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An Investigation of the Biodegradation Potential of Groundwater Contaminants

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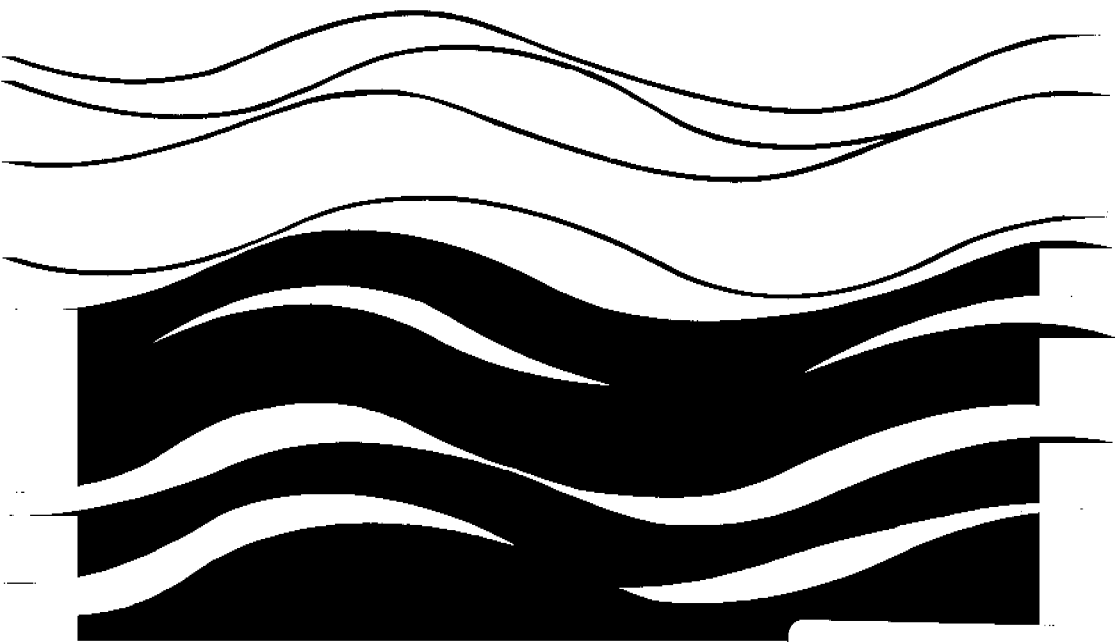

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John T. Novak
Gary T. Hickman
Mark A. Morris
Department of Civil Engineering
Robert E. Benoit
Department of Biology
Virginia Polytechnic Institute and State University

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ABSTRACT

The purpose of this research was to evaluate the rates, patterns, and pathways involved in the biodegradation of organic contaminants in subsurface environments. Subsurface material was obtained from several sites representing diverse environmental conditions. The overall goal was to gain a general understanding of biodegradative mechanisms rather than making site-specific measurements.

The biodegradation rates of methanol, phenol, and t-butanol (TBA) were evaluated in static soil/water microcosms. Biodegradation assays were conducted under ambient anoxic conditions, and with the addition of potential electron acceptors (nitrate, nitrite, sulfate) or metabolic inhibitors (molybdate, BESA) to promote different pathways of anaerobic microbial metabolism (nitrate respiration/denitrification, sulfate reduction, or methanogenesis).

In unamended systems, biodegradation rates varied considerably between sites. Methanol and phenol were degraded fairly readily. Rates generally ranged from 0.5 to 1.0 $mgL^{-1}d^{-1}$ for 20°C incubation. Disappearance of methanol and phenol followed zero- to first-order kinetics and was usually immediate, requiring no acclimation period. TBA was relatively recalcitrant in subsurface soils, disappearing at a rate of 0.1-0.3 $mgL^{-1}d^{-1}$ (20 °C). No biodegradation was evident, relative to sterile controls, in certain soils. The pattern of TBA degradation was typically biphasic: a long lag period of slow, linear removal was followed by an abrupt increase in removal rate (albeit still slow). Biodegradation rates were positively correlated with bacterial density for 12 soil samples from 3 sites within a localized area at Blacksburg, Virginia. However, this relationship did not exist between soils from diverse locations.

The prevailing electron acceptor conditions govern the catabolic pathways utilized in the anaerobic respiration of organic contaminants. The effects of the added electron acceptors and inhibitors on biodegradation rates varied between sites. Two general types of systems are indicated by relative biodegradation rates, characteristic responses to electron acceptor/inhibitor amendments, and general environmental conditions. "Fast" soils are characterized by a higher flux of water and nutrients, higher biodegradation rates, and rate enhancement upon adding nitrate or sulfate. In "slow" soils, organic contaminants are degraded at lower rates, rates are decreased by adding nitrate, sulfate, or BESA (which inhibits methanogenesis), and rates are increased by adding molybdate (which inhibits sulfate reduction). Nearly all soils tested were capable of sulfate-reducing and methanogenic metabolism, but those populations were more active, and competition between the two groups was less severe, in "fast" soils. In contrast, "fast" soils appeared to harbor an active population of nitrate respiring/denitrifying bacteria, whereas in "slow" soils that metabolic group was inactive, absent, or susceptible to nitrite toxicity.

Key Words: Groundwater, Contamination, Biodegradation, Gasoline

INTRODUCTION

The contamination of groundwater has received widespread attention in recent years because of concern about the deterioration in the quality of drinking water supplies. Since groundwater comprises more than 95 percent of all available freshwater in the United States and is used for drinking by almost 50 percent of the people, subsurface contamination poses significant health problems. Once in the groundwater, organic compounds may migrate with the flow of water or adsorb to soil particles. Remediation of contaminated aquifers usually requires costly treatment. In some cases, the aquifer must be abandoned in favor of alternative water supplies.

In recent years, researchers have searched for and attempted to culture bacteria capable of degrading man-made organic compounds as an inexpensive alternative to chemical and physical treatment of contaminated groundwater. While this research may eventually yield valuable results, it fails to address the fundamental question of why natural soil bacteria do not accomplish this task. The most widely held view is that soil bacteria are not acclimated to the organic compounds introduced into the environment and have not developed the enzymes necessary to degrade these chemicals. One would expect, however, that this acclimation period would not persist indefinitely and that a population of bacteria would develop in the subsurface which would eventually degrade xenobiotic compounds. This does not always appear to be the case. In reality, some compounds will degrade quite easily in the subsurface while others tend to persist. The key questions, therefore, are why do these compounds persist and can the subsurface environment be manipulated to encourage or accelerate subsurface degradation? These questions cannot be answered with the information currently available.

This study was designed to investigate the factors which control microbial degradation of subsurface contaminants. The study had two major focal areas. The first was to investigate the role of microbial populations in determining degradation. The second major area of interest was to determine the variations in degradability at various subsurface sites and to determine some of the factors which determined rates of degradation. Each of these major areas will be included as a separate section in this report.

LITERATURE REVIEW

I. Introduction

Groundwater contamination by man-made organic chemicals is recognized as a serious public health threat. In rural parts of the country, between 90 and 95 percent of the people drink groundwater with little or no treatment (Dyksen and Hess 1982). Groundwater was once thought to be pristine. This was found not to be the case when analytical capabilities were developed which allowed for the detection of trace quantities of contaminants. As a result, synthetic organic chemicals have been detected in many groundwater supplies. In 45 percent of the large public water supplies which utilize groundwater, volatile organic chemicals have been detected (Wilson et al. 1983; Wilson et al. 1983b). The problem is that the subsurface does not have an effective self-cleansing mechanism; therefore, groundwater may remain contaminated for many years. Magnifying the problem is our general lack of knowledge about the subsurface. Only within the last decade have methods been developed for analyzing the subsurface and determining the factors which control the transport and fate of anthropogenic chemicals in groundwater.

Most of the knowledge gathered thus far on biodegradation has been developed primarily using pure cultures of organisms degrading individual organic compounds or batch and column studies using seeded bacterial populations or enrichment cultures. The results from these studies may not translate well to the complex subsurface environment. Some studies have been performed using aquifer material and the indigenous bacterial population to study biodegradation. In each case, however, the outcome of the study was to determine whether compounds were degradable but little rate data has been generated. In most instances, it was determined that biodegradation was site specific. Since direct investigation of each man-made organic chemical at every site of interest would be prohibitively expensive, new approaches to the questions concerning biodegradation must be developed. One aspect of subsurface biodegradation that has received little attention is the role of the aquifer chemistry. The chemistry of an aquifer and its influence on the competing metabolic processes may be important in determining why some anthropogenic compounds persist. Such information may allow for defining methods of improving degradation, predicting the rates of degradation and evaluating the degradation potential in hazardous waste sites.

II. Methods to Study the Subsurface

The study of subsurface processes began with the development of techniques for recovering soil which avoided contamination by the sampling process. Procedures developed by Dunlap et al. (1977) and modified by Wilson et al. (1983b), Bengtsson (1985) and Novak et al. (1985) involved boring to a desired depth with an auger and collecting the sample in a sterile thin-walled core barrel. The core material from the tube was extruded and pared to remove any material which would be contaminated by the sampling operation. Ghiorse and

Balkwill (1983, 1985) determined that these samples could be kept at 4°C for 6 months or more without altering the bacterial population.

The primary method for studying degradation in the subsurface system is with microcosms. Bengtsson (1985) defined the microcosm as part of the ecosystem which can be controlled in the laboratory due to its reduction in size and complexity. It can be used to isolate various physical parameters and estimate the response of the larger system. This capability allows the researcher to collect a significant amount of information inexpensively. Ausmus et al. (1980), however have identified problems which should be taken into account when evaluating data obtained from microcosms. The first is the uncertainty of applying data obtained from the simplified system to the more complex natural system. The second problem is a lack of consistency in the size and structural characteristics of microcosms. This may affect the ability to reproduce and compare results obtained from microcosms.

Wilson and Noonan (1984) have described two broad classes of microcosms. The first focuses on the biological response to changes in the environment. The second emphasizes the pollutant and its interactions with the environment. This second type of microcosm can be designed either to imitate the response of the larger system or to obtain values for mathematical expressions which will be used to predict the response in the larger system. The design of a microcosm, therefore, depends on the type of information desired. Wilson, Noonan, and McNabb (1985) and Novak et al. (1985) described simple, inexpensive microcosms for monitoring the biodegradation of organic compounds by soil bacteria. These systems utilized screw-capped test tubes containing aquifer material mixed with the test compounds of interest. The disadvantage of this type of microcosm is that it does not simulate the flow of groundwater. It is, however, a quick and relatively inexpensive way of analyzing the fate of organic compounds and subsurface materials while easily maintaining anoxic conditions.

More complicated microcosms which simulate the flow of water have been developed by Dunlap et al. (1972). Such designs, however, are difficult to build and operate. The materials are expensive and the maintenance time is considerable. Anaerobic conditions are also difficult to maintain. The potential for contamination by outside organisms is increased because of the continual need to prepare feed solution.

III. Microbial Activity in the Subsurface

As a result of the development of techniques for aseptically sampling the subsurface, researchers have been able to characterize indigenous soil bacteria. Using electron microscopy, Ghiorse and Balkwill (1983) and Wilson et al. (1983) observed that a water-saturated sandy aquifer contained a morphologically diverse bacterial population with very few, if any, eukaryotic organisms. The bacteria found were generally smaller than laboratory-cultivated cells and contained both gram-positive and gram-negative types. Gram-positive bacteria

were more abundant. Cell numbers were determined by the acridine-orange (AO) fluorescent direct counting technique and the number of viable organisms were determined by plate-count techniques using two media. In each case, the number of colony-forming units (cfu) from the plate counts were less than the AO-fluorescent direct counts. This indicated that either many of the bacteria were not viable or that the media used were selective for certain bacteria.

At two Oklahoma sites and one in Louisiana, the number of cells determined by the AO epifluorescent direct count ranged from 1 to 10 million/gram of dry soil while the number of viable organisms determined by plate counts varied significantly depending on the site and the media used. At the Oklahoma site, the number of viable bacteria expressed as cfu/gram of dry soil ranged from 100 to 1,000 times less than the number of cells determined by the AO epifluorescent direct count. At the Louisiana site, the number of viable bacteria were about 10,000 times less than the total number. These results were determined using a medium containing peptone, yeast extract, and glucose. In each case, a dilute medium yielded more cfu than a concentrated medium. The authors attributed this result to the oligotrophic nature of subsurface bacteria.

Novak et al. (1985) and Goldsmith (1985) determined that in soil from Pennsylvania, New York, and Virginia, the number of cells determined by the AO epifluorescent technique did not vary significantly between sites or with depth. Viable cell counts using a soil extract medium, however, varied by as much as three orders of magnitude throughout the soil profile. The plate count values were one to four orders of magnitude less than the number of cells determined by direct counts. Goldsmith reported bacterial numbers as a function of depth for subsurface material collected at the three sites. Typical of the data, the results for the Williamsport, Pennsylvania, soil were $5.6 \pm 1.9 \times 10^7$ cfu/g soil at the surface, and $3.9 \pm 1.4 \times 10^7$ and $4.6 \pm 2.7 \times 10^7$ cfu/g soil at 12 and 30 feet, respectively, using the AO technique. Using plate counts with soil extract media, $3.0 \pm 0.3 \times 10^7$ cfu/g soil were measured at the surface, $3.5 \pm 2.1 \times 10^3$ cfu/g soil at 12 feet and $1.4 \pm 0.8 \times 10^5$ cfu/g soil at 30 feet. Plate counts with soil extract typically yielded counts averaging two orders of magnitude less than the direct counts. Goldsmith reported that the acridine-orange counts were more indicative of the actual bacterial population because of the biases introduced by plate-counting media.

White (1986) measured the bacterial population in a gasoline-contaminated soil from Philadelphia, Pennsylvania. At the surface, 6.8×10^7 organisms per gram soil were detected using the AO direct count while plate counts yielded 4.3×10^7 and 3.9×10^7 cfu per gram soil using soil extract and yeast extract media, respectively. The counts did not vary by more than one order of magnitude throughout the soil profile. White indicated that direct counts yielded misleading results in clay soil because of difficulty distinguishing between bacteria and clay particles. For nonclay soils, there was little difference between the AO and plate count results. Overall, however, plate counts were considered more reliable.

Bouwer et al. (1986) determined that the degradation of some halogenated aliphatic compounds was influenced by the anoxic electron acceptor. This study used fixed film continuous flow columns with glass beads inserted to simulate an aquifer matrix. Mixed bacterial cultures were obtained from primary sewage effluent. Anoxic conditions were produced by attaching two columns in series. The influent to the first column contained growth media of acetate resulting in an effluent with a dissolved oxygen concentration of less than 0.5 mg/l. The effluent of the first column then became the influent of the second column. Molybdate was added to the denitrifying column (0.25 mM) and the methanogenic column (1.5 mM) to inhibit growth of sulfate-reducing bacteria. 2-Bromoethanesulfanic acid (0.5 mM) was added to the sulfate-reducing column to inhibit methanogenesis. In strictly methanogenic conditions, tetrachloroethylene, chloroform, 1,1,2,2-tetrachloroethane, 1,1,1-trichloroethane, carbon tetrachloride, dibromochloromethane, bromodichloromethane, dibromochloropropane, bromoform and ethylene dibromide were biodegraded. Only 1,2-dichloroethane persisted. Under sulfate-reducing and denitrifying conditions, chloroform and tetrachloroethylene were not degraded. Ethylene dibromide, 1,1,1-trichloroethane, bromodichloromethane, dibromochloropropane, carbon tetrachloride and bromoform were biodegraded.

IV. Biodegradation of Organic Pollutants

Biodegradation of anthropogenic compounds may provide the most promising solution to the problem of groundwater contamination. Only within the last few years have researchers attempted to characterize the subsurface biodegradation potential and explain the processes that control the fate of organic compounds in groundwater. McCarty and his coworkers have attempted to model biodegradation in the subsurface as a biofilm reactor.The subsurface represents an environment which has a large surface area per unit volume and in many cases low nutrient conditions supporting attached growth. The biofilm model, therefore, may be valid (Rittmann et al. 1980). According to Bouwer and McCarty (1984), the biofilm model consists of four processes.

1. Substrate transport into the biofilm from the bulk liquid;

2. Substrate utilization with associated bacterial growth following Monod-type kinetics;

3. Substrate diffusion through the biofilm according to Fick's Law; and

4. Biofilm growth and decay.

An interesting aspect of the biofilm model is the concept of a minimum substrate concentration (S_{min}) below which no degradation will occur (Rittmann et al., 1980). S_{min} is defined as the concentration below which bacteria cannot obtain enough energy from utilization to support maintenance requirements. If valid, this concept would have significant effects on the ability of soil bacteria to degrade trace organic chemicals. Utilization of a compound below S_{min} may be

possible if the limiting compound is used simultaneously with another more abundant compound which supports the energy requirements of the organism. This process has been termed secondary utilization. The limiting substrate is called the secondary substrate and the substrate which supports growth is called the primary substrate. Using the biofilm model, Bouwer and McCarty (1983, 1983b, 1984) reported that chlorobenzene, 1,3-dichlorobenzene and 1,4-dichlorobenzene were biodegraded in aerobic conditions but were persistent in a methanogenic environment. On the other hand, halogenated aliphatics such as chloroform, carbon tetachloride and 1,1,1-trichloroethane were removed under methanogenic conditions but not in aerobic conditions. These experiments were conducted in a continuous flow column using glass beads to simulate the aquifer matrix. A mixed culture bacterial population was added and acetate was contained in the feed solution as a primary substrate to stimulate secondary utilization. While these experiments demonstrated the degradability of certain xenobiotic compounds, the relevance to actual subsurface conditions is uncertain.

Simkins and Alexander (1984) reported that the degradation response observed in mineralization of organic compounds was a function of bacterial population size and substrate concentration. Using *Pseudomonas* cultures, degradation rates of benzoate ranging in initial concentration between 10 ng/ml and 100μg/ml were measured. The data was then fit by nonlinear regression analysis to six models that were derived from Monod kinetics. As a result, Figure 1 was developed, which established kinetic regimes whose boundaries were defined only by the initial cell concentration and the initial substrate concentration. According to the authors, the zero order, Monod no-growth and first order kinetic regimes can be approximated from the general Monod equation when the population density is much greater than the initial concentration. In other words, the cell yield during substrate utilization is insignificant compared to the initial cell density. In addition, the zero order zone requires that initial concentration be much greater than the half saturation constant (K_s) while the first order regime assumes that the initial substrate is much less than (K_s). In the logarithmic, Monod (with growth) and logistic models, the growth of the population during mineralization is assumed to be significant. The logistic and logarithmic cases reflect the extreme substrate conditions where initial concentration is much less than (K_s) or much greater than (K_s), respectively.

