ENHANCEMENT OF BIODEGRADATION OF METHANOL AND TERTIARY BUTYL ALCOHOL IN GROUNDWATER SYSTEMS

by

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(ABSTRACT)

Groundwater contamination with organic compounds, especially leaked or spilled fuels, is a serious and growing environmental problem which is difficult to remediate. Alcohol additives in gasoline present more urgent problems because of their relatively high mobility in the subsurface.

This study focused on the subsurface biodegradation of two octane-boosting additives, methanol and tertiary butyl alcohol (TBA). A microcosm study was undertaken to determine the *in situ* biotransformation rates. While both alcohols were found to be amenable to biodegradation in the subsurface systems, the methanol removal rate was much greater than the TBA rate.

By using sets of microcosms, several chemical additives were used in an effort to determine their effects on biodegradation. Organic substrates were added to microcosms containing low concentrations of TBA (about 1 mg/L) without evidence of stimulation or inhibition. Variation of pH (from pH 5.1 to 8.8) in microcosms containing methanol and TBA was

part of the next experiment. This was combined with the addition of alternative electron acceptors, nitrate and sulfate.

Variation of pH in non-amended and sulfate-dosed (100 mg/L) microcosms did not enhance the biodegradation of either alcohol. Nitrate addition inhibited both methanol and TBA biodegradation at lower pH levels, but enhanced methanol degradation when combined with base. TBA degradation was not affected by base addition except in the highest (initial pH 8.8) base dose. These microcosms exhibited TBA removal rates which were uniformly higher than all other treatments.

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CHAPTER I

INTRODUCTION

Contamination of groundwater supplies by organic chemicals has been recognized as a serious and wide-spread environmental problem. A large part of this problem results from the leakage or spillage of gasoline and other fuels. The underground storage tanks commonly used to hold gasoline and other fuels have been identiied as a major source of groundwater pollution. Corrosion, ground movements, and poor sealing can cause leaks in underground storage tanks and piping which may not be detected for months or years. Most of the insoluble gasoline components are quickly sorbed to subsurface strata, but highly soluble alcohol octane enhancers may travel relatively quickly in an aquifer. Use of these additives has grown due to the restrictions recently imposed on lead compounds in gasoline. An added complication is the lower odor threshold of the alcohols relative to other gasoline components. Most gasoline contamination events are discovered when people nearby smell or taste gasoline hydrocarbons in their well water. Alcohols may reach toxic concentrations for humans before they are detected by taste or odor.

Two alcohol compounds were of interest in this study, methanol and tertiary-butyl alcohol. The IUPAC name for

tertiary-butyl alcohol is 2-Methyl-2-propanol and the common acronym, which is used throughout this report, is TBA. Methanol may be added to gasoline alone or together with TBA. Methanol is used as a fuel on an experimental basis, but methanol and methanol/TBA blends are added to unleaded gasoline to boost the octane rating. Both alcohols are also used in various chemical production processes.

Generally, organic compounds may persist for a much longer time in the subsurface compared to their persistence in surface soil or water. Reasons for this are: lack of mixing (slow water velocities), little to no volatilization, no photodegradation, and very little biological productivity. Biodegradation can be a major removal process for some compounds, depending on the solubility, structure, and other characteristics of the molecule. The state of the groundwater environment and microbial population will determine the rate and pathways of biodegradation. Degradation of both methanol and TBA have been demonstrated in subsurface microcosms, but the rate of TBA removal is very slow.

In this study attempts were made to evaluate the variations in degradation of alcohols in the presence of organic and inorganic additives. The effects of carbon substrates on the biodegradation rates for TBA were examined. environment. Also, alternative electron acceptors (nitrate and sulfate) were added to the oxygen-poor environment in an attempt to stimulate anaerobic respiration. Variation of the

pH of these microcosms was carried out to determine pH effects on nitrate-dosed, sulfate-dosed, and non-amended microcosms. The degradation rates of both methanol and TBA were studied.

The research objectives were:

1. Determination of the stimulatory or inhibitory effects of carbon and energy sources on TBA degradation in subsurface microcosms.

