figure 5) as the initial concentrations, before biodegradation. Recalculating the amounts on a percent basis revealed the effect of the hydrogen peroxide doses on the removal of toluene. The noticeable drop in toluene concentration for the microcosms containing bacteria (Figure 6) as opposed to the control groups without microorganisms (Figure 5) can be
Figure 6. Effect of hydrogen peroxide (H$_2$O$_2$) dose on the biodegradation of aqueous toluene by acclimated bacteria in soil microcosms. (a) Aqueous concentration vs. time; (b) Fraction of initial aqueous toluene concentration remaining (C/C$_0$) vs. time (C$_0$= aqueous concentrations on day 20).
directly attributed to biodegradation.

Replotted in Figure 7, the dose of H$_2$O$_2$ was shown to be proportional to the removal by microbial degradation. This result confirms the utility of adding hydrogen peroxide to enhance the biodegradation process. The intersection of the ordinate most likely reveals the initial presence of dissolved oxygen in all the microcosms which would result in some toluene removal even in the control group.

Based on these results, a decision was made to increase the H$_2$O$_2$ dose in subsequent experiments in order to achieve complete biodegradation of toluene. Due to possible toxicity from larger doses of hydrogen peroxide, 5 µL additions were to be made periodically instead of adding a single large dose. It was thought that the implementation of this procedure would be less stressful to the bacteria and would provide a more consistent supply of oxygen throughout biodegradation.

**Adsorbed Toluene**

Once a suitable dose of hydrogen peroxide for biodegradation had been determined, the next experiment was devised to determine the fate of the adsorbed toluene in the microcosms. Using the soil extraction procedure, it was possible to recover a majority of the toluene that was both adsorbed to the soil particles and dissolved in the pore water.
Figure 7. Dependence of percent removal of aqueous toluene by acclimated bacteria in soil microcosms on oxygen supply (dose of H₂O₂).
Dividing the microcosms into two groups, hydrogen peroxide was to be added either daily or every third day in 5 µL doses. All microcosms had been exposed to toluene for 3 weeks prior to the addition of both bacteria and H₂O₂ in order to assure that a majority of adsorption had occurred.

Improvements in the experimental procedure yielded a consistent initial toluene concentration in all the microcosms. By using a self-filling burette, which sped up the process considerably, and measuring the toluene solution concentration between every ten microcosms, it was determined that the initial concentration was within 5 percent for all the samples.

Another factor addressed in this experiment was the presence of the bacterial cells themselves. Since organic matter is known to influence adsorption, an equal amount of dead bacteria was added to the control group so that all microcosms received the same treatment throughout the experiment.

Results for the microcosms dosed with H₂O₂ every third day are presented in Figure 8 as aqueous concentration versus time and as extracted mass per amount of soil versus time. Both extracted and aqueous toluene values decreased noticeably after the fourth day of measurements. This data was important in that it was the first evidence that adsorbed toluene was biodegraded along with the aqueous component. It appeared that the microbes either underwent a lag period or four days were required before their population increased sufficiently to show
Figure 8. Biodegradation of both adsorbed and aqueous toluene in sterile soil microcosms seeded with acclimated bacteria. Microcosms were dosed with a 5 μL dose of \( \text{H}_2\text{O}_2 \) every third day.
measurable biodegradation.

Unfortunately, microcosms that were dosed with H2O2 daily failed to show any signs of toluene degradation (See Appendix D, Table D-1). It was suspected that daily H2O2 additions exceeded the toxic limit for P. putida. Britton's study on the effect of hydrogen peroxide on biodegradation supports this hypothesis (1985). He found that bacteria could tolerate a maximum concentration of only 0.050 percent (by volume). A 5 μL dose in a test-tube microcosm resulted in a concentration of approximately 0.021 percent. Thus, doubling the 5 μL dose would yield conditions that are close to the bacterial toleration limit for H2O2. This finding prompted the cessation of hydrogen peroxide dosing in the other microcosms after the third addition (day 6) in an attempt to minimize toxicity and maximize biodegradation. As seen on days 5 through 9, the bacteria in the microcosms were successful in reducing concentrations to levels near zero.