Only a very limited amount of information exists relating biodegradation to site-specific conditions. In a study of a creosote contaminated aquifer in St. Louis Park, Minnesota, Ehrlich et al. (1982) determined that phenolic compounds were biodegraded anaerobically. In contaminated wells, methane was detected and methane-producing bacteria were present in significant quantities. No methane or methanogenic bacteria were measured in uncontaminated wells. In laboratory reactors containing water from the contaminated well, phenol was degraded to methane. The fate of polynuclear aromatic hydrocarbons (PAHs) detected in the well was not determined. It was suggested, however, that sorption was an important removal mechanism for PAHs.

Wilson et al. (1986) determined that toluene and styrene were slowly biodegraded in microcosms containing soil from two previously uncontaminated sites. Chloroform, 1,1,1-trichloroethane and 1,1-dichloroethane did not degrade in either soil and chlorobenzene was utilized in soil from only one site. Each site was sampled in the unsaturated and saturated zones. If a compound degraded in the unsaturated soil of a site, it also degraded in the saturated soil. These results may conflict with those of Bouwer and McCarty (1983b, 1984) who reported the biodegradation of chloroform and 1,1,1-trichloroethane in a methanogenic biofilm reactor but not in a denitrifying system. The observation that the test compounds were either utilized slowly or not at all was attributed to the oligotrophic nature of the subsurface bacteria.

Goldsmith (1985) and Novak et al. (1985) examined the degradation of two gasoline additives, methanol and tertiary butyl alcohol (TBA), in subsurface samples collected from three previously uncontaminated sites. One of the sites was aerobic while the other two were anoxic. Methanol degraded rapidly at all three sites. TBA, on the other hand, was quickly utilized at only the aerobic site. At the anoxic sites, TBA degraded slowly following zero order kinetics in individual microcosms, but indicated a first-order response with respect to the initial concentration. The presence of benzene, toluene, and m-xylene did not affect the degradation rate of the alcohols. In each case, biodegradation in the saturated zone was greater than in the unsaturated zone.

White (1986) determined that TBA would degrade rapidly in aquifer material obtained from a site previously contaminated with that compound. Biodegradation of TBA was accompanied by bacterial growth and could be modeled by the Monod equation. TBA degradation in soil from an uncontaminated site was slow, exhibiting the same response observed by Goldsmith (1985). This slow rate could not be modeled adequately by Monod kinetics.

In a study of biodegradation enhancement, Wilson (1986) determined that the addition of nitrate to soil which did not contain an actively denitrifying bacterial population would inhibit the degradation of methanol due to the buildup of nitrite. This inhibition was relieved if the pH was raised above pH 6. The addition of sulfate inhibited methanol and TBA degradation at the site studied. Manipulation of the pH did not affect this condition. Variation of pH alone and the addition of organic substrates did not affect the rate of TBA degradation.

Suflita and Miller (1985) found that chlorophenolic compounds degraded in soil from an actively methanogenic site.The chlorinated compounds were degraded via reductive dehalogenation to phenol, which was subsequently mineralized.In nonmethanogenic soil, however, reductive dehalogenation did not occur, resulting in a persistence of the chlorophenols. Phenol degraded in both soils. The nonmethanogenic soil was characterized by a gray to black color and slight odor of sulfide, which led the authors to assume that active sulfate reduction occurred. These results suggested that reductive dehalogenation was slowed or inhibited by nonmethanogenic, possibly sulfate-reducing conditions. In a subsequent study, Gibson and Suflita (1986) reported that benzoate and phenol were

biodegraded in anaerobic microcosms containing either pond sediment, an-aerrobic digester sludge, methanogenic aquifer material or sulfate-reducing aquifer material. Chloroaromatic substrates were degraded in methanogenic aquifer soil but not in sulfate reducing soil. Once sulfate was biologically removed by the addition of acetate, degradation of the chlorinated compounds proceeded. When sulfate was added to the methanogenic aquifer material, the test compounds did not degrade. These results demonstrated that the recalcitrance of a chemical in the subsurface is not necessarily related to a lack of microorganisms capable of biodegradation. Smolenski and Suflita (1987) determined that biodegradation of various cresol isomers was favored in sulfate-reducing conditions but was inhibited in methanogenic conditions. Addition of sulfate stimulated degradation, whereas inhibition of sulfate reduction by the presence of MoO_4^{-2} reduced p-cresol mineralization.

Wilson et al. (1986) determined that soil from a shallow water table aquifer that had been contaminated with aviation gasoline would degrade benzene, toluene, m-xylene and o-xylene. Soil from the aerobic portion of the aquifer mineralized the test compounds within two weeks, whereas soil from the anaerobic region required at least 8 weeks. Methane was detected in the headspace of all samples except autoclaved controls.

In a similar study, Wilson, Smith, and Rees (1986) measured the rate of disappearance for five halogenated aliphatic hydrocarbons and four alkylbenzenes in methanogenic aquifer material collected near a sanitary landfill. All test compounds degraded relative to autoclaved controls. For the aliphatic compounds, 1,1-dichloroethylene, trans-1,2-dichloroethylene and trichloroethylene require long lag times before degradation began. Cis-1,2-dichloroethylene and 1,2-dibromoethane were readily consumed. Toluene was the only aromatic tested which did not require a significant acclimation period. Benzene, ethylbenzene and o-xylene degraded after a 20 week lag phase.

Smith and Novak (1987) determined that phenol and four of its chlorinated derivatives were readily degraded in soil from two previously uncontaminated sites. In each case, biodegradation followed first order kinetics with the rate of degradation proportional to the initial concentration. The degradation rates did not correlate well with the degree of chlorination. Biodegradation rates were site specific with bacteria population and nitrate concentration cited as important site variations.

RELATIONSHIP BETWEEN SUBSURFACE BIODEGRADATION RATES AND MICROBIAL DENSITY

I. Background

Reliable estimates of biodegradation rates of organic contaminants in subsurface systems are needed to assess the risk to public health from contaminant sources and to evaluate remediation strategies. The most common method for determining rates of biodegradation of organic chemicals in soil/groundwater systems is by the use of microcosm studies (Wilson et al., 1983b; Novak et al., 1985). For rapidly-degraded chemicals, the microcosm test may continue to be the best method for determining subsurface biodegradation rates, analogous to evaluating a biological process in a wastewater treatability study; however, many months or even years may be required to evaluate degradation rates of more refractory compounds by this method. Consequently, quicker, less expensive indicators of biodegradation potential are needed. And a microbiological measure should, logically, provide such an index.

Another reason for establishing the relationship between rates and biomass is to allow the development and application of kinetic models. Functional kinetic models of biodegradation have been developed for lab-scale aqueous, pure and enrichment cultures and for full-scale biological wastewater treatment processes. In these systems the active portion of the microbial community can be fairly accurately measured. Such measurements are often based on the assumption that all biomass present is active, and therefore involved in degrading the organic chemical(s) of interest. It is also assumed that specific utilization rates (rate of chemical removal per unit biomass) are essentially constant, even between different mixed cultures.

Our inability to accurately quantify the active microbial biomass in soil/groundwater systems is a major deficiency limiting our ability to predict subsurface biodegradation potential. Most measures typically used to quantify soil microbes consider a larger group of organisms, such as total cells, viable cells, or total biomass. The basis for comparing such measures to biodegradation rates is that they may be related to the active population — e.g., differences in total biomass between soils indicate proportional differences in active biomass.

Total (direct microscopical) counts are not good indicators of biodegradation potential of subsurface soils. In many cases, direct counts are strikingly similar in soil samples exhibiting substantially different biodegradation rates (Novak et al., 1985). *Adenosine triphosphate (ATP) content of subsurface soils* — a biomass measure based on the assumption that cells contain a relatively constant amount of ATP per unit of biomass (Grant and Long 1981) — was found to be directly related to the rate of toluene biodegradation in the same soils but not to chlorobenzene degradation rates (Wilson et al., 1986). Likewise, the maximum rate of phenol mineralization (v_{max}) was found to be positively correlated to total biomass (measured as phospholipid concentration) in subsurface soils (Dobbins, et al. 1987), although the relationship was not particularly strong (r=0.62).

Most Probable Number (MPN) techniques can be particularly instructive because they can be modified to enumerate specific groups of organisms, such as degraders of a specific compound. In adaptation studies, a positive relationship has been observed between biodegradation rates of p-nitrophenol and changes in numbers of p-nitrophenol degrading organisms over time within static microcosms (Aelion et al., 1987; Spain et al., 1984). MPNs of p-nitrophenol degraders generally increased as biodegradation rates increased in a variety of aqueous and sediment/water assays.

Plate-counting techniques are used to enumerate viable heterotrophic bacteria in environmental samples but, because of the inherent selectivity of agar media and incubation conditions, only a fraction of the total viable cells are recovered in any given application of this method. This appears to be a serious disadvantage if total viable numbers are desired. However, the results of Olsen and Bakken (1987) indicate that colony-formers may be representative for large cells ($>0.065\mu m^3$) in a community. The implication is that colony-forming cells may represent most of the bacterial biovolume (and biomass) in soils while non-colony-forming cells, although present in high numbers, represent a small fraction of the biovolume and energy flow through the soil ecosystem.

In this study, an examination was made of the relationship between biodegradation rates of methanol, t-butyl alcohol (TBA), and phenol in subsurface soils and measures of microbial density in those soils. The gradual phaseout of lead additives from gasoline has spawned the use of methanol, ethanol, TBA, and methyl t-butyl ether (MTBE) - collectively called oxygenates - as octane enhancers in unleaded gasoline sold in the United States and Europe. Gasoline constituents are leached into soils and groundwater from spills and leaking underground storage tanks. Once in the saturated zone, oxygenates create problems by: (I) being more mobile in groundwater than other gasoline constituents, resulting in a region of the plume distal from the source where only the oxygenate(s) are detectable; (II) being toxic, irritants, or containing toxic denaturants (in the case of ethanol), and (III) acting as cosolvents, thereby enhancing the spread of more hydrophobic (and more toxic) gasoline constituents. Phenol is a high-volume industrial chemical produced largely as an intermediate compound for the preparation of pharmaceuticals, pesticides, and synthetic polymers. Phenol is a common constituent of leachates from municipal and hazardous waste landfills. Its toxicity and water solubility can create a potential health hazard upon entering groundwater.

The objectives of this research were to assess rates of biodegradation of organic chemicals in subsurface soil samples collected from a variety of depths and locations within a localized area, and to investigate spatial and kinetic patterns of biodegradation. Also, several microbial enumeration techniques were employed and evaluated by linear regression analysis for their ability to indicate biodegradation rates.

II. Materials and Methods

A. Sites and Sampling

Soil samples were collected at three sites in a localized area — within 650 m of each other — on farmland near Blacksburg, Virginia. Sites 1 and 2 were located in the same cultivated field that had a history of atrazine application. Site 2 was positioned in a low area that received runoff from the surrounding fields and a nearby feedlot. Site 1 was on a rise approximately 260 m upslope from Site 2. Roughly 400 m from Site 1 and separated by a valley, Site 3 was located on a hill serving as pasture with no history of pesticide use. Thus, Site 3 was considered to be relatively pristine compared to the others, with Site 2 receiving the highest influx of organic matter. Nevertheless, none of the sites had a history of exposure to any of the chemicals examined in this study. Soil samples are identified by location and depth; for example, "soil 2-3.0" implies a sample obtained 3 meters below the surface at Site 2.

Soil profiles at the three sites were as follows:

> Site 1. The surface layer was dark grayish brown loam containing 3.6 percent organic carbon. The subsoil at 0.6 m was yellowish grey silt loam, and the substratum was yellowish brown, mottled with grey, clay loam at 1.5 m and 4.6 m.
>
> Site 2. The surface layer was dark grayish brown silt loam containing 5.9 percent organic carbon. The subsoil at 0.6 m was yellowish brown silt clay. The substratum was, at 1.5 m, yellowish brown, sticky and plastic clay, mottled with grey, and, at 4.6 m, grey, very sticky and plastic clay, mottled with yellowish brown.
>
> Site 3. The surface layer was grayish brown silt loam containing 2.2 percent organic carbon. The subsoil at 0.6 m was grayish brown silt loam; the substratum at 1.5 m was yellowish brown, mottled with grey, clay loam, and at 4.6 m was yellowish brown, sticky and plastic clay mottled with grey.

Sites 1 and 3 were characterized by Groseclose and Poplimento soils while Site 2 combined McGary and Purdy soil series. The water table at Site 2 was approximately 4 m below the surface; it was >7 m and >6 m deep at Sites 1 and 3, respectively.

Subsurface samples were collected in Shelby tubes and then extruded within 24 hours in the laboratory. The exposed ends of the soil cores and all surfaces in contact with the Shelby tube were aseptically pared off and discarded to prevent contamination from nonindigenous microorganisms. The remaining soil was then stored in autoclaved glass jars with Teflon lids at 10°C (the ambient

groundwater temperature) until used. Surface soils were placed directly into jars using sterile spatulas. All samples came from the unsaturated zone. Redox conditions were not controlled during sampling, since the soils were assumed to be generally aerobic (albeit with anoxic microsites) at the time of collection.

B. Biodegradation Rate Determination

Biodegradation rates were determined using static soil microcosms consisting of screw-capped, 13 mm x 100 mm, Kimax test tubes fitted with Teflon-lined, silicone septums. Microcosms were prepared by aseptically transferring approximately 5 g of soil to a tube, which was then filled with a sterile 100 mg/L aqueous solution of methanol, TBA, or phenol, capped precluding headspace, and vortex-mixed. Microcosms were sampled by withdrawing a few microliters of liquid through the septum using a syringe that was previously evacuated and heat-sterilized. Chemical disappearance was monitored over time by gas chromatographic (FID) analysis of aqueous samples. Methanol and TBA were analyzed using a flame ionization detector and either a stainless steel column (2 m x 2.1 mm I.D.) packed with 0.2 percent Carbowax 1500 on 80/100 mesh Caropack C or a glass SPB-5, 30 m x 0.75 mm (I.D.) wide bore capillary column (Supelco, Inc., Bellefonte, Pa.). Phenol was quantified using a glass column (2 m x 2 mm I.D.) containing 1 percent SP-1240-DA on 100/120 Supelcoport. Between sampling periods, microcosm caps were covered with paraffin wax in an attempt to mitigate any possible volatilization through the septum.

Microcosms prepared in this way represented saturated, anoxic conditions. Theoretical oxygen demand calculations indicate that any dissolved oxygen initially present would be depleted by aerobic metabolism of a small fraction (a few mg/L) of the supplied organic substrate. The microcosms were incubated at 5, 10, 15, 20, or 30°C.

Sterile control microcosms similar to live microcosms were prepared and monitored to distinguish biodegradation from abiotic losses. First, it was observed that microcosms containing either an autoclaved aqueous chemical solution and no soil or soil that had been combusted at 550°C for three hours in a muffle furnace showed, except for a few anomalous cases, virtually no chemical loss over time. These data indicated that chemical losses due to (1) leaks in the test system (e.g., volatilization through the septum), (2) biodegradation resulting from microbial contamination during sampling, or (3) chemical or physical transformations of the compound were minimal. However, controls such as these did not account for removal of the chemical from the liquid phase via sorption. Soils were sterilized by repeatedly autoclaving (45 minutes at 121°C and 15 psi) soil that had been ground with a pestle and spread into a thin layer in an enamelled pan. Soils for control microcosms were autoclaved at least six times over a period of several days.

C. Bacterial Enumeration

The soil microbial populations were quantified by:

1. Spread plate viable counts using 10 percent soil extract agar (100 mL soil extract, 15 g agar, 900 mL distilled water), and using yeast extract peptone agar (1 g yeast extract, 1 g peptone, 15 g agar, 1000 mL distilled water). Both media were adjusted to pH 6.8 to 7.0. Triplicate plates were incubated at 20°C for five days (aerobic) or three to four weeks (anaerobic) before counting colonies.

   a. Ambient moisture, aerobically incubated viable counts - plates inoculated immediately after mixing initial soil dilution bottle on shaker table for 30 minutes and making dilution series.

   b. Saturated, aerobically incubated viable counts - plates inoculated at various times after shaking the initial dilution.

   c. Ambient moisture, anaerobically incubated viable counts - plates inoculated as in a, but incubated in an anaerobic glovebox containing an atmosphere of 80 percent nitrogen and 20 percent hydrogen.

2. Substrate specific MPNs - Tubes (13 mm x 100 mm) inoculated with 1 mL of the appropriate soil dilution, filled to preclude headspace, and sealed with a septum cap (identical to microcosm setup). Media consisted of 100 mg/L of methanol, TBA, or phenol in a basal salts solution containing, per liter: 1.0 g NaCl; 1.0 g NH_4NO_3; 75 mg $CaCl_2 \cdot 2H_2O$; 200 mg $MgSO_4 \cdot 7H_2O$; 12 mg $FeSO_4 \cdot 7H_2O$; 20 mg Na_2EDTA; 1 mL Williams trace element solution; 1.0 mg thiamine hydrochloride; 1.0 mg biotin; and 0.5 mL of a stock phosphate buffer solution (1.6 g KH_2PO_4 and 2.4 g K_2HPO_4 per 100 mL). Control tubes were prepared without inoculum, and tubes were assayed based on substrate disappearance, determined by gas chromatography, compared to controls. MPN was determined from a table for 5-replicate, tenfold dilution analysis.