2. Determination of the effects of addition of alternative electron acceptors on methanol and TBA biodegradation.

3. Investigation of the effects of pH on degradation of both methanol and TBA in subsurface microcosms which were either dosed with nitrate, or sulfate, or neither.

CHAPTER II

LITERATURE REVIEW

The contamination of aquifers which supply drinking water has been recognized as a serious health problem. Groundwater is the primary source of drinking water for 40 to 50 percent of the people in the United States (1). Estimates of the extent of contamination range from 0.2 to 2 percent of usable aquifers (2,3). Although this seems to be a tiny fraction of the resource most contamination occurs near larger population centers so more people are involved than these numbers suggest. An Environmental Protection Agency (EPA) survey of groundwater-fed public water supplies found that larger water systems, serving more people, were more affected by volatile organic compounds (VOC) contamination than the more numerous small treatment plants (4).

THE SUBSURFACE

The subsurface environment is quite heterogeneous. The subsurface soil and rock vary widely in chemical composition and porosity (void volume percentage of the total volume). Water movement is generally quite slow underground. Groundwater velocities are proportional to the hydraulic conductivity, a property of the subsurface material. The

hydraulic conductivity is expressed in units of length per unit time and the range of values spans about ten orders of magnitude. Water in the groundwater, or saturated, zone primarily moves in horizontal or lateral directions at velocities that generally range from 1 to 500 meters (m) per year (5). Water in the vadose, or unsaturated, zone generally percolates downward at even slower velocities. The ionic composition of groundwater is directly tied to the geology of its subsurface system. Temperatures in the subsurface are more predictable, staying near the mean annual air temperature (4 to 25° C in the U.S.) to depths of about 18 m (60 ft.) (6). Below this depth the temperature increases roughly 30° C per 1000 m of depth (7). Another obvious but important constant is the lack of light in the subsurface. Because of adsorption and filtering effects in the upper soil and subsoil layers the organics concentration in groundwater is typically quite low. This results in an organic content in groundwater that is composed of more soluble and less sorbable compounds, this effect is accentuated with depth. The dissolved oxygen concentration may also be very low due to the isolation from the atmosphere and respiration of subsurface microbes. Human activities such as well pumping, waste disposal, and aquifer recharge may change many of the characteristics of the subsurface.

Until the last ten years or so, the subsurface was presumed to be devoid of life. It was, and still is to some

extent, common practice to pump groundwater directly into public water distribution systems without disinfection. Improved sampling techniques and growing interest have led to evidence of substantial microbial populations far below the surface soil layer (8,9). The nature and density of microbes in the subsurface are as variable as the geology itself. At least one study found bacterial numbers to be fairly constant with depth (8). Even macroinvertebrates, including worms, molluscs, insects, and crustaceans, have been identified in some aquifers (10). Identification of microbes is some what incomplete at this time but some of the genera which have been identified are: *Pseudomonas*, *Caulobacter*, *Agrobacterium*, *Nocardia*, *Flavobacterium*, *Acinetobacter*, and *Micrococcus* (11,12).

METHANOL AND TBA

Methanol and TBA are used in various chemical manufacturing processes and as fuels and fuel additives. The demand for these alcohols for enhancing gasoline octane ratings has been increasing due to restrictions on the use of lead additives. Methanol alone or methanol combined with TBA is blended with unleaded gasoline to produce a fuel with a maximum of 5 % methanol and a minimum of 2.5 % TBA (13). This adds a further complication to the serious problem of gasoline contamination of aquifers. Most components of gasoline

are hydrocarbons with a low aqueous solubility and high sorption affinity for subsurface materials. This limits the mobility of these constituents. These hydrocarbons are responsible for the characteristic gasoline taste and odor which is usually the first indication of a contamination incident. Persons using well water near a leak or spill can detect gasoline in that water at very low concentrations and many times alert authorities before a problem was suspected. Since these alcohols are completely soluble in water and do not sorb to soil they will move ahead of the gasoline hydrocarbons.