However, it appeared that the third dose of H2O2 may have been lethal to the bacteria. This was indicated by the increase in toluene concentrations for samplings after day 6. Apparently, once the bacteria were killed and biodegradation ceased, toluene slowly desorbed from the soil particles to equilibrate with the aqueous phase. This suggested that a portion of the toluene was strongly bound to the soil particles and was neither readily extractable nor available for biodegradation. Once biodegradation ceased, the concentration gradient between the adsorbed and aqueous fractions resulted in toluene desorbing back into
Some of the microcosms showed little or no biodegradation after addition of bacteria. It was assumed that the seed was insufficient to initiate biodegradation and that no viable culture developed.

Although the results of this experiment were not conclusive, these results provided some very useful information for the preparation of a final experimental study. First, the limitations of dosing with hydrogen peroxide were observed. Most importantly, the first evidence of biodegradation of adsorbed toluene was revealed. Finally, the results revealed the possibility of desorption playing an important role in the relationship between adsorption and biodegradation.

To prepare for the final microcosm experiment, an improved procedure was used to account for the mass of toluene in the microcosms. Most importantly, a consistent initial toluene concentration and a substantial bacterial seed were delivered to each microcosm. As shown in Figure 9, addition of both acclimated bacteria and 5 μL of H₂O₂, once rapid equilibrium was attained, successfully resulted in the removal of all measurable quantities of both dissolved and adsorbed toluene. The fact that concentrations were reduced to and maintained at zero differed considerably from the previous experiment. This was most likely due to
Figure 9. Complete biodegradation of all measurable toluene in sterile soil microcosms seeded with acclimated bacteria. Bacteria and 5 μL dose of H₂O₂ were added on day 21.
efforts to assure achievement of a substantial microbial seed (50 mg bacteria/L) in each microcosm. As a result, 5 µL of hydrogen peroxide was a sufficient oxygen source to accomplish complete biodegradation. Since only one dose of H₂O₂ was added, lethal conditions were not produced. Thus, a viable bacterial population was able to degrade all aqueous and extractable toluene and presumably any additional toluene that desorbed from the soil.

As shown in Figure 9, the aqueous concentration of the control group decreased slightly over the course of the experiment. This was attributed to toluene accessing additional adsorption sites and possibly to slow diffusion into internal pores. Similarly, the noticeable decrease in extractable toluene concentration with time could be explained by the diffusion of adsorbate into pores where it was unaccessible to the solvent. While these results are not conclusive, they certainly add to the evidence of the existence of adsorption sites that are accessed slowly and are not acted upon by solvent extraction.

Desorption

In the interests of better accounting for the unextractable toluene, it was decided to continue monitoring the solution concentration in previously extracted microcosms to reveal any slow desorption that might occur. After filling the microcosms with
methylene chloride for the soil extractions, all biodegradation was considered to have stopped. The solution toluene concentration was then periodically measured to quantify long-term desorption. Punctured septa were replaced after each sampling to eliminate volatilization losses. Typical microcosms were plotted in Figure 10 revealing that over the course of several months measurable toluene desorbed from the soil to reestablish equilibrium with the aqueous phase. This result could be explained by the slow diffusion of toluene from the soil particles.

Notably, all measurable toluene (aqueous plus extractable) in the microcosms had previously been biodegraded to zero. Since aqueous concentrations were readily measured, the only toluene unaccounted for was that adsorbed to the soil or contained in the pore water. Results presented in Figure 10 revealed that in all cases a portion of the unextractable toluene was desorbed to achieve a new solution equilibrium. Microcosms that had incubated longer after biodegradation had reduced solution levels to zero (Figure 9, day 23) yielded smaller equilibrium solution concentrations. It was postulated that toluene had been desorbing either to the soil surface or into solution where it became available to bacteria. Thus, as the incubation time increased, more desorption and more biodegradation occurred. Since desorption occurred at a slow rate, only small differences in final solution concentrations were revealed (0.3-0.7 mg/L). Nonetheless, as shown in Figure 10, a noticeably decreasing trend was apparent for microcosms that were incubated longer.
Figure 10. Desorption of toluene into methylene chloride from soil in microcosms after cessation of biological activity. Acclimated bacteria had previously biodegraded all measurable toluene to zero concentration on day 0 (day 0 equals day 23 in Figure 9). Temporal groupings denote the time after day 0 when soil microcosms were solvent extracted with methylene chloride.
These results revealed that the acclimated bacteria were not able to biodegrade all of the adsorbed toluene. Since the extraction procedure was not efficient enough to completely recover the adsorbed toluene, desorption studies were utilized in the hopes of recovering additional adsorbate. The observation of a slow increase in solution toluene in poisoned microcosms revealed that desorption of a component of sorbate occurred at a slow rate.