3. Acridine orange epifluorescence direct counts. The method used is identical to that of Wiggins et al. (1987).

III. Results and Discussion

A. Biodegradation Rates

Kinetics: Rates of methanol, phenol, and TBA biodegradation in the soil microcosms (incubated at 20°C) are summarized in Table 1. In general, concentration-time plots for methanol and phenol were concave downward but biodegradation was not clearly first or zero order. This is not surprising considering the heterogeneity of soil bacteria and microenvironments and the net degradation patterns that may be exhibited as a result of varying bacterial numbers and available substrate concentrations (Simpkins and Alexander, 1984). Also, the half-saturation constants, K_s, reported for biodegradation of methanol and phenol in subsurface soils (Novak et al., 1985) indicate that the present systems may have operated under mixed-order kinetics between zero and first order (i.e., substrate concentrations were not substantially different from reported K values). For this reason, degradation rates of methanol and phenol are reported in two forms in

Table 1: as initial disappearance rates measured over an initial, near linear portion of the concentration-time data (a zero order measure), and as first order reaction rate constants, determined by fitting a straight line through a semilog plot of the data. TBA degradation, on the other hand, occurred at a relatively constant rate; hence, only zero order reaction rate constants are reported. Plots of data shown in Figure 2 typify patterns of biodegradation for the three chemicals.

Methanol and phenol were biodegraded relatively rapidly in Blacksburg soils. Assuming first order decay, the half-life of methanol in the Blacksburg subsurface soils ranged from 58 to 263 days and the half-life of phenol ranged from 80 to 395 days ($t_{1/2}$= ln 2/K, where K = weight normalized 10°C first order rate constant). TBA was relatively recalcitrant in Blacksburg subsurface soils, although biodegradation clearly occurred, compared to sterile controls, in all but two soil samples. Assuming zero order decay, the half-life of TBA in subsurface soils ranged from 1.5 to 98 years ($t_{1/2} = 0.5C_o/K$; where the initial concentration, C_o = 100 mg/L and K = weight normalized 10°C zero order rate constant). The latter value pertains to soils 1-1.5 and 1-4.6. Virtually no biodegradation has occurred in those microcosms over two years of incubation. In general, rates determined for methanol, phenol, and TBA in Blacksburg soils fall within ranges reported for other uncontaminated sites (Goldsmith, 1985; White, 1986).

Biodegradation rates were measured using units that were convenient for the experimental system and consistent with common environmental engineering practice, that is, milligrams per liter per day and reciprocal days. These rates were divided by the exact soil weight in each tube for two reasons: to normalize individual microcosms for slight variations in soil weight and allow comparison between tubes, and to simulate a specific utilization rate measure using soil weight as a surrogate for biomass weight. Although static microcosms are commonly used to evaluate chemical degradation rates in soil/groundwater systems, they are not designed to mimic actual subsurface conditions; soil samples are diluted to higher liquid/solid ratios in the microcosms than occur under saturated soil conditions in the field. Consequently, the disappearance rates observed in the test systems may be expected to be slower than the rates expected to occur in the field under saturated conditions, but provide a relative measure of the rates. Caution must be used in applying laboratory rates to subsurface systems because of potential differences between the two environments and because such extrapolation has not been verified.

Acclimation: Acclimation, or adaptation, can be defined as a change in a microbial community effected by exposure to a chemical resulting in faster biotransformation of that substance. Acclimation is observed as an increased rate of biodegradation following a period of exposure during which degradation is slower or virtually nonexistent. This period of slow biodegradation is often referred to as an acclimation period or lag phase. Possible mechanisms for acclimation include: (1) enzyme induction, (2) mutation or genetic transfer, (3) growth of the active population(s), (4) preferential use of other organic substrates before the compound of interest (diauxie), (5) inactivation or degradation of

toxins or inhibitors, and (6) limitation of growth rates by nutrient supply or protozoan grazing.

Biodegradation of methanol and phenol in Blacksburg soils generally did not exhibit an acclimation period. Methanol and phenol were immediately degraded and instantaneous rates tended to decrease slightly rather than increase with increasing time of exposure. These results indicate that no adaptation was required for biodegradation. Similar results were observed for methanol and phenol in other soils (Novak et al., 1985; Smith and Novak, 1987). Soil 1-1.5, and to a lesser extent 1-4.6, were exceptions to the trend in that an acclimation period occurred in the biodegradation of methanol (Figure 3) and phenol. At 10°C, a lag period longer than 100 days occurred. After the acclimation period, biodegradation rates increased substantially but remained slower than rates measured in other Blacksburg soils. Increasing the incubation temperature shortened the duration of the acclimation period required by a given soil (Figure 3).

Biodegradation of TBA in the Blacksburg soils consistently required lengthy acclimation periods. At 10°C, lag periods ranging from 75 to 200 days occurred in soils 1-0.6, 2-0.6, 2-1.5, 2-3.0, 3-0.6, 3-1.5, and 3-4.6, while virtually no TBA disappeared from soils 1-1.5 and 1-4.6 over 600 days of incubation. It may be that no organisms capable of degrading TBA were present in soils 1-1.5 and 1-4.6 since no degradation was evident at any incubation temperature. Increasing incubation temperature tended to shorten the period of acclimation to TBA as it did for methanol and phenol. Similar patterns of lag periods followed by increased degradation rates of TBA were observed in several soils from Virginia, New York, and Pennsylvania, while others exhibited very slow, constant rates of TBA biodegradation, resembling a lengthy acclimation period which may have ended with the onset of faster degradation if the systems were monitored long enough (Goldsmith, 1985; White 1986).

Acclimation to p-nitrophenol has been observed in soil, pond sediment, lake water, and sewage (Aelion et al., 1987; Spain et al., 1984). Those studies suggested that acclimation was due to growth of the p-nitrophenol degrading portion of the microbial community. However, attributing acclimation to continuous growth of a small initial population of specific degraders becomes difficult to justify in situations characterized by an extended lag period followed by an abrupt, substantial increase in biodegradation rate.

This scenario describes a frequently observed pattern of TBA biodegradation in the present study. One possible explanation is that sequential utilization (diauxie growth) governs substrate biodegradation. An apparent diauxic pattern was observed in certain soil microcosms, dosed with both methanol and TBA, in which TBA was not degraded until methanol was depleted from the system (Goldsmith, 1985; Wilson, 1986). Consequently, it seems plausible that in soil/water systems dosed with TBA alone, naturally occurring organic carbon may be preferentially used as an electron donor, inhibiting TBA biodegradation,

and only after certain components of the native organic matter are depleted does the lag period end and TBA biodegradation begin. Considering the subsurface soils averaged approximately 0.1 percent organic carbon by weight, the mass of naturally-occurring organic carbon in soil microcosms exceeded the mass of added TBA-carbon by roughly 25 times. Wiggins et al. (1987) suggested that diauxie did not cause the lag period in p-nitrophenol mineralization; however, while the results of several experiments supported this hypothesis, other results were consistent with a sequential utilization pattern. For example, in some experiments with lake water and sewage, lag periods were shorter in aged than in fresh samples, and adding glucose increased the acclimation time.

Temperature Effects: Incubating soil microcosms at different temperatures had two effects on biodegradation. As incubation temperature increased (1) the duration of lag periods decreased (as previously described), and (2) biodegradation rates increased. Ambient groundwater temperatures in the mid-Atlantic United States are often 10 to 13°C. Since biodegradation rates are directly related to temperature, their assessment may require long periods of time at low temperatures. Biological reaction rates are often modified to reflect temperatures other than the one at which they were evaluated using a modification of the Arrhenius equation:

$$\ln\left(\frac{K_2}{K_1}\right) \frac{E_a(T_2 - T_1)}{RT_1T_2} \qquad [1]$$

where K_1 = reaction rate constant at temperature T_1, E_a = activation energy, and R = ideal gas constant. For many situations the quantity E_a/RT_1T_2 can be considered constant; hence the equation can be rewritten as:

$$K_2 = K_1\Theta^{(T_2 - T_1)} \qquad [2]$$

where the temperature characteristic term, $\Theta = e^{(E_a/RT_1T_2)}$. For a given system, $-E_a/R$ can be determined experimentally as the slope of a plot of ln K versus $1/T$.

Reaction rate constants of methanol and phenol biodegradation in Blacksburg subsurface soils were determined over a range of incubation temperatures. Over a temperature range of 10 to 30°C, the mean value of $\Theta = 1.04$ (SD = 0.02; n = 7) for both methanol and phenol biodegradation (Ghiorse et al., 1983). This temperature correction analysis provides a method for deriving ambient temperature rate constants from values assessed more quickly at higher temperatures. No temperature correction factor was determined for TBA biodegradation because it was unclear how to meaningfully incorporate the effect of temperature on lag periods into the analysis. (For the same reason, data from soils 1-1.5 and 1-4.6 were omitted from the methanol and phenol analyses).

Spatial Variation: Biodegradation rates varied considerably over small distances both horizontally and vertically (Figure 4), highlighting the heterogeneous nature of the microbial activity in soils. The variation in biodegradation rates among subsurface samples was generally less than the variation in rates between

subsurface and surface soils. Compared to subsurface soils, methanol and phenol were degraded more quickly, while TBA was degraded more slowly, in surface soils. This may occur because the soil surface, receiving a more or less continuous supply of various organic materials, develops a microbial community over time which is acclimated to using relatively easily metabolized organics, whereas subsurface microorganisms are more adapted to using the remaining, more refractory organics which are transported to them from overlying soil horizons. Other researchers have reported negative correlations between general microbial activity and depth ($r = 0.88$) (Federle et al., 1986) and between maximum rate of phenol mineralization and depth ($r = 0.58$) (Dobbins et al., 1987) in subsurface soils collected from depths ranging from 0.03 to 1.87 m. This pattern clearly was not followed in Blacksburg subsoils, where, in many cases, biodegradation rates tended to increase with increasing depth. Goldsmith (1985) measured utilization rates for methanol and TBA in soils collected at depths ranging from 3.4 to 31.1 m. Rates generally differed by less than fivefold for a given initial substrate concentration (C_o) over the depth range, and rates were positively correlated with depth.

In general, the site previously exposed to higher organic influx (Site 2) exhibited slightly higher biodegradation rates for all three chemicals.

B. Bacterial Density

Bacterial numbers determined by the various counting techniques appear in Tables 2 and 3. Every soil sample supported a substantial bacterial density. The lowest bacterial numbers, according to all indirect counting procedures, occurred in sample 1-1.5, which also exhibited the lowest biodegradation rates for all three chemicals (Table 1). Aerobic viable counts on soil extract agar (SEA) and yeast extract peptone agar (YEPA) (not shown individually) were very similar. The literature reports significantly higher recovery of soil bacteria on low-nutrient agar media, compared to conventional, nutrient-rich, agar media (Ghiorse and Balkwill, 1985; Olsen and Bakken, 1987). The YEPA used in this study, however, is more comparable to the nutrient-poor media used in the referenced studies than to their "rich" media.

Two modifications of the standard spread-plate technique and a substrate specific MPN test were employed in an attempt to find better indicators of biodegradation potential in soils; methods were designed to resemble conditions of bioassay microcosms or to target the active population. Soils were saturated with water and incubated for different periods of time before plating to produce saturated viable counts. Saturating soils had the effect of increasing viable counts by roughly an order of magnitude (Figure 5). Viable counts became relatively constant after 2 to 5 days of saturation. Adding water to soils enhances nutrient availability and relieves moisture stress, which may lead to growth of active microorganisms and/or germination and growth of inactive forms. Since soils are saturated in the microcosms, this measure was expected to be more representative of viable bacterial numbers in the biodegradation assays. However, the saturation effect on microbial density indicates that biodegradation

rates determined in saturated microcosms may not be representative of rates in unsaturated subsurface environments. It also suggests that unsaturated systems may harbor dormant biodegradative potential which may be activated upon saturation.

The anaerobically incubated viable counts were 1-3 orders of magnitude lower than respective aerobic counts. In fact, numbers were so low that it was necessary to count dilutions in which soil particles partially obscured the agar surface. This complicated counting and attached a degree of uncertainty to these data. Numbers of anaerobic bacteria were expected to be more representative of the microbial population active in the anoxic microcosms.

MPN tests were designed to mimic the bioassay microcosms. Methanol, TBA, or phenol was added to MPN tubes as the sole carbon source in an attempt to quantify the bacterial population responsible for degrading that chemical in the soil. Note that this technique may not detect organisms that contribute to degradation only through co-metabolism or secondary utilization (Bouwer and McCarty, 1984) where a primary substrate is needed to support metabolism. MPNs decreased as resistance of the substrate to biodegradation increased: methanol > phenol > TBA. In some cases (soils 1-1.5, 1-4.6, 3-1.5, and 3-4.6), methanol MPNs were similar to the saturated viable counts, suggesting that a large portion of the viable bacterial community (or at least the culturable part) was capable of degrading methanol.

The total (direct) counts were very similar at the three locations measured. Ambient moisture viable counts were 0.1 to 10 percent of their respective total counts in the same soils. The soil exhibiting slowest degradation of all three chemicals, site 1-1.5, showed the highest cell density of the three soils evaluated by the direct microscopical method.

Profiles of bacterial density (Figure 6) show that bacteria were more numerous in surface than in subsurface soils, but that there was no consistent relationship between numbers and depth in subsurface soils. A similar pattern was observed in Dumfries, Virginia, soils where bacterial numbers decreased from a maximum in the surface layer to a minimum at 3.4 m, then remained relatively constant from 4.3 to 31.1 m in depth (Wilson et al., 1986). Biomass declined with increasing depth ($r = 0.88$) in four Alabama soils, but patterns of decrease varied and the maximum depth studied was only 1 to 2 m (Federle et al., 1986).

C. Rate-Density Relationship

To determine if the microbial measures employed were indicative of biodegradation rates of organic chemicals in soils, biodegradation rates in Table 1 were regressed (least squares linear regression) against bacterial densities in Tables 2 and 3, the underlying assumption being that the relationship between rates and numbers could be approximated by a linear function. Correlation coefficients (r) resulting from the linear regression analysis are shown in Table 4. Correlation coefficients are reported for regressions including subsurface data alone ($n = 9$ or 7) and surface and subsurface data combined ($n=12$ or 10).

The regression analyses showed that a direct (positive) relationship generally existed between biodegradation rates and bacterial densities measured by aerobic plate-counting techniques (AMVC and SVC). Correlation coefficients for these analyses (Table 4) were low when the subsurface soils were considered alone, indicating considerable deviation of the data from linearity, but were fairly high for methanol and phenol, while being negative for TBA, when the surface soils were included in the analyses. Biodegradation rates were negatively correlated with anaerobic plate-count densities of subsurface soils alone; for surface plus subsurface soils, r-values were strongly positive for methanol, lower but still positive for phenol, and negative for TBA. Regressions with MPNs gave ambiguous results with very low coefficients of determination (r^2), indicating that substrate-specific MPNs accounted for almost none of the variability in biodegradation rates.

In general, including surface soil data in the regression analyses had a dramatic effect on correlation coefficients (as well as on linear regression intercepts and slopes), increasing them for methanol and phenol while decreasing them for TBA. It is because of this relatively high influence of the surface soil data on the character of the regressions that the statistics are reported separately — i.e., subsurface alone and surface and subsurface combined. Data that are far removed from other cases typically exert a relatively high influence on regression analyses. Therefore, the reliability of the "combined" regression equations is dependent on the validity of considering the surface and subsurface soil data together. The data available are insufficient to make such a judgment — for example, whether to consider all data in Figure 7 together or as two separate groups.

Differences in the biodegradation potential of subsurface and surface soils are demonstrated by the rate-density data. Such differences are likely due to differential historical exposure to the various soil horizons resulting in the development of microbial communities acclimated to using organic substrates of differing recalcitrance. The net result is that the microbial community of the surface layer, compared to subsurface zones, has a higher percentage of organisms capable of using easily-degradable substrates (e.g., methanol and phenol), while the microbial communities of subsurface soils have a relatively higher percentage of organisms able to use more resistant chemicals (e.g., TBA). For the Blacksburg sites, soils collected from depths as shallow as 0.6 m (2 ft) were representative of subsurface soils with respect to biodegradation rates and bacterial numbers. In fact, except for the anomalously low degradation rates measured for TBA at sites 1-1.5 and 1-4.6 where TBA-degrading microorganisms may have been absent, biodegradation rates of methanol, phenol, and TBA in subsurface soils were generally within a factor of 3 of rates determined for the 0.6 m depth for a particular chemical and site.

The ambient moisture and saturated viable counts were generally the best indicators of biodegradation rates for all three chemicals. At best, coefficients of determination indicate that aerobic viable counts accounted for 87 percent, 92 percent and 37 percent of the variability in biodegradation rates of methanol, phenol, and TBA, respectively. Neither anaerobically incubating spread plates,

saturating soils prior to plating, nor enumerating specific degraders enhanced the correlation with biodegradation rates, in general, over the conventional spread plate counting technique (AMVC). First order reaction rate constants and initial disappearance rates rendered comparable regression results.

Other researchers have failed to find a high correlation between biodegradation rates of organic chemical and microbial measures. A similarly low correlation between maximum phenol mineralization rate and biomass ($r = 0.62$) led Dobbins et al. (1987) to conclude that microbial biomass was a poor predictor and that direct measurement (e.g., microcosm assay) remains the best method for determining biodegradation potential. Methanol and TBA biodegradation rates reported for five soil depths near Dumfries, Virginia, were poorly (sometimes negatively) correlated with bacterial numbers in soils measured by both soil extract plate counts and total direct counts (Goldsmith, 1985).

The reasons for the lack of high correlation between rates and microbial density in subsurface soils are not clear. It may be that microbial measuring techniques currently available are not sensitive or accurate enough to distinguish the differences in biodegradation rates observed in subsurface soils. Regression analysis using nonsubstrate-specific measures (e.g., total biomass, viable numbers, total numbers) actually tests two inherent assumptions: (I) that the microbial measure is indicative of the active biomass, and (II) that active biomass is an index of biodegradation rate — that similar quantities of active biomass in different soils will result in similar degradation rates, even though the species composition and metabolic regime may differ. The potential shortcomings of both of these assumptions are apparent. The fact that substrate-specific MPNs did not enhance the biodegradation rate-microbial density correlation, but rather produced lower r-values than viable counts, implies that the second assumption may be important in limiting the strength of the correlation. Certainly, a profusion of microbial diversity occurs in subsurface soils and groundwater.