Ingestion of these alcohols can lead to serious health problems and even death. Methanol has an odor threshold (just perceptible) vapor concentration of 5900 parts per million (ppm) and a permissable exposure limit of 200 ppm in air as recommended by the National Institute for Occupational Safety and Health (14). The odor threshold of TBA is not known, but, by comparison of vapor pressure and other properties with similar compounds, it seems likely that it should be near the permissable exposure limit of 100 ppm (14).

Dermal absorption of these alcohols could be a significant exposure pathway. Blindness and an infant death have resulted from skin exposure to methanol, TBA is less active in skin absorption studies than methanol (14). Acute symptoms of exposure to these alcohols range from headaches and nausea to loss of vision and death. Chronic exposure to

methanol may cause optical atrophy and enlargement of the liver in humans (14).

BIODEGRADATION

Biodegradation of methanol has been demonstrated under a variety of conditions by microorganisms known as methylotrophs. These microbes use methanol and other organics without carbon-carbon bonds (C_1 compounds) as carbon sources (15). Methylotrophs produce an enzyme, methane monooxygenase, which has broad substrate specificity. This implies that the methylotrophic bacteria could degrade organics besides C_1 compounds via co-metabolism (16). The commonly accepted degradation pathway begins with the oxidation of methanol to formaldehyde by methanol dehydrogenase. Then the formaldehyde is either assimilated via a ribulose monophosphosphate (RMP) pathway or dissimilated with formaldehyde dehydrogenase and formate dehydrogenase (17). Methanol, being a one-carbon compound, cannot be oxidized via the tricarboxylic acid (TCA) cycle.

Biodegradation of TBA has not been studied as extensively as methanol degradation. TBA is more recalcitrant, i.e. less amenable to biodegradation, than methanol due to its closely-packed tertiary molecular structure. It is known that tertiary alcohols are more readily dehydrated than secondary or primary alcohols and that tertiary alcohols are

stable to oxidation under normal conditions (18). Several investigations have found little to no TBA biodegradation in aerobic, activated sludge systems (19,20,21). One study did show aerobic biodegradation of TBA using long-term enrichment culture techniques and continuous flow, completely mixed reactors (22). Microcosm studies using subsurface soil and groundwater under anoxic conditions have shown TBA to be slowly biodegradable in several different systems. Previous contamination of the natural system appeared to result in more rapid biodegradation (23,24).

The longer methanol and TBA remain in an aquifer, the farther they can travel. Increased rates of biodegradation could limit the extent and severity of the contamination. Before determining a method of enhancement the degradation process must be understood.

The common pathways for carbon utilization are fermentation, aerobic respiration, and anaerobic respiration. Fermentation does occur in groundwater systems but yields little energy and may result in incomplete degradation if an organic molecule is the electron acceptor. Fermentation of methanol results in the formation of methane, carbon dioxide, and water. Aerobic respiration produces the most energy per mole of substrate and the most complete mineralization of all the pathways considered. Low oxygen concentrations in groundwater limit aerobic respiration.

Addition of oxygenated water or peroxides has been used to stimulate aerobic biodegradation in aquifers (26).

In the absence of molecular oxygen, respiration may still occur by use of an alternative electron acceptor in the electron transport system. Acceptors which may be used are nitrate, sulfate, and carbon dioxide. Anaerobic respiration will also typically result in the complete mineralization of an organic substrate (electron donor) to carbon dioxide. The energy yield per mole of substrate is less for anaerobic respiration than for aerobic respiration because of a less complete electron transport chain. Aerobic respiration can produce three moles of the energy storage compound adenosine triphosphate (ATP) per mole $NADH_2$, but only two moles of ATP can be produced per mole $NADH_2$ with anaerobic respiration (27).

Complete reduction of nitrate to molecular nitrogen is termed denitrification. This process yields less energy than aerobic respiration, but yields the most energy of the anaerobic respiration processes. The first step of denitrification is the reduction of nitrate to nitrite. If the resulting nitrite is not further reduced it may reach concentrations toxic to the microbes. Nitrite toxicity increases with decreasing pH and temperature (28).