Because the bacteria were able to biodegrade both the aqueous and extractable toluene rapidly, it was speculated that the microbes were acting on the substrate in the aqueous phase only. Given the rapid biodegradation of the aqueous toluene, the proceeding concentration gradient would have resulted in the desorption of adsorbed toluene and its subsequent removal. Thus, both fractions of toluene would eventually be completely biodegraded.

Multiple Water Extractions

It was decided to determine whether adsorbed toluene could desorb quickly enough during the initial two days of rapid biodegradation to become available in solution. Thus, unseeded microcosms from the final biodegradation experiment were repeatedly extracted with water to simulate the desorption and subsequent removal of aqueous toluene by
biodegradation. This procedure consisted of repeatedly decanting the supernatant and replacing it with distilled water over a two day period. Microcosms were vortex-mixed and stored for approximately twenty minutes between samplings for GC quantification. The period of two days was selected to reflect the observed rapid biodegradation of all measurable toluene over this same time period (Figure 8). In a manner similar to adsorption, desorption exhibited an initially rapid stage, where large amounts of toluene were recovered (water extractions number one and two) followed by continued slow desorption. After two days, 90 to 95 percent recovery of toluene was achieved indicating that desorption occurred extensively enough to account for the previous biodegradation results (Figure 11, Table 3).

The fact that more total toluene was recovered with water extractions than methylene chloride was a noteworthy result. Since toluene is a nonionic compound, one would expect it to more readily partition into the non-polar solvent, methylene chloride. This was exhibited in the last extraction (Figure 11) where a much larger incremental amount was recovered. Thus, methylene chloride appeared to be better able to extract the small amount of adsorbed toluene that remained after long-term desorption/biodegradation.

However, greater total recovery of toluene with water than with methylene chloride was at odds with expectations. The deficiencies of extracting with methylene chloride were attributed to detrimental interactions with the soil. After two or three extractions, the use of
Figure 11. Multiple water extractions of toluene from sterile soil microcosms over a two day period (12 total). Water extractions were followed by a single methylene chloride extraction to quantify the resistant toluene fraction.
Table 3. Recovery of adsorbed & aqueous toluene from sterile soil microcosms by multiple water extractions. \(^a\) Comparison to mean recovery by solvent extraction.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>#1</th>
<th>#2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial mass of toluene added [µg]</td>
<td>214.0</td>
<td>205.0</td>
</tr>
<tr>
<td>Toluene extracted by water within two days [µg]</td>
<td>192.3</td>
<td>195.2</td>
</tr>
<tr>
<td>Percent toluene recovered</td>
<td>89.9%</td>
<td>95.2%</td>
</tr>
<tr>
<td>Resistant toluene removed by single methylene chloride extraction. [µg]</td>
<td>3.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Mean recovery of total amount of toluene in soil microcosms by solvent extraction procedure. (^b)</td>
<td>75.2 - 83.6%</td>
<td></td>
</tr>
</tbody>
</table>

a: See Figure 11.
b: See Table B-1.
methy1ene ch1or1de resu1ted 1n the c1ump1ng of so11 w1th1n the microcosms. Subsequently, vortex-mix1ng and centr1fugat1on of the soil were much more difficult. The outcome of this interaction was the incomplete mixing of the solvent and soil. Thus, the usefulness of methy1ene ch1or1de as an extractant was limited due to the resultant agglomerat1on of the soil. Water differed from methy1ene ch1or1de because soil could be resuspended and thoroughly mixed in the microcosms even after two days of repeated water extractions. Thus, although water was not as strong of a solvent, to1uene recovery was more complete because soil agglomerat1on did not occur.