EFFECTS OF SITE VARIATIONS ON SUBSURFACE BIODEGRADATION POTENTIAL

I. Introduction

Indigenous soil microorganisms have a considerable capacity for degrading many organic chemicals, and in situ biological restoration of contaminated aquifers is a potentially cost-effective and environmentally acceptable remediation technique. As Wilson et al. (1986) point out, many potentially hazardous situations are naturally remediated by indigenous soil microorganisms. In situ biodegradation may be stimulated by enhancing the degradative capacity of the native microbial community or by adding biomass that has been acclimated to using a particular contaminant. While the potential for biodegrading many organic contaminants is inherent in subsurface systems, the actual biodegradation rates realized in situ are influenced by myriad factors: the nature of the contaminant and its concentration, the presence of other organic substrates, oxidation-reduction potential, dissolved oxygen or alternate electron acceptors, pH, temperature, inorganic nutrients, soil moisture, salinity, toxic or inhibitory compounds, and the composition and condition of the microbial community. The availability of terminal electron acceptors and the resulting oxidation-reduction potential governs catabolic pathways used by microorganisms, and therefore may have a profound effect on biodegradation of organic pollutants in subsurface systems. Where replenishment of dissolved oxygen is restricted, decomposition of organic matter causes the redox potential to decrease.

Oxidation reactions involved in metabolizing organic compounds are coupled with microbially mediated reduction reactions that proceed in a characteristic sequence as redox potential declines. This sequence is paralleled by a succession of metabolic strategies used by the microorganisms responsible for active decomposition in the order: aerobic respiration, denitrification, fermentation, sulfate reduction, and methanogenesis (Stumm and Morgan, 1981). Microorganisms use the energy liberated by the coupled redox reactions to produce ATP. The standard free energy change associated with decomposition of organic carbon decreases along the ecological succession of respiratory mechanisms. Far more energy per electron transferred is yielded via aerobic respiration, denitrification, and nitrate reduction than by fermentation, sulfate reduction, and methanogenesis.

In anoxic systems containing nitrate, sulfate reduction and methanogenesis are strongly inhibited and the more energy-efficient nitrate reduction/denitrification pathway predominates. When oxidation-reduction potential becomes sufficiently low, it is thermodynamically possible for fermentation, sulfate reduction, and methanogenesis to occur simultaneously. While many methanogenic substrates are largely unfermentable, sulfate reducers and methanogens utilize some of the same electron donors. Consequently, in aerobic systems lacking nitrate a competitive situation may develop between coexisting sulfate reducers and methanogens.

Sulfate-reducing bacteria can outcompete methanogenic bacteria for hydrogen and acetate — the major methanogenic substrates present in nature — because they are kinetically more efficient at using those substrates. Inhibition of methane production by sulfate-reducing bacteria has been demonstrated repeatedly in sediment systems. Methane is produced from competitive substrates such as hydrogen in combination with CO_2 or acetate only after sulfate is depleted from the system. Some evidence suggests that certain substrates, including methanol, methylamine, and methionine, are noncompetitive and may be oxidized by the two mechanisms simultaneously (Beeman and Suflita, 1987).

The objectives of this portion of the study were to: (1) evaluate and compare biodegradation rates of several organic chemicals, primarily methanol and tertiary butyl alcohol (TBA), in subsurface soils from several locations, and (2) to compare degradation rates in those soils under ambient anoxic, nitrate-reducing/denitrifying, sulfate-reducing, and methanogenic conditions. The different metabolic conditions were encouraged only by adding a potential electron acceptor or specific pathway inhibitor to the natural soil. The overall goal of this research was to add to the understanding of intersite variation in biodegradation potential, and to assess the importance of different metabolic pathways to biodegradation of organic contaminants in subsurface systems.

II. Methods and Materials

A. Site Description

Soil samples were collected from five locations believed to be relatively free from previous anthropogenic contamination by organic chemicals. Study sites were located near: Wayland, New York; Williamsport, Pennsylvania; Dumfries, Virginia; Blacksburg, Virginia; and Newport News, Virginia.

The Wayland site was on flat terrain within 370 m of a small lake. Soils at the site consisted of dark brown loam interspersed with marl down to a depth of 0.6m, where a 15 cm-thick continuous marl layer was underlain by a mixture of glacial material and silty clay. The water table at the Wayland site was at approximately 1.2 m.

The Williamsport site was located within 90 m of the Susquehanna River on level terrain at the base of a mountain range. The soil profile at the site consisted of loamy silt to a depth of 3.7 m, clean sand from 3.7 m to 4.9 m, dense sand mixed with gravel from 4.9 m to 9.1 m, and coarse sand to 11.6 m, underlain by rock. The water table existed at a depth of 3-4 m at the times of sampling the Williamsport site.

The Dumfries site was sampled to a depth of 31 m. Subsurface material consisted of alternating layers of sand and silty clay, and the water table was at 13 m.

The site at Blacksburg has been described previously in this report.

Soils were collected at two locations at the Newport News site. Site 1 was located within 100 m of a water-supply reservoir dam. The soil surface was at lower elevation than the water level in the reservoir, resulting in a shallow (38 cm deep) water table. Surface soils were dark brown loam interspersed with grey plastic clay (probably fill material from the dam core). The subsoil was greenish brown sand mixed with shell fragments and small-diameter gravel. Site 2 was located on a high area adjacent to the reservoir, where the water table was 4.2 m below the surface. The substratum at this location was dense brown sand.

B. Sampling

Soils were obtained using previously described methods (Goldsmith, 1985; White, 1986). All sample collection and handling procedures were conducted in a manner that minimized contamination from nonnative microorganisms. Most samples were acquired in Shelby tubes which were extruded either onsite through a sterilized paring ring or in the laboratory where all exterior surfaces of the soil core were pared away with sterile spatulas and discarded. The remaining, uncontaminated soil was stored in autoclaved glass jars at 10°C — the ambient subsurface temperature at most sites — until used.

C. Microcosm Setup

Biodegradation of organic chemicals by indigenous soil microorganisms was evaluated in static soil/water microcosms. This research focused on the degradation of methanol and TBA but other alcohols, phenol, and 2,4-dichlorophenol were also assessed. These chemicals are common groundwater contaminants, entering the subsurface environments through accidental spills, leaking storage tanks, and leachate from municipal and hazardous waste landfills. Microcosms were prepared by aseptically transferring soil and an aqueous solution of the desired organic chemical (plus a potential electron acceptor and/or metabolic inhibitor in certain experiments) to glass test tubes or bottles, which were sealed with a Teflon-lined septum cap. Prepared microcosms were vigorously mixed to distribute the added chemical constituents through the soil. Earlier experiments (using Wayland, Williamsport, and Dumfries soils) differ from more recent ones (on Blacksburg and Newport News soils) in that sterilized groundwater collected at the sites was used in preparing the dosing solutions in the former while sterile distilled/deionized water was used in the latter.

Control microcosms were prepared and monitored parallel to live microcosms to distinguish between biological and non-biological chemical disappearance. Moist soils for control microcosms were spread into a thin layer in an enameled metal pan (aggregates were broken up with a pestle) and were repeatedly autoclaved over several days.

Anoxic conditions were produced in microcosms by: (1) filling test tube microcosms completely with liquid to eliminate headspace and allowing aerobic metabolism of an added organic substrate to deplete incidental dissolved oxygen, or (2) purging liquid and headspace of bottle microcosms with helium.

D. Biodegradation Rate Assay

Biodegradation rates were determined by monitoring the disappearance of organic substrates from microcosms over time and computing the slope of the concentration-time data. Test tube microcosms were prepared with approximately equal weights of soil; however, rates were divided by the exact dry weight of soil in each particular tube to facilitate intercomparison.

E. Electron Acceptor and Inhibitor Experiments

The effect of potential electron acceptors on biodegradation rates was determined by adding nitrate, nitrite, or sulfate to a soil microcosm, along with the organic substrate, at a concentration sufficient to sustain oxidation of the added electron donor via anaerobic respiration. To evaluate the importance of the sulfate reduction and methanogenic pathways in the biodegradation of organic chemicals, two specific inhibitors were added to microcosms: sodium molybdate which inhibits the sulfate reduction pathway (Taylor and Oremland, 1979) and 2-bromoethanesulfonic acid (BESA) which blocks methanogenesis (Smith and Mah, 1978). Initial concentrations of chemicals in solutions used to dose microcosms are listed in Table 5. Ammonium was added in certain electron acceptor experiments to provide a readily available nitrogen source and insure that disappearance of nitrate and nitrite could be attributed to the dissimilatory process.

F. Analytical Methods

Groundwater constituents were measured by atomic absorption spectrophotometry (cations) and ion chromatography (anions). Since no groundwater was collected at the Blacksburg or Newport News Site 2, aqueous constituent levels for those soils were characterized by measuring concentrations in the filtrate of a 3.33:1 deionized water/soil mixture which was shaken for one hour. This procedure was designed to approximate constituent levels occurring in the microcosms. Aqueous samples were withdrawn from microcosms through the septum using a syringe. Organic chemical concentrations were determined by flame ionization detector gas chromatography. Alcohols were separated on a 2m x 3mm stainless steel column packed with 0.2 percent carbowax 1500 or 80/100 mesh Caropak C at 80-120°C isothermal. Phenols were measured using a 2m x 2mm glass column filled with 1 percent 5P 1240-DA on 100/120 Supelcoport at 120-140°C isothermal. Anions were separated with a HPIC-AS3 column.

Further information on sampling, microcosms preparation, and analytical procedures can be found elsewhere (Novak et al., 1985; Smith and Novak, 1987).

III. Results and Discussion

Groundwater characteristics for the studied sites are given in Table 6. Groundwater at Wayland and Newport News had relatively high pH. The Williamsport

groundwater was aerobic, while groundwater at other sites was anoxic; Williamsport was also the only nitrate-rich site. Wayland and Williamsport groundwaters contained significant sulfate concentrations. While groundwater analysis revealed low sulfate levels for Newport News, greater than 50 mg SO_4^{-2}/L was commonly measured in amended Newport News Site 1 microcosm water following equilibration with the soil.

A. Bacterial Density

The densities of viable bacteria and total microbial cells in the studied soils are given in Table 7. Substantial viable numbers of bacteria existed at each site and depth evaluated, including to a depth of 31 m at Dumfries, Virginia. Predictably, viable counts were 2-3 orders of magnitude lower than direct counts, because viable counting techniques only enumerate organisms capable of growing under a single combination of medium and incubation conditions whereas the direct microscopical procedure presumably counts all viable and nonviable cells. Direct counts were remarkably similar between sites and depths, as has been observed elsewhere (Beeman and Suflita, 1987; Trolldenier, 1973; Webster et al., 1985). While biodegradation rates were directly related to viable bacterial density in soils from within the Blacksburg location, it appears that such a relationship does not exist between sites from different locations. The intrinsic variation in microbial communities precludes such a correlation based on gross viable numbers of microorganisms.

B. Comparison of Biodegradation Rates

Subsurface biodegradation rates for methanol and TBA, averaged over many microcosms per site, are shown in Figure 8. The inherent difference in recalcitrance between the two chemicals is evident. Biodegradation rates for a given chemical vary considerably between sites. However, the sites can be grouped into two relative categories:Dumfries, Wayland, and Blacksburg sites 1 and 3 exhibited relatively low biodegradation rates for both methanol and TBA, whereas Blacksburg Site 2, Newport News Site 1, and Williamsport soils biodegraded methanol and TBA at relatively high rates. Newport News Site 2 degraded methanol at a relatively high rate but degraded TBA relatively slowly. Similar trends have been observed for other chemicals which were evaluated at selected sites. Phenol and 2-chlorophenol were degraded much faster in Williamsport soil than in Dumfries (saturated) soil (Smith and Novak, 1987). Phenol biodegradation in Blacksburg soils followed the order: Site 2 > Site 3 > Site 1. Ethanol, propanol, 1-butanol, 1-pentanol, and 2,4-dichlorophenol were all degraded much more rapidly in Newport News Site 1 soil than in Blacksburg Site 1 soil (Morris, 1988).

The wide variation in biodegradation rates between sites could not be predicted from the presence or absence of potential electron acceptors in the groundwater, pH, soil type, moisture content, or viable or total (direct) microbial counts.

C. Effect of Electron Acceptor on Biodegradation Rates

These experiments were designed to evaluate the effect of adding potential electron acceptors on biodegradation rates of organic chemicals, compared to nonamended rates, and to infer which metabolic pathways may be important in biodegrading organics in natural soils. Data are presented in the following sections to demonstrate trends (i.e., rate enhancement or inhibition) for a given site; after presenting the data, intersite differences in trends are discussed.

Dumfries, VA Site: Adding nitrate to Dumfries soil initially enhanced, and then inhibited, methanol biodegradation (Figure 9). The data in Figure 9 shows that nitrite accumulated in the microcosm and then remained relatively constant in concentration. Cessation of methanol utilization in this microcosm roughly corresponded to the leveling off of the nitrite concentration. This effect was reproduced in several microcosms. Increasing the pH of microcosm water to $\geq$ 6.0 by adding hydroxide or bicarbonate caused methanol degradation rates to exceed rates in microcosms with similar pH adjustment but no nitrate addition (Wilson, 1986). When methanol disappeared completely, no nitrate or nitrite could be measured in nitrate/high pH microcosms.

These data indicate that nitrate-reducers initially degraded methanol resulting in enhanced biodegradation rates, but that the concomitant production of nitrite inhibited further utilization. The low natural pH of these systems coupled with nitrite may have created a toxic situation. Nitrite toxicity has been found to be inversely related to pH and artificially raising pH allowed denitrification metabolism to proceed in soil microcosms.

Adding nitrate, likewise, did not stimulate TBA biodegradation in Dumfries soils (Wilson, 1986). No inhibition was apparent after 200 days of incubation. It is possible that TBA utilization is so slow that if it is metabolized by the nitrate-reduction pathway denitrifying organisms are able to prevent nitrite accumulation by converting it to gaseous products as fast as it is produced.

When sulfate was added to Dumfries soil microcosms, methanol disappeared slightly more slowly than in nonamended assays (Figure 10). Again, increasing pH in sulfate-dosed microcosms enhanced rates compared to similar no-sulfate microcosms (Wilson, 1986). Mitigation of hydrogen sulfide toxicity was hypothesized as an explanation.

Sulfate addition had no apparent effect on TBA utilization, but the small amount of TBA utilized during the 200-day experiment (2 mg/L) probably was insufficient to deplete sulfate present in the groundwater added to nonamended microcosms, so no effect would be expected.

Wayland, NY Site: Neither adding nitrate nor sulfate appeared to affect biodegradation of methanol or TBA in Wayland soil microcosms (Figure 11).

Groundwater at the Wayland site (which was added to the microcosms) was nitrate-poor and sulfate-rich (Table 6). The observation that nitrate addition had

no effect on methanol or TBA degradation rates indicates that nitrate-respiring bacteria may not be important in removing those compounds in Wayland soil. If any nitrate were converted to nitrite during the experiment (e.g., serving as an electron acceptor for anaerobic respiration of methanol, TBA, or organics occurring naturally in the soil), the higher pH of the system would likely preclude the apparent nitrite inhibition observed in Dumfries soil. It is not too surprising that adding sulfate did not affect methanol or TBA utilization rates. The sulfate present in non amended microcosms (along with sulfate expected to be released from the soil as aqueous-phase sulfate is used) likely would be sufficient to obscure any effect of the added quantity of sulfate, if sulfate-reducing bacteria were indeed active in degrading the added organic substrates.

Williamsport, Pennsylvania Site: Methanol biodegradation rate in Williamsport soil was not enhanced by adding nitrate or sulfate initially, but was increased upon redosing with methanol (Figure 12). Likewise, TBA was biodegraded faster when either nitrate or sulfate was added to microcosms (Figure 13). Both methanol and TBA disappearance was negligible from microcosms amended with nitrite.

The nitrate and sulfate naturally present in the Williamsport subsurface (Table 6) may account for the initial absence of biodegradation rate enhancement from adding those electron acceptors, and the subsequent rate increases observed upon redosing, as the endemic quantities were depleted. While nitrite did not accumulate to inhibitory levels in nitrate-amended microcosms, the high nitrite concentrations in the nitrite-amended systems proved entirely inhibitory to the biodegradation of methanol and TBA in the low pH Williamsport soil. The Williamsport data imply that nitrate reduction/denitrification and sulfate reduction are both potentially active biodegradative pathways. The presence of nitrate and sulfate in Williamsport groundwater reflects the preclusion of these anaerobic pathways by dissolved oxygen.

Blacksburg, Virginia Site 1: Adding nitrate to Blacksburg Site 1 microcosm slowed methanol degradation substantially, while nitrite addition precluded methanol removal (Figure 14). Biodegradation of methanol was slightly slower in sulfate-amended than in non amended microcosms (Figure 14).

The Blacksburg subsurface contained very little naturally occurring nitrate and sulfate and had a pH of 4.2 (Table 5). The low pH creates a potential for nitrite toxicity, which apparently occurred in the nitrite-amended microcosms. While nitrite did not accumulate to high levels in the nitrate-amended microcosms —1.8 mg/L being the highest concentration measured — inhibition of methanol biodegradation was apparent.

Blacksburg, Virginia Site 2: Nitrate and sulfate both increased methanol biodegradation rates when added to Blacksburg Site 2 soil, while nitrite slowed methanol biodegradation (Figure 15).

Inhibition of methanol degradation as observed in the nitrite-amended microcosms was expected considering the low pH of the site. In nitrate-amended systems, nitrite formed during methanol biodegradation accumulated to fairly high levels (12.6 mg/L) apparently without inhibiting the reaction. In Blacksburg Site 2 soil, as in Williamsport soil, both nitrate reduction and sulfate reduction appear to be potentially active pathways which can be stimulated.

Newport News, Virginia Site 1: In Newport News Site 1 soil, methanol biodegradation rates followed the order: nitrate-amended > nitrite-amended > sulfate-amended > non amended (Figure 16). Both sulfate and nitrate increased the rate of TBA degradation compared to the non amended rate, while nitrite slowed TBA removal (Figure 17).