Sulfate is used as an electron acceptor by microorganisms known as sulfate reducers. Sulfate reducers are obligate anaerobes and require an oxidation reduction

potential of less than -100 millivolts (mV). Although the subsurface environment is often thought to be devoid of oxygen, low concentrations of dissolved oxygen may persist. Even a small amount of oxygen is toxic to obligate anaerobic microbes. The sulfate-reducing bacteria grow slowly because this process provides relatively little energy and because the end product, sulfide, is toxic and will precipitate out essential iron as iron sulfide or FeS (29).

Carbon dioxide (CO_2) reduction to methane is called methanogenesis and is carried out by methanogenic bacteria or methanogens. Biodegradation of recalcitrant organics has been demonstrated under methanogenic conditions at rates higher than those seen under aerobic conditions (30).

Biodegradation in subsurface systems may be limited by insufficient amounts of essential nutrients. In many aquatic systems the limiting nutrient is either nitrogen or phosphorus. Trace metals such as nickel, cobalt, molybdenum, and iron may also be limiting factors for bacterial growth and activity (31).

MICROCOSM STUDIES

While laboratory studies can yield much valuable information, they also cannot completely duplicate *in situ* conditions. Microbial growth rate, an important factor for biodegradation, can be very slow in the environment because

of low temperature, nutrient limitations, etc. in the subsurface. Pure culture or enrichment culture methods give a highly specialized view of the degradation process and cannot be used to accurately predict the results of interactions within a diverse microbial community.

The presence of more than one substrate in a system leads to competition between species of degraders, but not necessarily to dominance of a single species (32). Some pollutants are degraded incidentally, providing no carbon or energy, by microbes using another compound as substrate. This situation is called co-metabolism. Some subsurface bacteria are oligotrophs, especially adapted to nutrient-poor conditions. Oligotrophs find it advantageous to have a low specificity for substrates and a low selectivity for slowly metabolized or excess substrates (33). It is important to use low concentrations of substrate in experiments designed to predict biodegradation rates in the subsurface. High concentrations of organics can inhibit or kill oligotrophic microbes or may cause other changes in the population (34,35).

Microorganisms in a heterogeneous population may work in concert to degrade a compound in a stepwise fashion. Presence of more than one contaminant may lead to preferential use of the more easily metabolized compound, when it has been degraded the next most easily utilized compound is used. This is termed diauxie growth. Bouwer and McCarty have pro-

posed that below a certain low concentration an organic compound cannot be degraded because it will not provide enough energy (36). This theory was developed using the assumption of no co-metabolism and no interspecies interactions.

Microorganisms in the subsurface grow as biofilms attached to soil particles, less than 1 % of the population is free-floating in the groundwater (37). Studies using an artificial surfaces such as glass beads or using only groundwater without solid media for bacterial attachment will not truly model the subsurface environment.

Microcosm studies using native microflora under simulated subsurface conditions and low levels of organics will avoid many of the problems of laboratory studies. Data from microcosms is as representative as possible in the laboratory and does take into account interactions within a microbial population. It is important to remember that the results of microcosm studies are somewhat site-specific and not absolutely transferable to natural systems.

CHAPTER III

METHODS AND MATERIALS

In this research project microcosms were used to predict the *in situ* biodegradation of methanol and TBA. Every effort was made to prevent the introduction of surface bacteria into the microcosms. The alcohols concentrations in most of the microcosms were monitored over a period of several months until degradation was complete or static.

SOIL AND GROUNDWATER

All the studies described herein used subsurface soil collected on March 20 and 21, 1984 from an uncontaminated area near Dumfries, Virginia. The samples were collected from depths of 7 to 9 m (20 to 30 ft.) below the surface in Potomac River terrace deposits. This stratum was made up of fine sand with some clayey silt and was described as wet or moist, probably due to a perched water table (38).

Subsurface soil samples were obtained with an Osterberg piston sampler and Denison core barrel. The core was extruded from the barrel through a flame sterilized paring ring to trim away the layer of soil which had been in contact with the sampling apparatus. Ends of the core were pared away with sterile instruments. The core material was placed

in sterile glass jars on wet ice for transport to and storage in the Virginia Tech Environmental Engineering Department laboratories. The soil was stored at 10° C, the temperature of the subsurface. Groundwater was collected from a well near the drilling site and stored in a similar manner. Microbial enumeration of the soil samples was conducted by soil extract agar plate count and acridine orange direct count methods. Groundwater temperature, dissolved oxygen, and pH were measured in the field immediately upon sample collection. Chemical analyses for various ions, total organic carbon (TOC), total organic halides (TOX), and alkalinity were carried out at the laboratories at Virginia Tech. More complete sampling and analysis descriptions have been detailed by Goldsmith (23).