Another interesting finding resulted from the add1t1onal methy1ene chloride extraction of the microcosms that had been cont1nuously extracted with water for two days (Figure 11, Table 3). The fact that a substantially larger amount of to1uene was liberated by methy1ene chloride revealed that the desorption of to1uene into water, a polar solvent, was rate-limited at the concentrat1on that was present after two days. As expected, the relatively non-po1ar solvent was much more effective at extracting the non-po1ar chem1cal from the soil. However, because no measurable to1uene was extracted in any microcosms after biodegradation to zero concentrat1on (Figure 9), it would appear that bacteria were possibly degrad1ng to1uene that was still adsorbed to the soil surface. Due to the relative ineff1c1enc1es of the water extraction procedure (incremental vs. continuous), this postulation could not be supported conclusively. In summary, the to1uene from the
final methylene chloride extraction, the resistant fraction, was considered to have been either more strongly bound or more unavailable than the toluene extracted at the beginning.

**Resistant Toluene Component**

The presence of a more strongly bound or more unavailable component of toluene could be equated to the amount of toluene that was earlier shown to desorb from the soil after biodegradation (Figure 10). Since at least as much toluene desorbed into water over two days of extractions as was biodegraded during a similar period, multiple water extractions was considered to have effectively simulated the removal of toluene by biodegradation. In both cases, a resistant component of adsorbed toluene was recovered by desorption, or extraction, into methylene chloride after removal appeared to have been complete.

As seen in Table 3, the final extraction with methylene chloride recovered approximately 3 µg of toluene. Multiplying the equilibrium desorption concentration (Figure 11) of the "0 day" microcosms by the volume of toluene solution resulted in a value of 4.6 µg (0.7 mg/L * 6.5 mL = 4.6 µg). Thus, the amount of toluene that desorbed from the soil was comparable to the amount extracted with methylene chloride. It was postulated that these amounts were representative of the same resistant component of adsorbed toluene whose desorption into water was rate-
limited.

Further support of this explanation was evident by examining Figure 10 in more detail. Replotted as Figure 12, the equilibrium solution concentration was seen to decrease with time and was attributed to the biodegradation of desorbing toluene. By plotting the curves from Figure 10 through the origin, it was apparent that toluene was initially desorbing at the same rate, given by the slope of the dashed line, for these three groups of microcosms. Thus, for each temporal grouping, by starting on the x-axis at the respective time (after biodegradation to zero concentration), the y-coordinate, determined from the intersection of the dashed line, equaled the toluene concentration that would have been present if no biodegradation had been occurring. Assuming that the bacteria were still active, this amount of toluene was consumed and should have equaled the difference in equilibrium concentrations between the 0 day group and any other group. As revealed in Table 4, the amounts in columns 4 and 5 were comparable. These results are presented as evidence of the slow desorption of toluene from the soil and the subsequent biodegradation of the desorbed substrate.

The fact that the data for the 38 day group did not follow the same trend could be explained by the cessation of biodegradation. If toluene was not completely removed, the equilibrium concentration would have been larger than expected as was evident in Figure 10.
Figure 12. Slow desorption of toluene into methylene chloride from soil in microcosms after cessation of bacterial activity. All soil microcosms were solvent extracted on day 0. Temporal groupings denote the time after acclimated bacteria had biodegraded all measurable toluene to zero concentration (day 23 in Figure 9).
Table 4. Comparison of predicted solution concentrations calculated from the initial desorption rate at time t to measured difference in equilibrium concentrations (amount biodegraded during time t). a

<table>
<thead>
<tr>
<th>Temporal group (t days)</th>
<th>$C_e$ [mg/L]</th>
<th>difference in equil. conc.</th>
<th>calculated sol’n conc. from rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.70</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>0.60</td>
<td>0.10</td>
<td>0.16</td>
</tr>
<tr>
<td>16</td>
<td>0.49</td>
<td>0.21</td>
<td>0.30</td>
</tr>
</tbody>
</table>

a: See Figure 12.