Nitrate affected biodegradation of methanol and TBA differently in Newport News Site 1 soil. Addition of nitrate enhanced biodegradation rates of both organics even though considerable concentrations of nitrite appeared in the microcosms during the experiments: as much as 18.9 mg/L and 9.3 mg/L in methanol and TBA bioassays respectively.However, in nitrite-amended microcosms, methanol degradation was faster, while TBA degradation was slower, than in nonamended systems. The absence of nitrite inhibition can possibly be explained by the mitigating effect of the relatively high pH of the systems, but this does not explain the lower rates for TBA in nitrite-amended microcosms.

It is also difficult to explain, without further study, why sulfate addition did not change the rate of methanol degradation while it dramatically increased TBA removal. Nevertheless, the general pattern of biodegradation enhancement achieved by adding nitrate or sulfate was similar to that observed for Williamsport and Blacksburg Site 2 soils.

Newport News, Virginia Site 2: Methanol and TBA biodegradation rates in Newport News Site 2 soil followed the pattern: non amended > nitrate-amended > sulfate-amended > nitrite-amended (Figure 18). Adding nitrate slowed biodegradation slightly, while sulfate retarded removal considerably and no degradation was apparent in nitrite-amended systems.

The responses observed at Newport News Site 2 resemble those for Dumfries and Blacksburg Site 1 — very different from Newport News Site 1 results.

D. Effect of Inhibitors on Biodegradation Rates

The potential importance of sulfate reduction and methanogenesis in biodegrading organic chemicals in two soils was investigated using specific inhibitors known to block those metabolic pathways: molybdate and BESA, respectively.

Adding molybdate to Blacksburg Site 1 soil increased biodegradation rates, especially the TBA rate which increased 1,390 percent (Figure 19), while adding BESA generally decreased biodegradation rates. This trend was observed for phenol, 2,4-dichlorophenol, and 1-5 carbon n-alcohols (Morris, 1988). A signifi-

cantly higher half-saturation coefficient (K_s) was determined for TBA biodegradation in the unamended Blacksburg system (9600 mg/L) than in the Blacksburg system amended with molybdate (365 mg/L), indicating the higher affinity, and concomitant faster biodegration, for TBA produced by adding molybdate (Morris, 1988). If the observed changes in biodegradation rates can indeed be attributed to altering electron flow through the system, the results of inhibitor experiments imply that in Blacksburg Site 1 soil the organic compounds studied were biodegraded faster via methanogenesis than via sulfate reduction. Faster removal of certain halogenated aliphatic compounds (tetrachloroethylene, chloroform, 1,1,1-trichloromethane, and ethylene dibromide) has been reported for continuous-flow, fixed-film columns operating under methanogenic than under sulfate-reducing conditions. Although the former required longer acclimation periods to develop an active methanogenic culture while no difference in removal rate was reported for other compounds (carbon tetrachloride, di-bromochloropropane, bromodichloromethane, and bromoform) (Bouwer et al., 1986). In studies involving aquifer material, several phenoxy acetates, chlorinated benzoates, and chlorinated phenols were biodegraded in microcosms containing methanogenic soil but not in microcosms containing sulfate-reducing soil from the same aquifer nor in the methanogenic material to which sulfate had been added (Wilson 1986; Suflita and Miller, 1985).

In contrast to Blacksburg Site 1 soil, adding molybdate or BESA to Newport News Site 1 soil generally did not affect biodegradation rates (Figure 20). Molybdate did not significantly change the degradation rate of TBA, phenol, 2,4-dichlorophenol, or the 1 through 5 carbon n-alcohols (Morris, 1988). BESA slowed methanol biodegradation slightly (48 percent) but did not alter the rate of degradation of other alcohols or phenol. In unamended Newport News Site 1 systems the appearance of a black precipitate presumed to be a metal sulfide such as FeS and an intermediate gas chromatographic peak presumed to be methane (based on identical elution times) during experiments indicated that both sulfate reduction and methanogenesis were potentially active biodegradative pathways in that soil. No black precipitate was formed in molybdate-amended microcosms and no methane peak appeared in BESA-amended microcosms. These observations imply that the inhibitors exerted the desired effects. Nevertheless, biodegradation rates did not vary when either the sulfate reduction or methane production pathway was inhibited.

IV. Discussion and Implications

The results of this study are summarized in Table 8. The data from numerous microcosm experiments using subsurface material from eight sites and five geographical locations suggest that the soils may be categorized into two general types. One type is characterized by a relatively high influx of water, carbon, and inorganic nutrients and relatively fast biodegradation of organic compounds; the second type is typified by relatively low nutrient inflow and biodegradation rates. Table 9 lists characteristics of the two types of sites observed in the rate evaluation, electron acceptor, and inhibitor experiments of this study.

In "fast" soils, a variety of metabolic pathways are potentially available and may be operative in biodegrading organic compounds. Inhibiting either sulfate reduction or methanogenesis causes a different pathway to be functional without any decrease in degradation rate. Biodegradation rates can be enhanced by adding nitrate or sulfate for use as electron acceptors for anaerobic respiration (apparently, the availability of electron acceptors limits biodegradation in nonamended systems). Adding nitrate in high concentrations is inhibitory but nitrate accumulating from nitrate reduction does not preclude the stimultory effect of nitrate addition on biodegradation rates.

Adding potential electron acceptors to "slow" soils does not enhance biodegradation and often decreases biodegradation rates. Nitrate addition decreases degradation rates except in soil with relatively high pH due, at least in some cases, to accumulation of inhibitory nitrite levels. Nitrite added in high concentration is always inhibitory to biodegradation. The negative effect of nitrate on biodegradation rates in certain soils has important practical implications with respect to proposals to: (1) add nitrate to aquifers to enhance biodegradation of organic contaminants and (2) add organic substrates such as methanol to groundwater to stimulate denitrification in drinking water supplies.

Adding molybdate produces the only stimulatory effect observed in "slow" soils. This effect is presumptively attributed to blockage of the sulfate-reducing mechanism. Either adding sulfate to the systems or inhibiting methanogenesis with BESA slows degradation rates. Because of the competitive nature of the sulfate-reduction and methane-production reactions, it may be reasonable to hypothesize, based on the results, that methanogenesis is a potentially important biodegradative pathway in "slow" soils.

Aside from molybdate-amended systems, the fastest "slow" soil degradation rates occur in nonamended microcosms. It is possible that the availability of electron acceptors is not limiting, although addition of nitrate to Dumfries soil did seem to enhance biodegradation prior to the onset of nitrite inhibition. This outcome does not rule out methanogenesis as an important pathway since its electron acceptor, carbon dioxide, is not likely to be limiting in the soil/water microcosms. However, methanogenesis was not confirmed by detecting methane in the microcosms. Consequently, another metabolic pathway may contribute to the biodegradation of organic chemicals in "slow" systems (e.g., fermentation or anaerobic respiration using the ferric iron, functional groups on humates, etc., as a terminal electron acceptor).

SUMMARY AND CONCLUSIONS

The major goal of this study was to relate the characteristics of subsurface environments to the potential for biodegradation of organic contaminants. The initial set of experiments, detailed in the section beginning on page 11, focussed on the relationship between microorganism counts and biodegradation rates. For easily degradable compounds, a correlation between bacterial numbers and rates existed for soils from the same general location. For more refractory organics, no such correlation could be made. This is probably because most enumeration techniques do not provide an indication of substrate-specific organism populations, but rather, measure gross organism numbers or those organisms that grow on a specific medium. For refractory substrates, organisms grown on these may be so low in numbers or reproduce so slowly that enumeration becomes much less useful than direct degradation studies.

Therefore, based on the data discussed in this section, it is unlikely that organism counts can be used to predict biodegradation rates for organic groundwater contaminants. The major value of organism counts is to indicate that viable organisms are present, thereby offering the possibility that biodegradation is a possible mechanism for the reduction of subsurface contaminants.

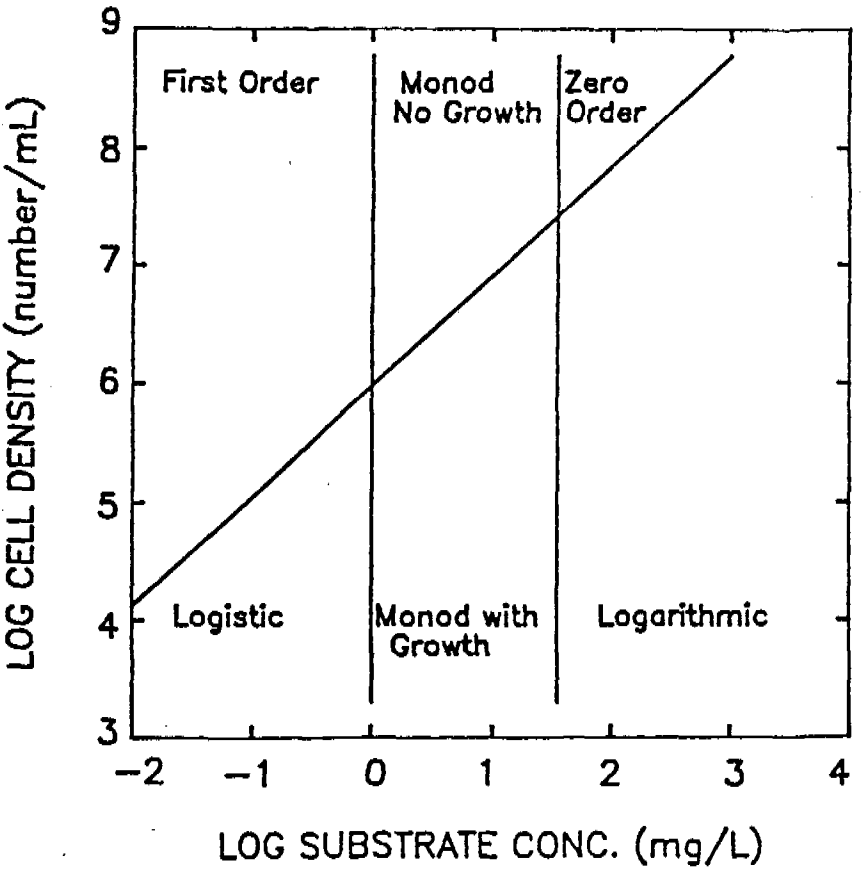
With regard to site variations, it appears that subsurface environments which undergo change due to a high water flow will develop a diverse and responsive microbial population. At such sites, characterized in the section beginning on page 23 as "fast" sites, degradation of a variety of organics was found to be rapid. Moreover, addition of nitrate resulted in stimulation of degradation, probably because a substantial active denitrifying population of organisms was present. In addition to denitrification it appears that a number of metabolic pathways are potentially available for biodegrading organics in these soils.

At "slow" sites, characterized by a limited transport of water through the site, it appeared that sulfate-reducing and methanogenic organisms dominated. Addition of molybdate often stimulated growth, which is indicative of an environment where sulfate-reducing organisms outcompete other organisms for hydrogen. Addition of nitrate to these sites either had no effect or inhibited degradation due to the buildup of nitrite.

Of particular significance in the comparison between fast and slow sites was that no differences in organism numbers were evident and there was no water chemistry parameter that could be measured that would "key" the identification of each type of site. Where a normally slow site was exposed to both the additional flow of water and enrichment as occurred in one of the Blacksburg sites as a result of runoff from a feedlot, the site took on many of the characteristics of a fast site.

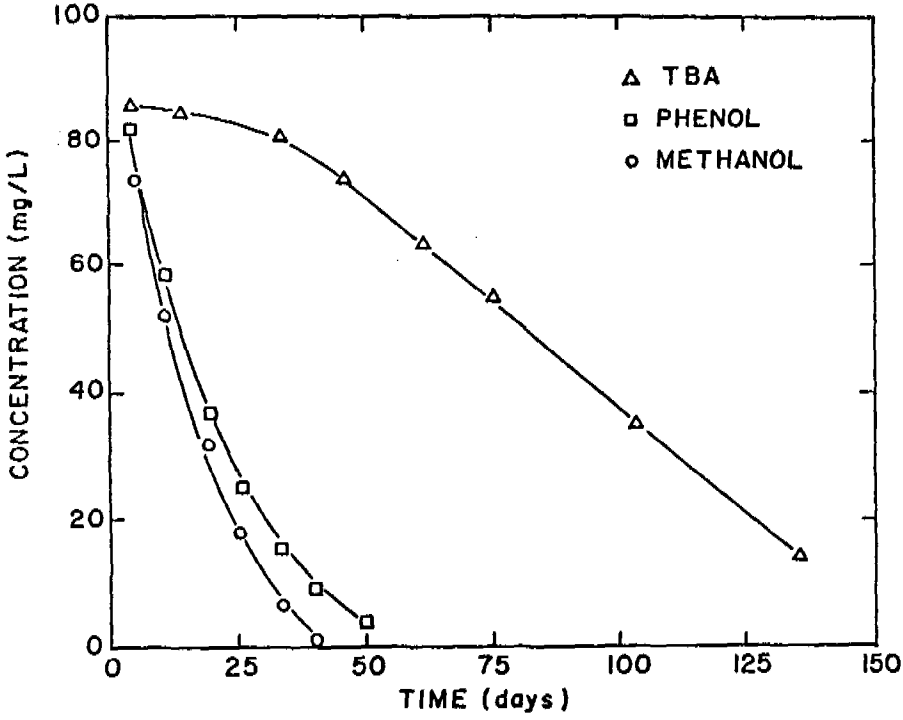
FIGURES

FIGURE 1
Variation in Kinetic Response as a Function of Initial Substrate Concentration and Initial Cell Concentration[1]



[1] After Simkins and Alexander 1984

FIGURE 2
Typical Biodegradation Patterns in Substrate Soils[1]