MICROCOSMS

Microcosms consisted of glass, screw-top test tubes (13 x 100 mm) filled with subsurface soil and autoclaved groundwater dosed with the desired compounds. The cap of each microcosm was fitted with a teflon-backed silicone septum to allow aqueous sampling over time with a microliter syringe. Microcosm tubes were stored in a refrigerator at a constant temperature of 10° C.

Microcosm tubes were acid-washed and rinsed with distilled water. Tubes, caps, and septa were autoclaved at 120°

C. and 15 pounds per square inch (psi) pressure for 20 minutes. Groundwater was autoclaved at the same temperature and pressure for 30 minutes. Transfer of subsurface soil to the microcosm tube was carried out with flame-sterilized instruments, groundwater transfer was effected with a sterile disposable syringe.

Compounds to be added to the microcosms were mixed with the groundwater to maintain uniform concentrations. A sample of soil was weighed, dried at 300° C for 45 minutes, then reweighed to determine the percent moisture by dividing the difference in mass (water mass) by the original mass. This moisture was considered when making up the dose concentration in the added groundwater. The dosing concentration in the groundwater was greater than the desired microcosm concentration by a factor equal to the combined soil water and added water volume divided by the added water volume.

A measured amount (approximately 10 grams) of subsurface soil was added aseptically to each microcosm tube. The tube was then filled with dosed groundwater and capped with a solid cap. The contents were thoroughly mixed with a vortex mixer then centrifuged to leave a free water layer above the soil. Air bubbles were excluded by refilling if necessary and replacing the solid cap with a septum fitted open cap. To ensure complete mixing, vortex mixing and centrifuging were repeated after it was determined that there were no air bubbles in the tube.

LOW TBA/ SUBSTRATE ADDITION EXPERIMENT

The first sets of experiments involved addition of organic substrates to microcosms dosed with approximately 1 milligram per liter (mg/L) of TBA. The substrate compounds chosen and their concentrations in the microcosms were:

- Bacto-peptone (Difco) - 10 mg/L.
- Pyruvate - 100 and 500 mg/L as pyruvate.
- Sodium citrate - 1 and 5 mg/L as citrate.
- Ferric citrate - 1 and 5 mg/L as citrate.
- Reference - only TBA added.

A sterile control microcosm was prepared for each treatment. Subsurface soil was autoclaved (120° C, 15 psi) in aluminum foil several times for 30 to 60 minutes each time for a total autoclave time of 400 minutes (6 hr., 40 min.). Except for the use of sterilized soil, the control microcosms were prepared in the same manner as others in the set.

ALTERNATIVE ELECTRON ACCEPTOR, VARIED PH EXPERIMENT

The next experimental matrix involved the addition of nitrate and sulfate along with variation of pH. Each microcosm was dosed so as to produce a methanol concentration of approximately 100 mg/L and a TBA concentration of 5 mg/L. Sets of replicate microcosms were either dosed with nitrate or sulfate or neither (non-amended). Potassium nitrate (KNO_3) was used as the nitrate source, the nitrate concentration was 100 mg/L (as nitrate) in the dosed microcosms. Sulfate dosing to 100 mg/L (as sulfate) was effected with potassium sulfate (K_2SO_4).

Each treatment was set up at four different pH levels. To raise the pH, sodium hydroxide (NaOH) was added to the groundwater in proportions which produced microcosm normalities of 4, 10, and 20 milliequivalents of base per liter (meq/L). To determine the microcosm pH for each treatment, vials of soil and dosed groundwater were prepared which had the same water to soil ratio as the microcosms. After mixing, the pH in each vial was measured with a Fisher Scientific (Pittsburgh, PA) model 610a pH meter and glass pH electrode.