$C_e = \text{equilibrium concentration}$
**Slow Adsorption/Desorption**

In an attempt to equate slow desorption to slow adsorption, a comparison was made between the results from the desorption study and the initial adsorption experiment. In Figure 3, it was noted that the linear isotherms increased in slope with time. This change was attributed to the slow adsorption of a small amount of toluene. It was possible to calculate the rate of change in the adsorbed concentration by dividing the difference between the slopes for the 1 week and 2-5 week isotherms by the amount of time to affect that change for the same aqueous concentrations as in Figure 12 (0.12-0.25 mg/L). Multiplying this value by the amount of soil in the micrososms resulted in a mass rate for slow adsorption of 0.04-0.20 ug/day (Table 5). A range of values resulted since the second isotherm was plotted for a 2 to 5 week range of data.

From Figure 12, the slope of the initial linear portion of the curve was calculated to be 0.019 mg/L/day. Multiplying this value by the volume of solution resulted in a mass rate of slow desorption of 0.12 ug/day. These values for the rate of slow adsorption and slow desorption were exceptionally close. Thus, it appeared that slow sorption was occurring at the same rate in both directions. A more detailed study would have to be undertaken to fully explain these results.
Table 5. Comparison of the initial rate of slow adsorption and slow desorption. Slow adsorption rate determined from the change in slope of the linear adsorption isotherm (Figure 3). Slow desorption rate calculated as the initial slope of the desorption curve (Figure 12).

<table>
<thead>
<tr>
<th>Desorption:</th>
<th>Initial Rate</th>
<th>Solution Volume</th>
<th>Soil Mass</th>
<th>Slow Desorption Rate [µg/g/day]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.019 mg/L/day * 6.5 mL ÷ 7.3 g = 0.017</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adsorption:</th>
<th>Change in Adsorbed Conc.</th>
<th>Time Period</th>
<th>Slow Adsorption Rate [µg/g/day]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 µg/g ÷ 1-5 wks = 0.007-0.036</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In conclusion, the slow sorption phenomenon appeared to be the controlling factor in the fate of toluene in the subsurface. Earlier experiments showed that bacteria were able to biodegrade all measurable aqueous and adsorbed toluene that rapidly desorbed but could not utilize a small fraction of adsorbate. The presence of a resistant component was revealed through the desorption studies on biodegraded microcosms. The existence of a resistant component could be explained by the adsorption and subsequent diffusion of solute to sites where neither bacteria nor solvent could physically interact with it. Another possibility could be strong chemical bonding of the resistant toluene fraction to molecules within the soil structure. Permanently bound toluene could result for the case where chemical bonding to soil is thermodynamically favored over dissolution. In either case, toluene would only become available for biodegradation or extraction after slow desorption and possibly not at all. However, the fact that the rate of slow sorption was about the same in both the forward and reverse directions suggested that the process was reversible.
This research was undertaken using batch soil microcosms to study the relationship between sorption and biodegradation of organic chemicals in subsurface soils. The binding of toluene to soil was a two-stage process with rapid and extensive adsorption occurring during the first two days. The subsequent slow stage entailed the adsorption of a much smaller amount of toluene to the soil over a period of several months. Toluene appears to require at least several months to approach absolute sorption equilibrium.

In a manner similar to adsorption, desorption exhibited fast and slow stages. Slow sorption rates were essentially the same in both directions.

For the concentration range studied, toluene adsorbed linearly to the Newport News soil. The organic carbon distribution coefficient derived from the slope of the line was comparable to values predicted by established empirical equations. This finding supports the use of the empirical relationships for predicting adsorption of organic compounds to soils.

Hydrogen peroxide was also studied as a potential amendment for supplying oxygen to the aerobic, toluene-degrading bacteria. Small doses of hydrogen peroxide successfully provided oxygen to support aerobic biodegradation at concentrations less than the toxicity limit.
The following specific conclusions were derived from this research:

1) Soil bacteria such as *P. putida* are not able to immediately biodegrade the total amount of toluene adsorbed to soil. These bacteria can readily transform the aqueous toluene but biodegradation is ultimately limited by the rate-controlled desorption of adsorbed toluene.

2) Slow adsorption and slow desorption proceed at a similar rate over a lengthy period of time. Thus, treatment methods for complete removal of adsorbed toluene in subsurface soils including *in situ* biodegradation and groundwater pumping will have to address the slowly desorbing component.
BIBLIOGRAPHY


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Pfaender, F., 1987, "Aquifer Microbial Communities Do Adapt to Pollutants; The Main Question Which Persists Is 'At What Rate?'", ESE Notes (Research Update), Dept. Env. Sci. & Eng., UNC, V.23, no.4, 3-4.