[1] Soil2-3.0, 20°=C

FIGURE 3
Biodegradation of Methanol in Soil 1-1.5 Showing Acclimation

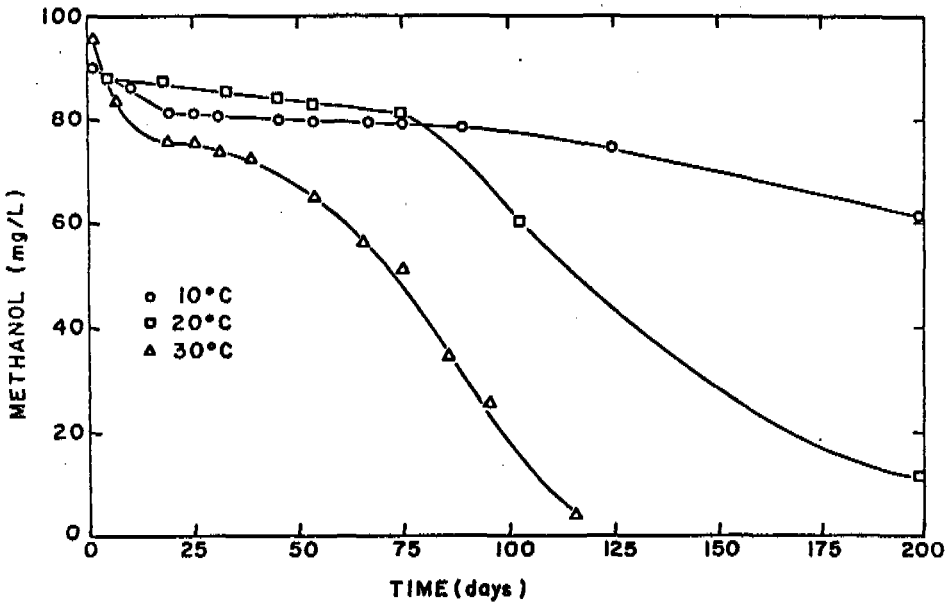


FIGURE 4
Depth Profiles of Biodegradation Rates

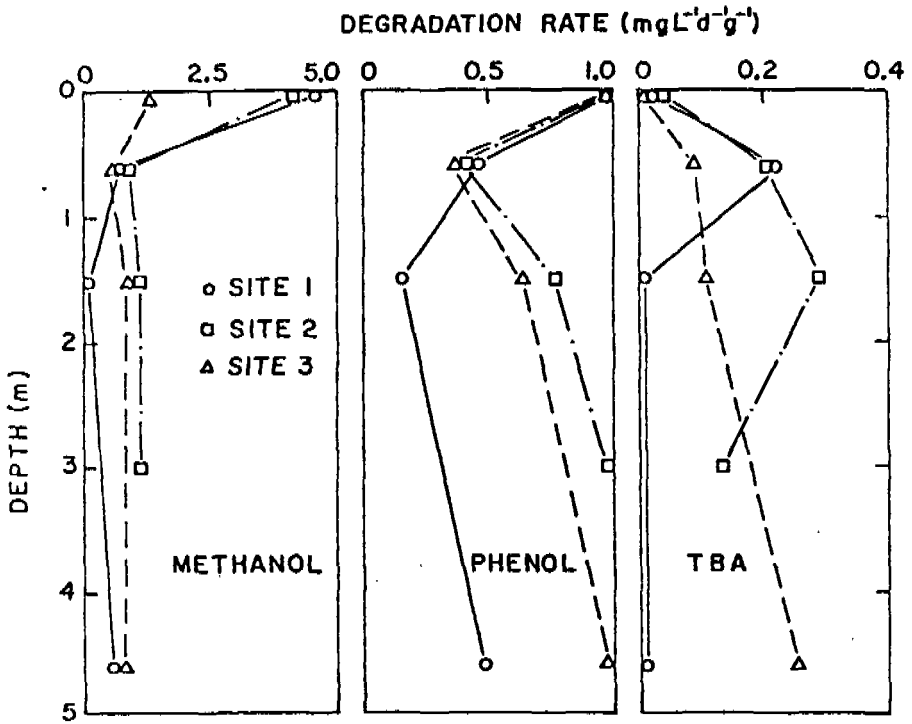


FIGURE 5
Effect of Saturation on Viable Counts

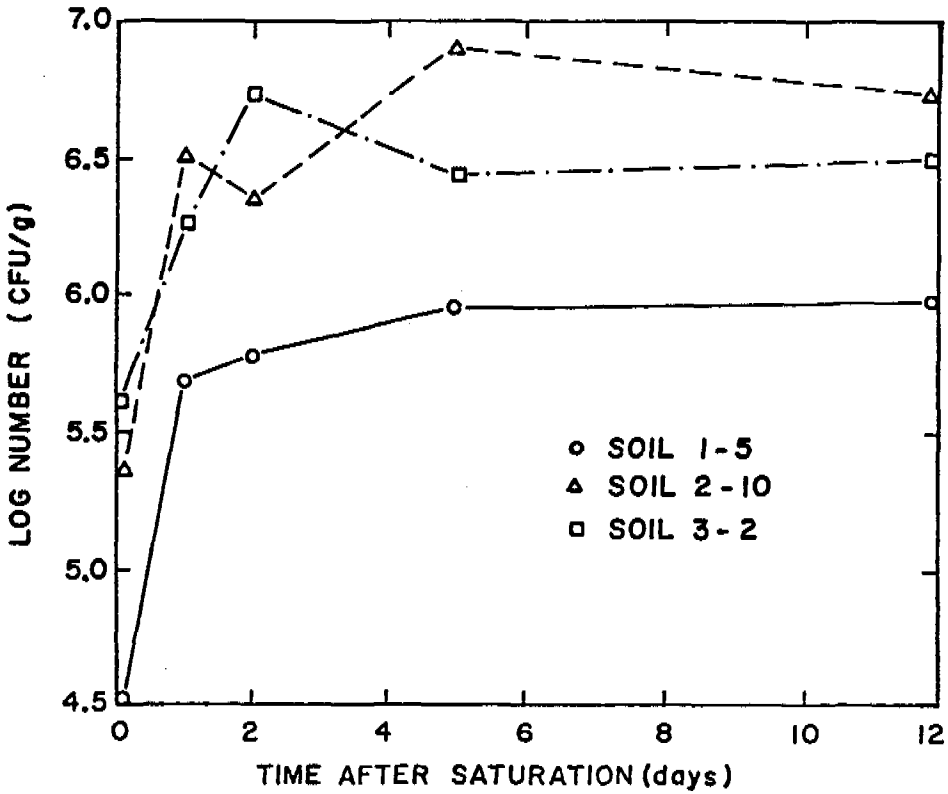


FIGURE 6
Depth Profiles of Ambient Moisture Viable Counts

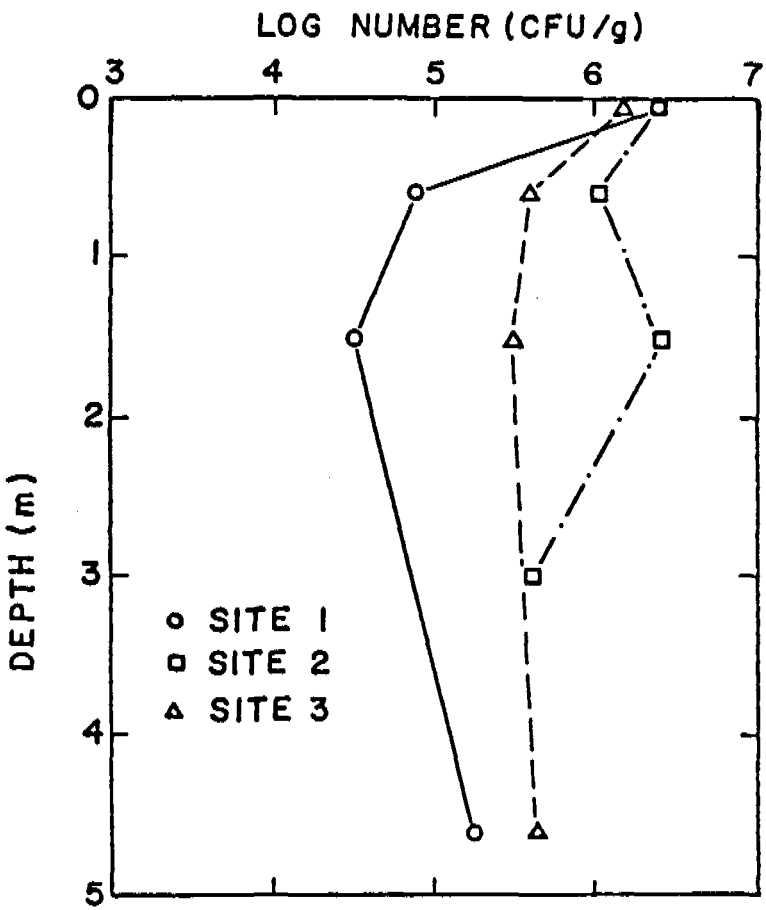


FIGURE 7
First Order Reaction Rate Constant for Methanol Biodegradation Versus Ambient Moisture Viable Count, Showing Regression Lines Through Surface and Subsurface Soil Data Individually and Combined

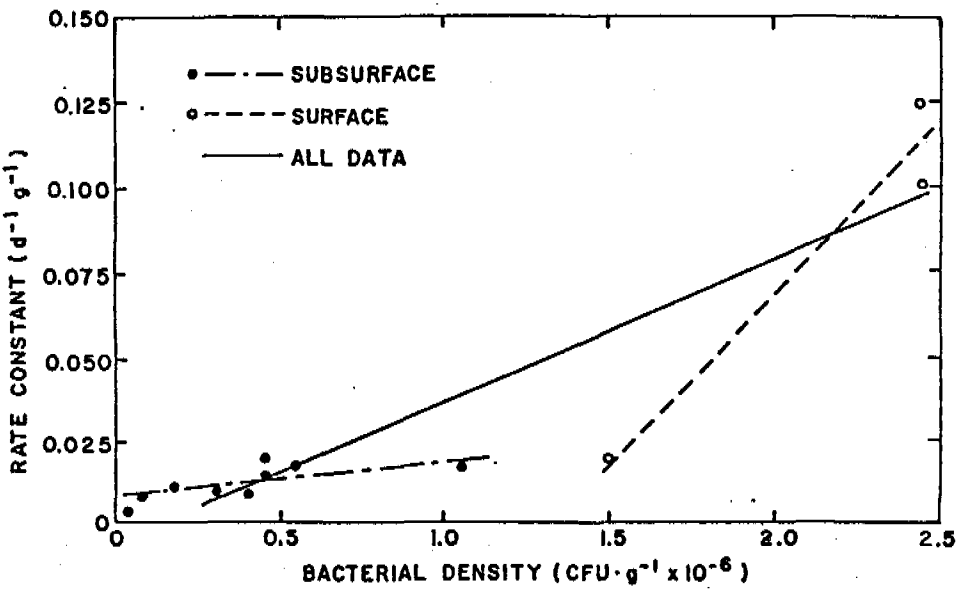


FIGURE 8
Intersite Comparison of Biodegradation Rates

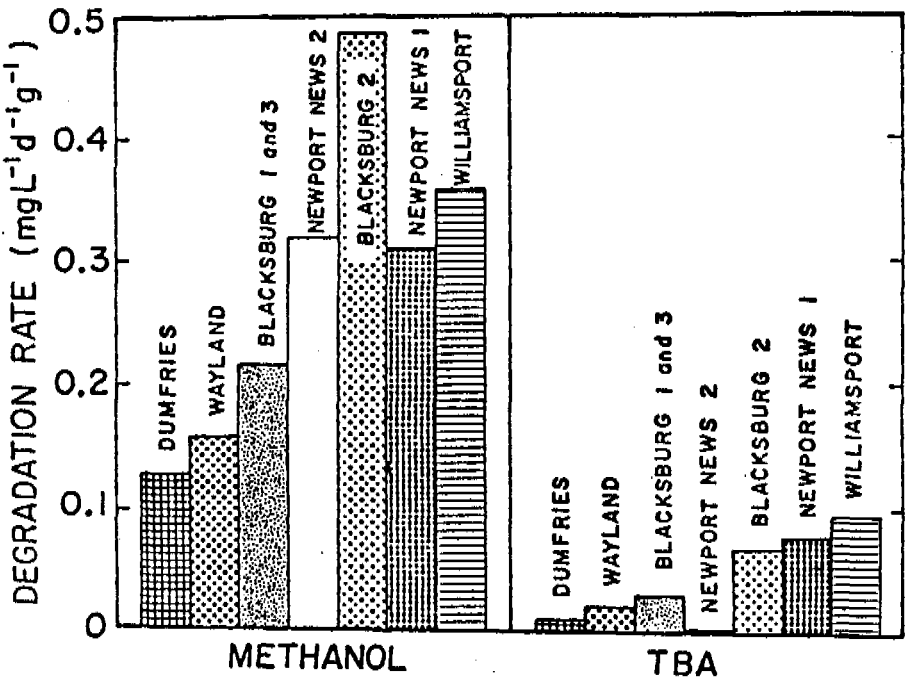


FIGURE 9
Effect of Nitrate on Methanol Degradation in Dumfries Soil, Showing Nitrite Accumulation

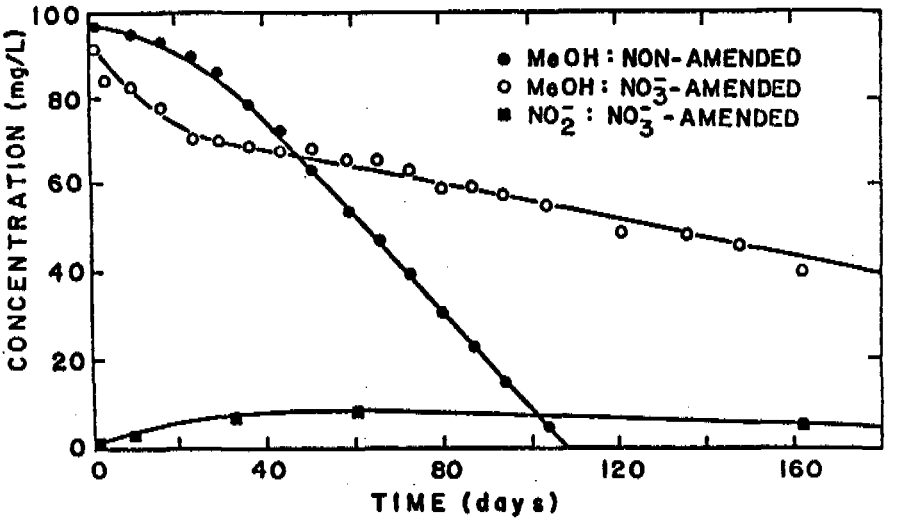


FIGURE 10
Effect of Sulfate on Methanol Degradation in Dumfries Soil

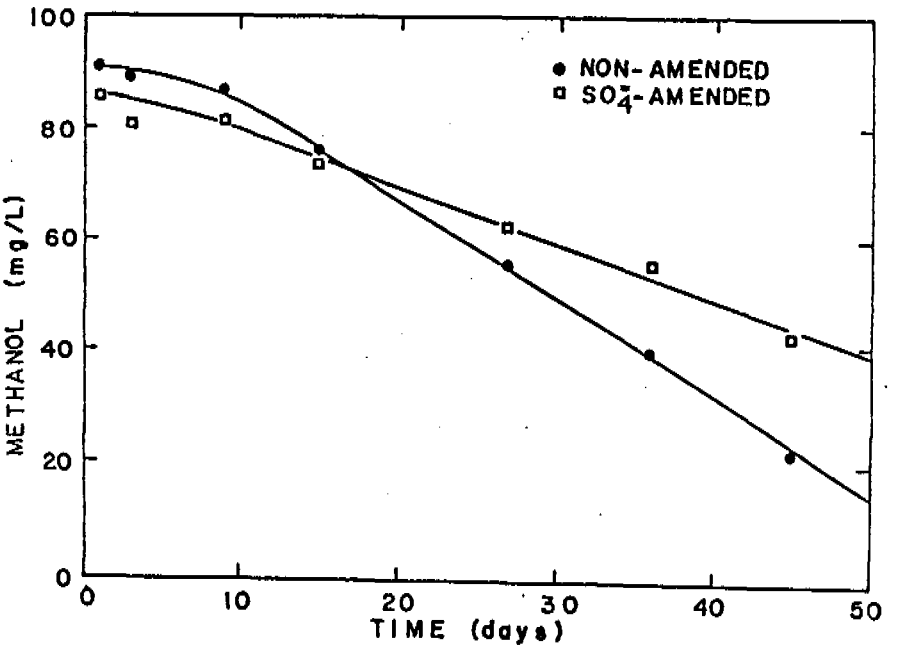


FIGURE 11
Effect of Nitrate and Sulfate on Methanol Degradation in Williamsport Soil, Showing Re-dose

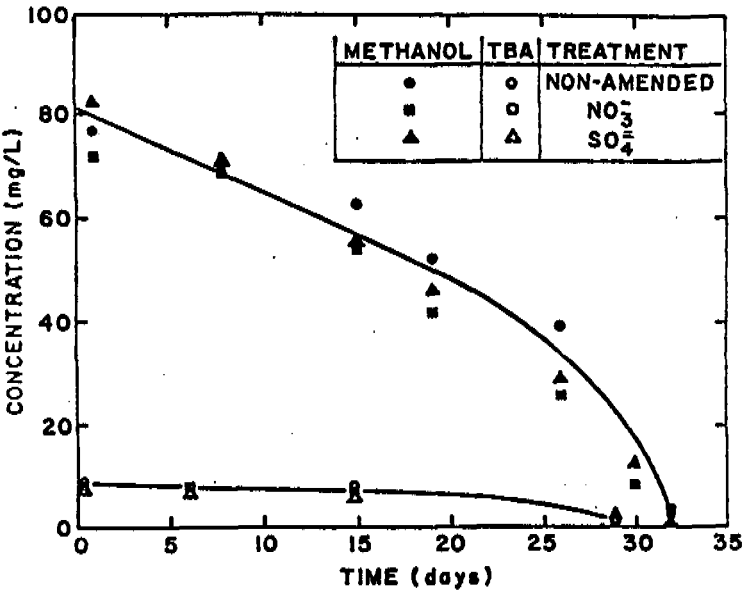


FIGURE 12
Effect of Nitrate and Sulfate on Methanol Degradation in Williamsport Soil, Showing Re-dose

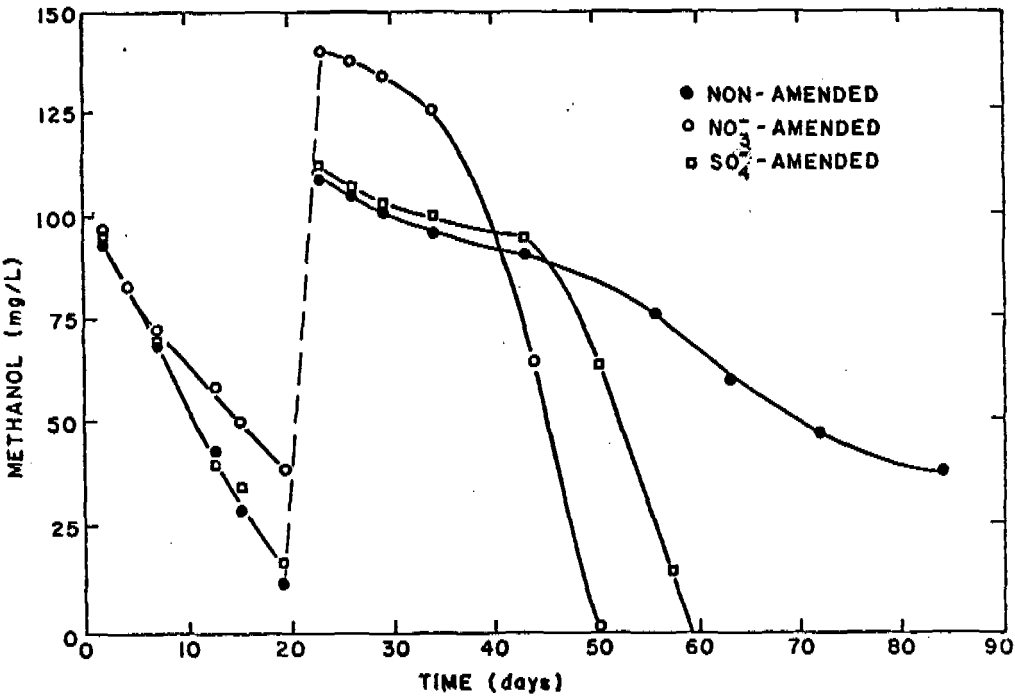


FIGURE 13
Effect of Nitrate and Sulfate on TBA Degradation in Williamsport Soil

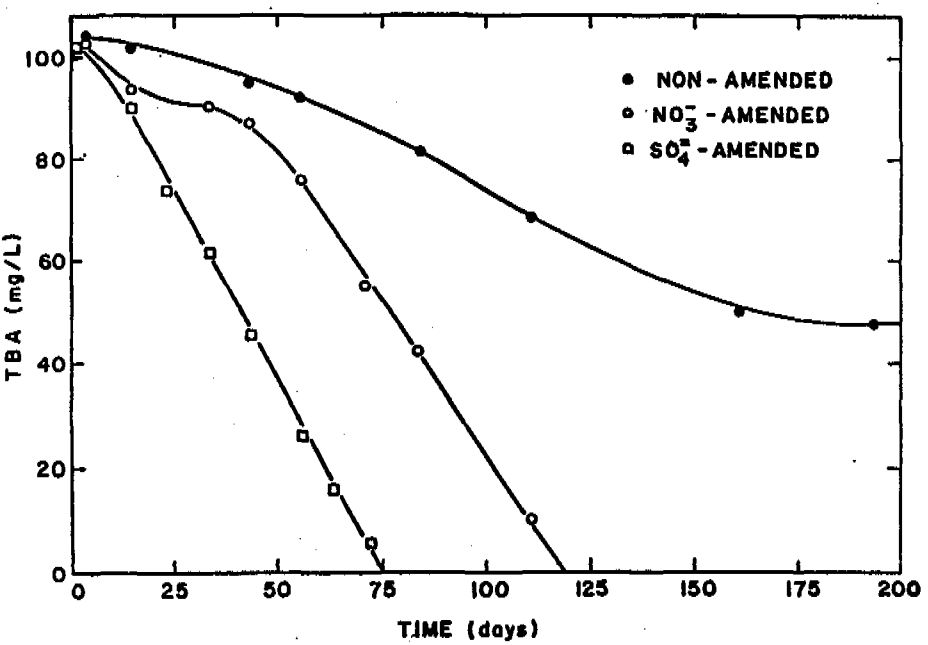


FIGURE 14
Effect of Nitrate, Sulfate, and Nitrite on Methanol Degradation in Blacksburg Site 1 Soil