Sterile controls for these experiments were initially prepared in the same way as in the low TBA concentration/substrate addition experiment. Contamination was suspected, however, and the controls were redone. The

new controls were filled with soil autoclaved for 60 to 120 minutes per time, at 120° C and 15 psi as before, for a total autoclaving time of 14 hours. The microcosm tubes were autoclaved once more after being capped to inactivate microbes which could have entered during filling. Some groundwater escaped from the sealed microcosms due to the heat and pressures of the autoclaving process, but the lost volume (at most 1 mL) did not affect sampling and the caps remained in place.

ANALYSIS PROCEDURES

At regular time intervals, normally 7 to 10 days, each microcosm was sampled and the alcohols concentrations were determined. Samples were collected by withdrawing several microliters of water from the free water layer with a syringe inserted through the septum. The syringe was rinsed with distilled water, vacuum cleaned, and heat sterilized at 200° C before insertion into the microcosm to prevent contamination. The septa were wiped with a paper towelette moistened with isopropyl alcohol (70%) before analyses to further ensure against introduction of bacteria into the microcosms.

The 2 micoliter sample was then injected into a Hewlett-Packard (Santa Clara, CA) 5880A gas chromatograph for determination of methanol and TBA concentrations. The gas

chromatograph uses a flame ionization detector (FID). The injection port temperature was 125° C, detector temperature was 250° C. The detector flame hydrogen feed rate was 30 cubic centimeters (cc) per minute, air flow rate to the flame was 400 cc/ minute. Most analyses were conducted with a 2.13 m (7 ft.) by 3.2 millimeter (mm) (1/8 inch) stainless steel column packed with 0.2% Carbowax 1500 on 80/100 mesh Carbopack C. The oven temperature for this column was a constant 120° C. The nitrogen carrier gas flow rate was set at 25 cc/minute. Other analyses were conducted using a wide-bore glass capillary column (30 m by 0.75 mm) with Supelco SPB-5 phase and 1 micron film thickness. The carrier gas (nitrogen) flow rate was 2 cc/minute, make-up gas flow rate was 25 cc/minute. The oven temperature began at 90° C, remained isothermal for 2 minutes, then increased at the rate of 8° C/minute until the oven reached 130° C.

Before each analysis session the gas chromatograph was calibrated with a standard solution containing methanol and TBA at concentrations near those in the microcosms. Fresh standard solutions were prepared at least monthly and were stored in sterile containers in the refrigerator. Variation in replicate runs of more than 5% was considered unacceptable during calibration and analysis.

Nitrate-dosed microcosms were sacrificed at the end of the experiment in order to measure the concentrations of nitrite and nitrate in the water. All free water from the

microcosm (approximately 2 mL) was injected into a Dionex (Sunnyvale, CA) 2010i ion chromatograph (IC) for anion analysis. The IC was calibrated with fresh standard solutions before analysis.

CHAPTER IV

RESULTS AND DISCUSSION

SUBSURFACE ENVIRONMENT

The microbial population of the subsurface soil samples was analyzed by plate count and direct count bacterial enumeration methods. Results of these analyses are presented in Table 1 (23). The soil used in these studies was from approximately the 9 m (30 ft.) depth. Populations remained relatively constant with depth. The moisture content of the soils used ranged from 16 % to 20 %. This soil was described as "wet" or "moist" in the driller's report (38).

Physical and chemical characteristics of the groundwater are summarized in Table 2 (23).

The subsurface at the 6 to 9 m depth at the Dumfries, VA site is characterized by an extremely low oxygen concentration, constant 10° C temperature, nutrient-poor conditions, a substantial bacterial population, and low concentrations of nitrate and sulfate. Although the groundwater analyses indicated no that no phosphorus was present, there may be insoluble phosphorus compounds (and other nutrients) adsorbed to the soil.

Table 1. Bacterial populations in the subsurface at Dumfries, VA (23).