Figure A-1. Linear GC FID response curve for toluene.
Figure A-2. Typical GC chromatogram of toluene (RT 5.12)
APPENDIX B
Table B-1. Efficiency of the extraction of adsorbed toluene from soil.

<table>
<thead>
<tr>
<th>Initial Concentration</th>
<th>10 mg/L</th>
<th>50 mg/L</th>
<th>100 mg/L</th>
</tr>
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<tbody>
<tr>
<td>Group 1</td>
<td>61.4</td>
<td>71.7</td>
<td>69.3</td>
</tr>
<tr>
<td>Group 2</td>
<td>50.5</td>
<td>68.7</td>
<td>66.7</td>
</tr>
<tr>
<td>Group 3</td>
<td>72.1</td>
<td>62.5</td>
<td>77.0</td>
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<tr>
<td>Group 4</td>
<td>54.1</td>
<td>64.2</td>
<td>62.8</td>
</tr>
</tbody>
</table>

Mean Recovery of Adsorbed Fraction
\(\pm\) coeff. of var.
- \(59.5 \pm 19.0\%\)
- \(66.8 \pm 8.8\%\)
- \(68.9 \pm 9.0\%\)

Mean Recovery of All Toluene
\(\pm\) coeff. of var.
- \(75.2 \pm 8.8\%\)
- \(83.6 \pm 4.9\%\)
- \(83.4 \pm 5.4\%\)
APPENDIX C

"MYSTAT" statistical analyses of linear isotherm data.
**MODEL CONTAINS NO CONSTANT. DATA FOR WEEK 1**

<table>
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<th>STD COEF</th>
<th>TOLERANCE</th>
<th>T</th>
<th>P(2 TAIL)</th>
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<td>0.024</td>
<td>0.999</td>
<td>1.00E+01</td>
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**ANALYSIS OF VARIANCE**

<table>
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<th>F-RATIO</th>
<th>P</th>
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**MODEL CONTAINS NO CONSTANT. DATA FOR WEEKS 2 THROUGH 6**

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<tr>
<td>X</td>
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<td>0.995</td>
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**ANALYSIS OF VARIANCE**

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<td>RESIDUAL</td>
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Press ENTER <- or RETURN
MODEL CONTAINS NO CONSTANT.

DATA FOR MONTH 7

DEP VAR: Y  N: 3  MULTIPLE R: .999  SQUARED MULTIPLE R: .998
ADJUSTED SQUARED MULTIPLE R: .998  STANDARD ERROR OF ESTIMATE: 1.245

VARIABLE  COEFFICIENT  STD ERROR  STD COEF  TOLERANCE  T  P(2 TAIL)
X          1.420  0.046  0.999  .100E+01  30.807  0.001

ANALYSIS OF VARIANCE

SOURCE  SUM-OF-SQUARES  DF  MEAN-SQUARE  F-RATIO  P
REGRESSION  1470.242  1  1470.242  949.050  0.001
RESIDUAL    3.098  2   1.549

Press ENTER <-' or RETURN
APPENDIX D
### Table D-1. Toluene data for soil microcosms that failed to exhibit biodegradation when given daily 5 µL H₂O₂ doses.

<table>
<thead>
<tr>
<th>DAY</th>
<th>Aq. Toluene Conc. in Biologically Active Microcosms (mg/L)</th>
<th>Aq. Toluene Conc. in Controls (mg/L)</th>
<th>Mass of Toluene Extracted from Soil in Active Microcosms (µg)</th>
<th>Mass of Toluene Extracted from Soil in Controls (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25.6</td>
<td>25.6</td>
<td>95.3</td>
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</tr>
<tr>
<td>1</td>
<td>21.3</td>
<td>22.7</td>
<td>89.9</td>
<td>84.7</td>
</tr>
<tr>
<td>2</td>
<td>23.0</td>
<td>22.5</td>
<td>102.0</td>
<td>94.1</td>
</tr>
<tr>
<td>4</td>
<td>20.1</td>
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<td>70.4</td>
<td>89.5</td>
</tr>
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<td>17.4</td>
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<td>91.6</td>
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