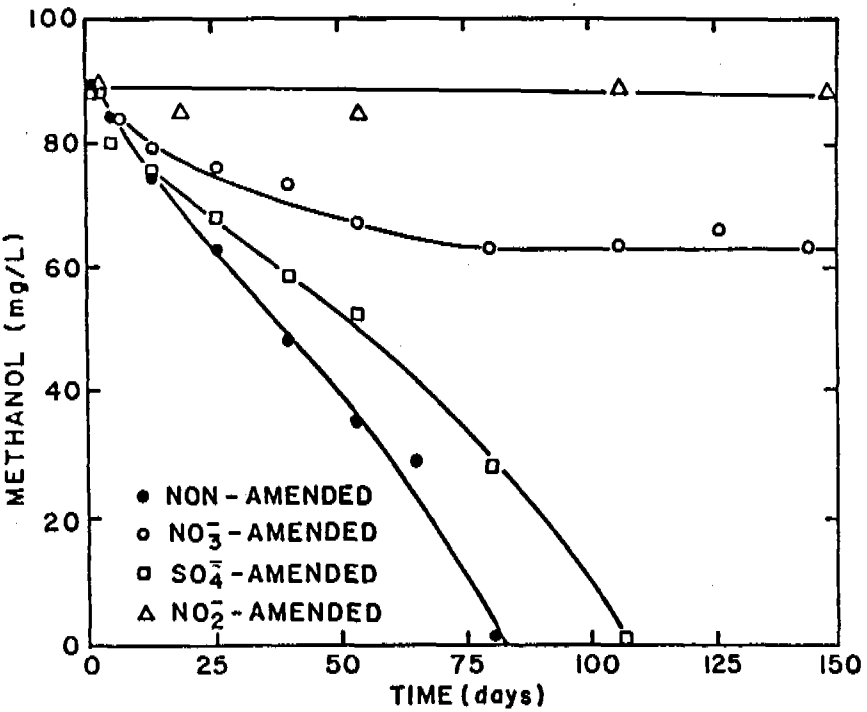


FIGURE 15
Effect of Nitrate, Sulfate, and Nitrite on Methanol Degradation in Blacksburg Site 2 Soil

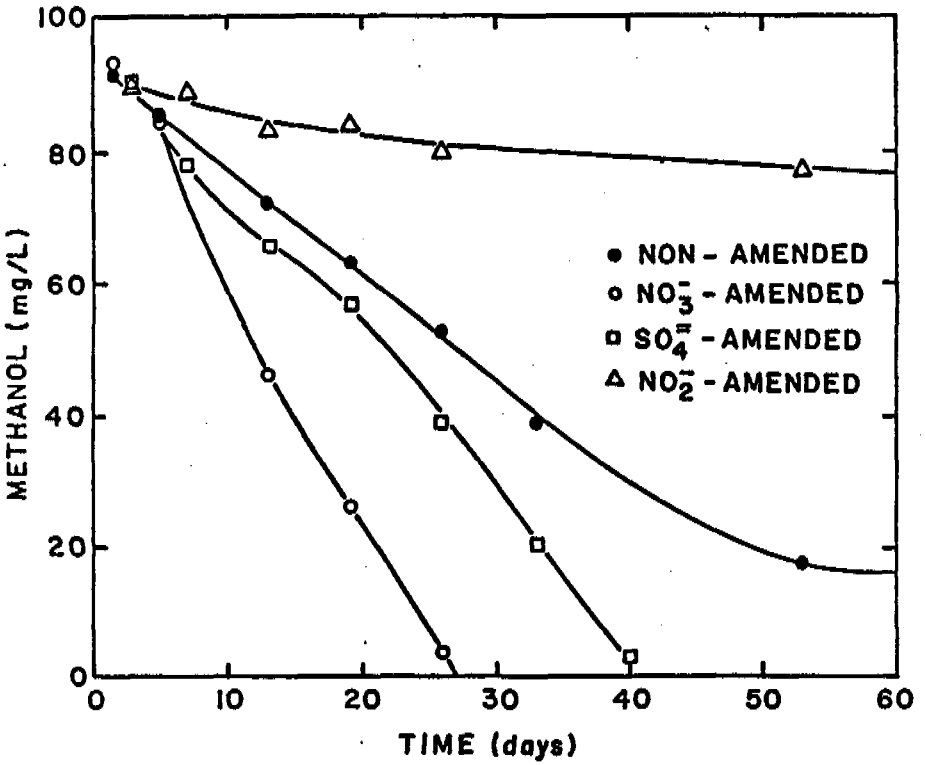


FIGURE 16
Effect of Nitrate, Sulfate, and Nitrite on Methanol Degradation in Newport News Site 1 Soil

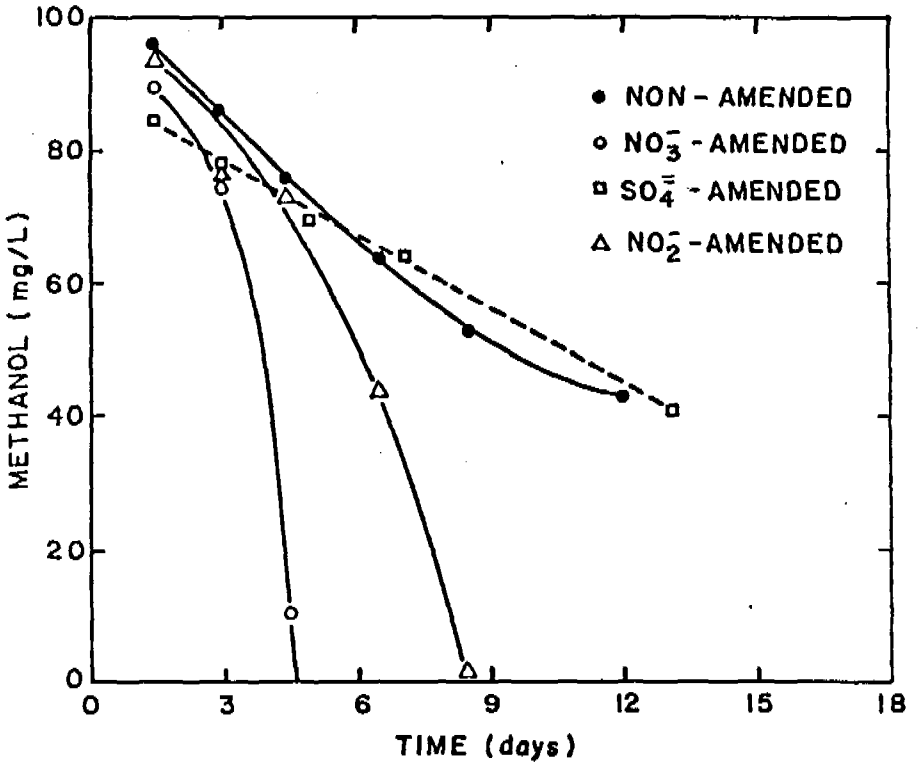


FIGURE 17
Effect of Nitrate, Sulfate, and Nitrite on TBA Degradation
in Newport News Site 1 Soil

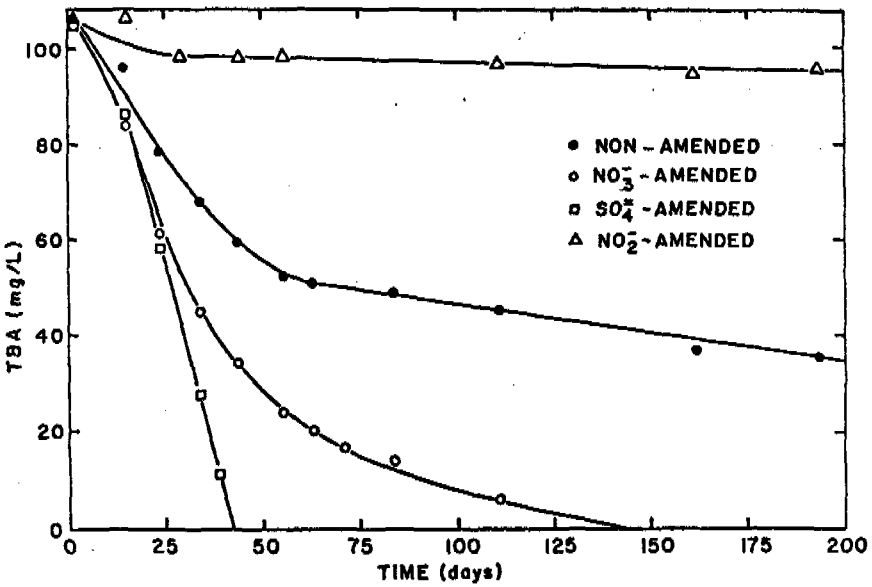


FIGURE 18
Effect of Nitrate and Sulfate on TBA Degradation in Newport News Site 2 Soil

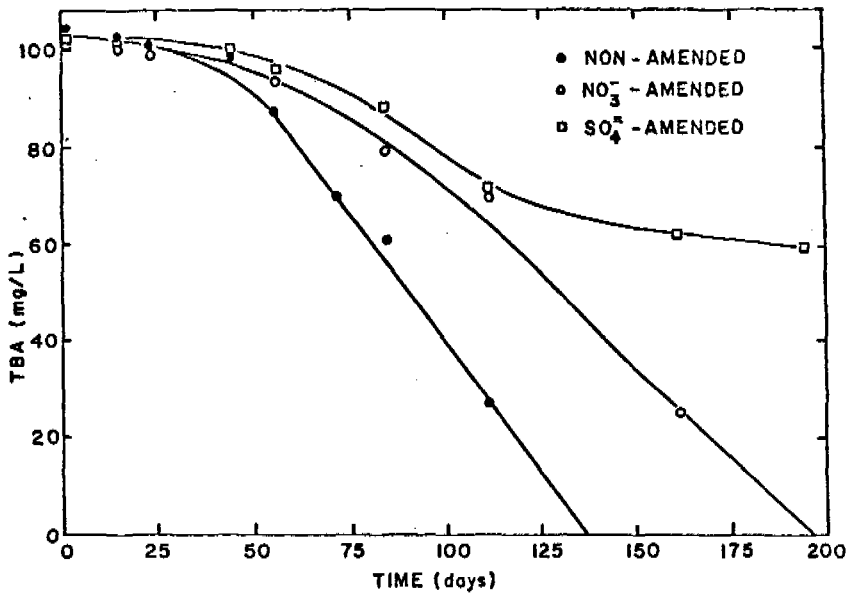


FIGURE 19
Effect of Molybdate on TBA Degradation
in Blacksburg Site 1 Soil

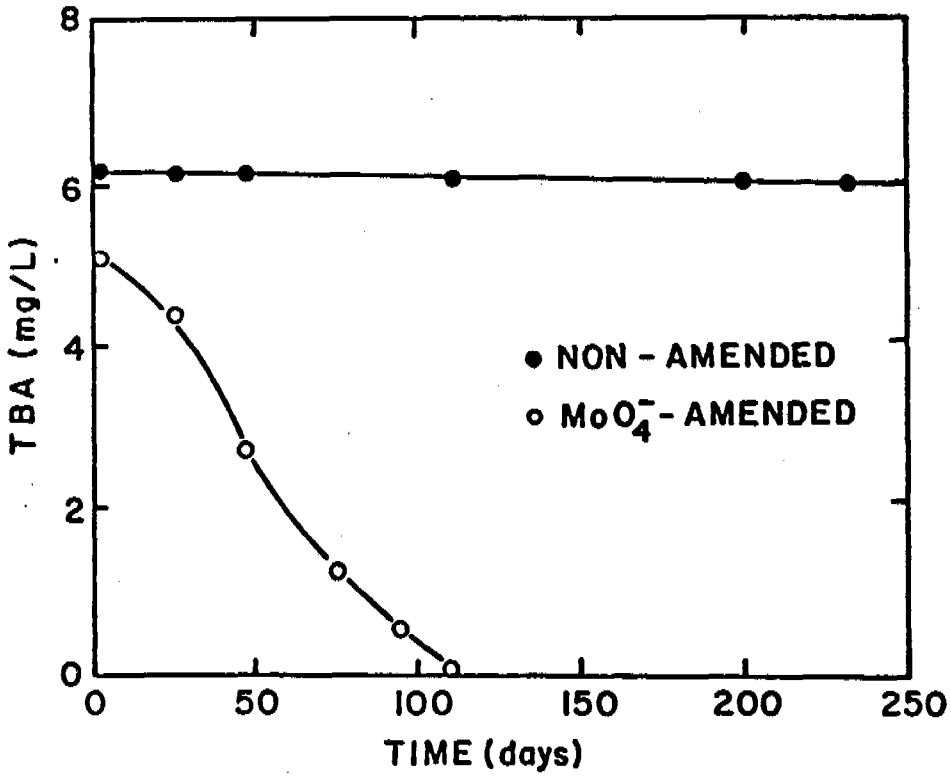
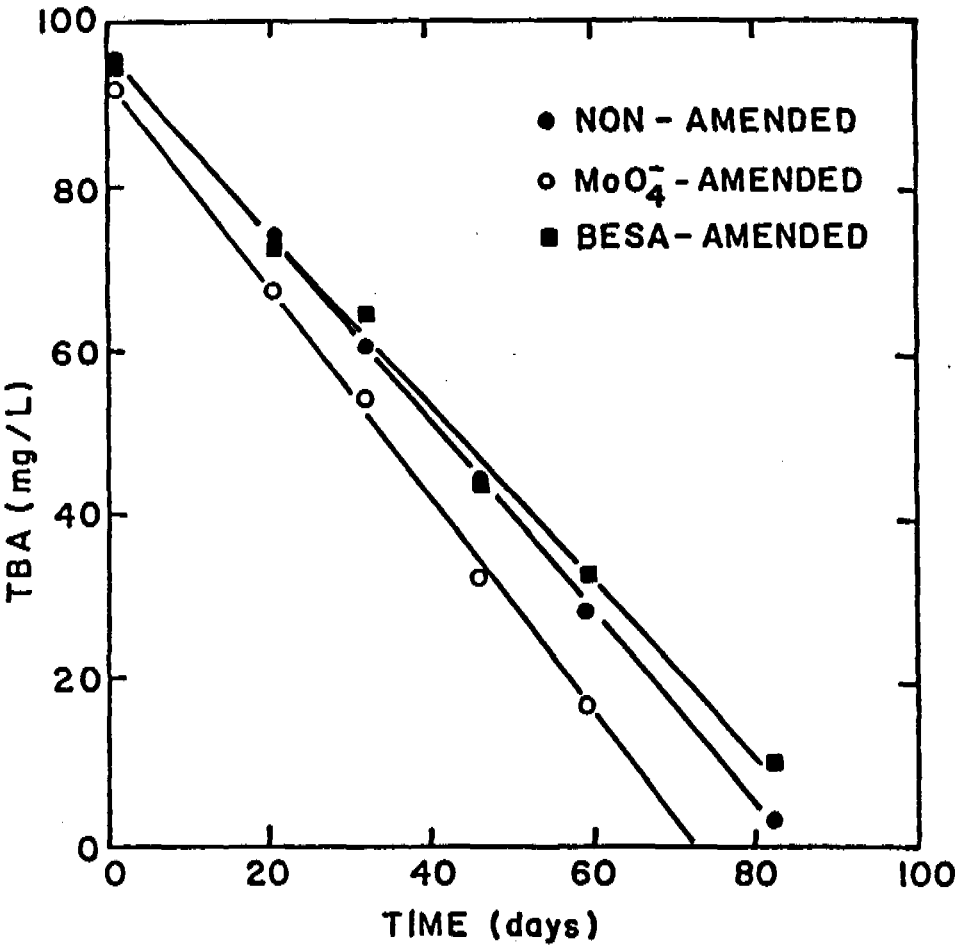


FIGURE 20
Effect of Molybdate and BESA on TBA Degradation
in Newport News Site 1 Soil



TABLES

TABLE 1
Degradation Rates in Blacksburg Soil Microcosms[a]

Soil site-depth (m)	First order reaction rate constant ($d^{-1}g^{-1}$)		Initial disappearance rate(mg $L^{-1}d^{-1}g^{-1}$)		Zero order reaction rate constant (mg $L^{-1}d^{-1}g^{-1}$)
	Methanol	Phenol	Methanol	Phenol	TBA
1-0	0.1251	0.0383	4.56	1.01	0.016
1-0.6	0.0083	0.0049	0.63	0.47	0.217
1-1.5	0.0039	0.0026	0.06	0.15	0.009
1-4.6	0.0122	0.0116	0.54	0.48	0.009
2-0	0.1007	0.0676	4.21	1.07	0.034
2-0.6	0.0155	0.0063	0.94	0.42	0.201
2-1.5	0.0180	0.0140	1.05	0.77	0.290
2-3.0	0.0201	0.0190	1.13	1.08	0.133
3-0	0.0203	0.0173	1.33	1.08	0.010
3-0.6	0.0089	0.0054	0.58	0.38	0.087
3-1.5	0.0103	0.0082	0.83	0.64	0.108
3-4.6	0.0149	0.0176	0.73	1.05	0.255

[a]Average of 2 or 3 microcosms; values determined at 20°C and normalized by dry weight of soil in the microcosm; initial substrate concentration = 100 mgL^{-1}

TABLE 2
Bacterial Density in Blacksburg Soils
Viable Counts

Soil Site-depth (m)	Viable Counts, CFU/g (S.D.)[a]		
	Aerobic		Anaerobic
	Ambient-moisture	Saturated[b]	Ambient-moisture
1-0	$2.43(\pm0.26)\times10^{6}$	$4.23(\pm0.70)\times10^{8}$	$6.56(\pm1.56)\times10^{5}$
1-0.6	$7.29(\pm5.08)\times10^{4}$	$1.25(\pm0.13)\times10^{7}$	$6.31(\pm1.85)\times10^{3}$
1-1.5	$3.41(\pm0.48)\times10^{4}$	$5.44(\pm0.67)\times10^{5}$	$<1.56\times10^{2}$
1-4.6	$1.74(\pm0.29)\times10^{5}$	$7.21(\pm1.32)\times10^{6}$	$<1.70\times10^{2}$
2-0	$2.45(\pm0.36)\times10^{6}$	$8.46(\pm0.33)\times10^{8}$	$9.59(\pm3.44)\times10^{5}$
2-0.6	$1.12(\pm0.32)\times10^{7}$	$2.31(\pm0.15)\times10^{7}$	$3.58(\pm1.91)\times10^{3}$
2-1.5	$5.33(\pm2.18)\times10^{7}$	$2.65(\pm0.52)\times10^{7}$	$9.37(\pm8.95)\times10^{2}$
2-3.0	$4.45(\pm0.60)\times10^{5}$	$5.03(\pm0.87)\times10^{6}$	$4.02(\pm1.52)\times10^{3}$
3-0	$1.52(\pm0.32)\times10^{6}$	$9.32(\pm1.01)\times10^{7}$	$2.18(\pm0.44)\times10^{5}$
3-0.6	$4.10(\pm2.85)\times10^{5}$	$3.34(\pm0.41)\times10^{6}$	$5.54(\pm1.90)\times10^{3}$
3-1.5	$3.13(\pm0.93)\times10^{5}$	$2.16(\pm0.21)\times10^{6}$	$2.63(\pm1.14)\times10^{3}$
3-4.6	$4.46(\pm1.08)\times10^{5}$	$1.38(\pm0.31)\times10^{6}$	$2.25(\pm0.79)\times10^{2}$

[a]Average of SEA and YEPA counts
[b]Soils saturated 2-5 days before plating

TABLE 3
Bacterial Numbers in Blacksburg Soils
MPNs and Direct Counts

Soil Site-depth (m)	Most Probable Number		AO Direct Counts cell/g (S.D.)
	Substrate[a]	MPN/g	
1-0.6	B	7.43×10^{2}	
1-1.5	M	1.78×10^{5}	$3.41(\pm 3.75) \times 10^{7}$
	P	1.44×10^{3}	
1-4.6	M	2.47×10^{6}	
	P	3.08×10^{2}	
	B	<30.8	
2-0.6	M	2.88×10^{5}	
	P	2.04×10^{4}	
2-1.5	M	4.11×10^{5}	$2.48(\pm 3.60) \times 10^{7}$
	P	2.62×10^{4}	
	B	<24.6	
3-0.6	M	9.40×10^{4}	
	P	3.93×10^{4}	
3-1.5	M	6.62×10^{6}	$3.35(\pm 3.34) \times 10^{7}$
	P	3.93×10^{4}	
	B	<24.6	
3-4.6	M	1.37×10^{6}	
	P	8.02×10^{3}	
	B	1.52×10^{3}	

[a] M=methanol; P=phenol; B=tertiary butyl alcohol.

TABLE 4
Correlation Coefficients (r) for Linear Regression of Biodegradation Rate Versus Bacterial Density in Soils

		Biodegradation Rate[a]				
		Methanol		Phenol		TBA
Microbial Measure[b]	n	FORRC	IDR	FORRC	IDR	ZORRC
AMVC	9	0.61	0.62	0.18	0.20	0.46
AMVC	12	0.90	0.93	0.84	0.60	-0.36
SVC	9	0.46	0.51	0.01	-0.02	0.60
SVC	12	0.88	0.90	0.96	0.47	-0.38
AAVC	7	-0.54	-0.40	-0.65	-0.62	-0.55
AAVC	10	0.92	0.95	0.85	0.50	-0.65
MPN	7	-0.07	0.19	-0.15	0.12	------

[a] FORRC = first order reaction rate constant ($d^{-1}g^{-1}$); IDR = initial disappearance rate (mg $L^{-1}d^{-1}g^{-1}$); ZORRC = zero order reaction rate constant ($mgL^{-1}d^{-1}g^{-1}$).

[b] AMVC = ambient moisture viable count; SVC = saturated moisture anaerobic viable count; MPN = substrate specific most probable number.

TABLE 5
Initial Concentrations in Microcosms

Constituent	Concentration
Organic Substrates	
Methanol, TBA, etc.	100 mg/L
Electron Acceptors	
Nitrate	5 mM
Nitrite	7 mM
Sulfate	3 mM
Nutrient	
Ammonium	0.64 mM
Inhibitors	
Molybdate	1 mM
BESA	5 mM

TABLE 6
Groundwater Characteristics at Studied Sites

Parameter (mg/L)[b]	Location						
	Wayland NY	Williamsport PA	Dumfries VA	Blacksburg VA[a]		Newport News VA	
				Site1	Site2	Site1	Site2
NO_3^-	1.54	53.4	0.11	4.8	3.6	nd[d]	nd
NO_2^-	1.20	nd	0.56	nd	nd	nd	nd
SO_4^{2-}	52.0	27.6	8.10	nd	nd	7.6	5.3
PO_4^{3-}	nd	nd	nd	nd	nd	nd	nd
Fe (total)	0.05	0.44	4.23	<0.03	0.06	7.0	0.05
TOC[c]	1.7	1.0	1.3	1.3	1.7	--[e]	2.1
Dissolved Oxygen	0.7	6.7	0.2	--	--	0.1	--
Temperature, °C	10.0	11.0	10.0	--	--	24.0	16.5
pH	7.8	4.7	4.5	4.7	4.7	6.5	5.4

[a] Measured in the filtrate of a 3.33:1 water/soil mixture.
[b] All parameters have units of mg/L except temperature and pH.
[c] TOC = total organic carbon.
[d] nd = none detected.
[e] -- = not analyzed.

TABLE 7
Bacterial Density in Subsurface Soils

Location	Soil Extract Viable Count,CFU/g		Direct Count, Cell/g	
	Geometric Mean	Range	Geometric Mean	Range
Wayland NY	1.0×10^6	9.3×10^5 - 1.1×10^6	7.8×10^7	7.6×10^7 - 8.0×10^7
Williamsport PA	2.2×10^4	3.5×10^3 - 1.4×10^5	4.2×10^7	3.9×10^7 - 4.6×10^7
Dumfries VA	1.5×10^6	5.6×10^5 - 5.2×10^6	5.9×10^7	3.1×10^7 - 1.1×10^8
Blacksburg VA				
Site 1	1.1×10^5	3.6×10^4 - 4.0×10^5	3.4×10^7	--
Site 2	8.0×10^5	5.9×10^5 - 1.4×10^6	2.5×10^7	--
Site 3	4.5×10^5	3.9×10^5 - 6.0×10^5	3.4×10^7	--
Newport News VA				
Site 1	8.4×10^5			
Site 2	8.8×10^4			

TABLE 8
Variation in Microbial Response at Several Sites to Additives

	Relative Rate of Degradation		Effect on Degradation Rate				
Site	Methanol	TBA	NO_3^-	NO_2^-	SO_4=	MoO_4=	BESA
Dumfries, VA	low	low	a	a	a		
Williamsport, PA	high	high	b	a	b		
Wayland, NY	low	low	c		c		
Blacksburg, VA (1+3)	low	low	a	a	a	b	a
Blacksburg, VA (2)	high	high	b	a	b		
Newport News, VA (1)	high	high	b	b,a	c,b	c	c
Newport News, VA (2)	high	low	a	a	a		

a rates slower than non-amended rates
b rates faster than non-amended rates
c rates approximately equal to non-amended rates

TABLE 9
Site Characterization Based on Microbial Response

	Fast Sites	Slow Sites
Representative Sites	Williamsport, PA Newport News, VA (1) Blacksburg, VA (2)	Wayland, NY Dumfries, VA Newport News, VA (2) Blacksburg, VA (1+3)
Relative Degradation Rates (Methanol and TBA)	High	Low
Nitrate Addition	Increases Rates	Decreases Rates (Low pH sites) or No Effect (High pH sites)
Nitrite Addition	Inhibitory	Inhibitory
Sulfate Addition	Increases Rates	Decreases Rates or No Effect (Sulfate Present)
Inhibit Sulfate Reduction (Molybdate Addition)	No Effect	Increases Rates
Inhibit Methanogenesis	No Effect	Decreases Rates
Apparently Important Metabolic Pathway(s)	Nitrate Respiration Denitrification, Sulfate Reduction, Methanogenesis	

REFERENCES

Aelion, C.M., C.M. Swindoll, and F.K. Pfaender. 1987. "Adaptation to and Biodegradation of Xenobiotic Compounds by Microbial Communities from a Pristine Aquifer." *Applied and Environmental Microbiology*. 53:2212-2217.

Ausmus, B.B., G.K. Eddleman, S.J. Draggan, J.M. Giddings, D.R. Jackson, R.J. Luxmore, E.G. O'Neill, R.V. O'Neill, M. Ross-Todd, and P. Van Voris. 1980. "Microcosms as Potential Screening Tools for Evaluating Transport and Effects on Toxic Substances." Athens, GA: U.S. Environmental Protection Agency. (EPA-600/3-80-042).

Beeman, R.E., and J.M. Suflita. 1987. "Microbial Ecology of a Shallow Unconfined Ground Water Aquifer Polluted by Municipal Landfill Leachate." *Microbial Ecology*. 14:39-54.

Bengtsson, G. 1985. "Microcosms for Groundwater Research." In *Ground Water Quality*, ed. C.H. Ward, W. Giger, and P.L. McCarty, 330-341. New York: John Wiley & Sons.

Bouwer, E.J., and P.L. McCarty. 1983. "Transformation of Halogenated Organic Compounds under Denitrification Conditions." *Applied and Environmental Microbiology*. 45:1295-1299.

Bouwer, E.J., and P.L. McCarty. 1983b. "Transformations of 1and 2-carbon Halogenated Aliphatic Organic Compounds under Methanogenic Conditions." *Applied and Environmental Microbiology*. 45:1286-1294.

Bouwer, E.J., and P.L. McCarty. 1984. "Modeling of Trace Organics Biotransformation in the Subsurface." *Ground Water*. 22:433-440.

Bouwer, E.J., J.P. Wright, and G.D. Cobb. 1986. "Anoxic Transformation of Trace Halogenated Aliphatics." In *Toxic and Hazardous Wastes, Proceedings of the 18th Mid-Atlantic Industrial Waste Conference*, 545-553. Lancaster, PA: Technomic Publishing Co.

Dobbins, D.C., J.R. Thornton-Manning, D.D. Jones, and T.W. Federle. 1987. "Mineralization Potential for Phenol in Subsurface Soils." *Journal of Environmental Quality*. 16:54-58.

Dunlap, W.J., R.L. Cosby, J.F. McNabb, B.E. Bledsoe, and M.R. Scalf. 1972. "Probable Impact of NTA on Ground Water." *Ground Water*. 10:107-117.

Dunlap, W.J., J.F. McNabb, M.R. Scalf, and R.L. Cosby. 1977. "Sampling for Organic Chemicals and Microorganisms in the Subsurface." Ada, OK: U.S. Environmental Protection Agency, Office of Research and Development, Robert S. Kerr Environmental Research Laboratory. (EPA-600/2-77-176).

Dyksen, J.E., and A.F. Hess. 1982. "Alternatives for Controlling Organics in Groundwater Supplies." *Journal of the American Water Works Association*. 74:394-403.

Ehrlich, G.G., D.F. Goerlitz, E.M. Godsy, and M.F. Hult. 1982. "Degradation of Phenolic Contaminants in Ground Water by Anaerobic Bacteria." *Ground Water*. 20:703-710.

Federle, T.W., D.C. Dobbins, J.R. Thornton-Manning, and D.D. Jones. 1986. "Microbial Biomass, Activity, and Community Structure in Subsurface Soils." *Ground Water*. 24:365-373.

Ghiorse, W.C., and D.L. Balkwill. 1983. "Enumeration and Morphological Characteristics of Bacteria Indigenous to Subsurface Environments." *Developments in Industrial Microbiology*. 24:213-224.

Ghiorse, W.C., and D.L. Balkwill. 1985. "Microbiological Characterization of Subsurface Environments." In *Ground Water Quality*, ed. C.H. Ward, W. Giger, and P.L. McCarty, 387-401. New York: John Wiley & Sons.

Gibson, S.A., and J.M. Suflita. 1986. "Extrapolation of Biodegradation Results to Goundwater Aquifers: Reductive Dehalogenation of Aromatic Compounds." *Applied and Environmental Microbiology*. 52:681-688.

Goldsmith, C.D. 1985. Biodegradation of Methanol and Tertiary Butyl Alcohol in Previously Uncontaminated Subsurface Systems. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Grant, W.D., and P.E. Long. 1981. *Environmental Microbiology*. New York: John Wiley & Sons.

Morris, M.S. 1988. Biodegradation of Organic Contaminants in Subsurface Systems: Kinetic and Metabolic Considerations. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Novak, J.T., C.D. Goldsmith, R.E. Benoit, and J.H. O'Brien. 1985. "Biodegradation of Methanol and Tertiary Butyl Alcohol in Subsurface Systems." *Water Science Technology*. 17:71-85.

Olsen, R.A., and L.R. Bakken. 1987. "Viability of Soil Bacteria: Optimization of Plate-Counting Technique and Comparison between Total Counts and Plate Counts within Different Size Groups." *Microbial Ecology*. 13:59-74.

Rittman, B.E., P.L. McCarty, and P.V. Roberts. 1980. "Trace Organics Biodegradation in Aquifer Recharge." *Ground Water*. 18:236-243.

Simkins, S., and M. Alexander. 1984. "Models for Mineralization Kinetics with the Variables of Substrate Concentration and Population Density." *Applied and Environmental Microbiology*. 47:1299-1306.

Smith, J.A., and J.T. Novak. 1987. "Biodegradation of Chlorinated Phenols in Subsurface Soils." *Water, Air and Soil Pollution*. 33:29-42.

Smith, M.R., and R.A. Mah. 1978. "Growth and Methanogenesis by *Methanosarcina* Strain 227 on Acetate and Methanol." *Applied and Environmental Microbiology*. 36:870-879.

Smolenski, W.J., and J.M. Suflita. 1987. "Biodegradation of Cresol Isomers in Anoxic Aquifers." *Applied and Environmental Microbiology*. 53:710-716.

Spain, J.C., P.A. Van Veld, C.A. Monti, P.H. Pritchard, and C.R. Cripe. 1984. "Comparison of p-Nitrophenol Biodegradation in Field and Laboratory Test Systems." *Applied and Environmental Microbiology*. 48:944-950.

Stumm, W., and J.J. Morgan. 1981. *Aquatic Chemistry*, 2nd Ed. New York: John Wiley & Sons.

Suflita, J.M., and G.D. Miller. 1985. "Microbial Metabolism of Chlorophenolic Compounds in Ground Water Aquifers." *Environmental Toxicology and Chemistry*. 4:751-758.

Taylor, B.F., and R.S. Oremland. 1979. "Depletion of Adenosine Triphosphate in *Desulfovibrio* by Oxyanions of Group VI Elements." *Current Microbiology*. 3:101-103.

Trolldenier, G. 1973. "The Use of Fluorescence Microscopy for Counting Soil Microorganisms." In *Modern Methods in the Study of Microbial Ecology*, ed. T. Rosswall. *Bull. Ecol. Res. Comm.* 17:53-59. Stockholm: Swedish Nat. Sci. Res. Council.

Webster, J.J., G.J. Hampton, J.T. Wilson, W.C. Ghiorse, and F.R. Leach. 1985. "Comparison of Microbial Cell Numbers Determined by Microscopic and Biochemical Measurements on Subsurface Samples." *Ground Water*. 23:17-25.

White, K.D. 1986. A Comparison of Subsurface Biodegradation Rates of Methanol and Tertiary Butanol in Contaminated and Uncontaminated Sites. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Wiggins, B.A., S.H. Jones, and M. Alexander. 1987. "Explanations for the Acclimation Period Preceding the Mineralization of Organic Chemicals in Aquatic Environments." *Applied and Environmental Microbiology*. 53:791-796.

Wilson, B.H., B.E. Bledsoe, D.H. Kampbell, J.T. Wilson, J.M. Armstrong, and J.H. Sammons. 1986. "Biological Fate of Hydrocarbons at an Aviation Gasoline Spill Site." Conference on prevention, detection, and restoration of groundwater contamination. Houston, TX: National Water Well Association and the American Petroleum Institute.

Wilson, B.H., G.B. Smith, and J.F. Rees. 1986. "Biotransformations of Selected Alkylbenzenes and Halogenated Aliphatic Hydrocarbons in Methanogenic Aquifer Material: A Microcosm Study." *Environmental Sciences and Technology*. 20:997-1002.

Wilson, J.T., and M.J. Noonan. 1984. "Microbial Activity in Model Aquifer Systems." In *Groundwater Pollution Microbiology*, ed. G. Bitton and C.P. Gerba. New York: John Wiley & Sons.

Wilson, J.T., L.E. Leach, M. Heenson, and J.N. Jones. 1986. "*In Situ* Biorestoration as a Ground Water Remediation Technique." *Ground Water Monitoring Review*. Fall:56-64.

Wilson, J.T., J.F. McNabb, D.L. Balkwill, and W.C. Ghiorse. 1983. "Enumeration and Characterization of Bacteria Indigenous to a Shallow Water-Table Aquifer." *Ground Water*. 21:134-142.

Wilson, J.T., J.F. McNabb, B.H. Wilson, and M.J. Noonan. 1983b. "Biotransformation of Selected Organic Pollutants in Groundwater." *Developments in Industrial Microbiology*. 24:225-233.

Wilson, J.T., G.D. Miller, W.C. Ghiorse, and F.R. Leach. 1986b. "Relationship between the ATP Content of Subsurface Material and the Rate of Biodegradation of Alkylbenzenes and Chlorobenzene." *Journal of Contaminant Hydrology*. 1:163-170.

Wilson, J.T., M.J. Noonan, and J.F. McNabb. 1985. "Biodegradation of Contaminants in the Subsurface." In *Ground Water Quality*, ed. C.H. Ward, W. Giger, and P.L. McCarty, 483-492. New York: John Wiley & Sons.

Wilson, W.G. 1986. Enhancement of Biodegradation of Methanol and Tertiary Butyl Alcohol in Groundwater Systems. Master of Science Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA.

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Virginia Water Resources Research Center
Virginia Polytechnic Institute and State University
617 North Main Street
Blacksburg, Virginia 24060-3397
Phone (703) 231-5624