Depth (m)	Soil extract (cfu/g)	AO Direct Count
	NEW YORK SOIL	
0	$1.0 \times 10^7 \pm 4.0 \times 10^6$	$1.0 \times 10^8 \pm 4.1 \times 10^7$
1.8	$9.3 \times 10^5 \pm 1.1 \times 10^5$	$7.6 \times 10^7 \pm 3.8 \times 10^7$
3.7	$1.1 \times 10^6 \pm 8.5 \times 10^4$	$8.0 \times 10^7 \pm 6.4 \times 10^7$
	PENNSYLVANIA SOIL	
0	$3.0 \times 10^7 \pm 0.3 \times 10^7$	$5.6 \times 10^7 \pm 1.9 \times 10^7$
3-4	$3.5 \times 10^7 \pm 2.1 \times 10^3$	$3.9 \times 10^7 \pm 1.4 \times 10^7$
9	$1.4 \times 10^7 \pm 0.8 \times 10^5$	$4.6 \times 10^7 \pm 2.7 \times 10^7$
	VIRGINIA SOIL	
0	$9.7 \times 10^6 \pm 0.6 \times 10^6$	$1.0 \times 10^8 \pm 4.1 \times 10^7$
3	$\leq 10^3$	$7.3 \times 10^7 \pm 3.7 \times 10^7$
4.5	$3.3 \times 10^6 \pm 0.4 \times 10^6$	$3.1 \times 10^7 \pm 2.2 \times 10^7$
9	$5.6 \times 10^6 \pm 0.9 \times 10^6$	$3.9 \times 10^7 \pm 3.4 \times 10^7$
15-17	$5.2 \times 10^6 \pm 0.9 \times 10^6$	$1.0 \times 10^8 \pm 6.4 \times 10^7$
24-25	$9.8 \times 10^5 \pm 0.5 \times 10^5$	$7.3 \times 10^7 \pm 5.6 \times 10^7$
31	$1.1 \times 10^5 \pm 0.3 \times 10^5$	$4.8 \times 10^7 \pm 4.5 \times 10^7$

Table 2. Characteristics of the groundwater at three locations (23)

Water-Quality Analysis	Water-Quality Characteristics at Locations Shown		
	New York	Pennsylvania	Virginia
Cl^-	8.00 mg/L	9.94 mg/L	12.24 mg/L
Br^-	0.06	ND	0.32
NO_3^-	1.54	53.40	0.11
NO_2^-	1.20	ND	0.56
SO_4^{2-}	52.00	27.64	8.10
PO_4^{3-}	ND	ND	ND
Fe	0.05	0.44	4.23
Ca	73.80	22.60	2.88
Mg	14.40	4.65	3.88
Na	33.40	2.78	13.50
K	3.50	1.09	4.54
TOC	1.7	1.0	1.3
TOX	none	none	50.0 µg/L
Alkalinity	180	none	none
Dissolved Oxygen	0.7	6.7	0.2
Temperature (°C)	10.0	11.0	10.0
pH	7.81	4.73	4.50

ADDITION OF CARBON SUBSTRATES

Microbial growth and activity requires energy to proceed. That energy is provided by organic compounds which are utilized in the metabolic pathways which produce ATP. The subsurface environment is frequently a carbon-poor system due to its isolation from the surface and the sorption and utilization of organics in the upper soil layers. The sampling site at Dumfries, Virginia was no exception to this general rule, having a TOC of 1.3 mg/L.

Addition of organic compounds to this environment may have many direct and indirect effects on biodegradation. The compounds will provide carbon and energy for increased bacterial growth and activity. This in turn leads to a larger microbial population, but the relative dominance of species in the population may change. Oligotrophic microbes are adapted to an environment with only 1 to 15 milligrams of carbon per liter and may be inhibited at higher concentrations (33). Facultative oligotrophs may be able to adapt to levels of organics which are not very much higher than the oligotrophic range but would not effectively compete with organisms which could effectively utilize these concentrations. The effect on biotransformation would be dependent on the type(s) of degrading bacteria and their relative response to the organic addition.

The effects of these population changes on the degradation of methanol and TBA may be direct or indirect. A general stimulation of all microbes should result in enhanced biodegradation indirectly due to a higher level of uptake of all organics. Another indirect effect could be the stimulation of activity of bacteria which can degrade the alcohols by co-metabolism. The most direct effect would be enhancement of the growth and activity of an organism which has an enzyme system specific for methanol or TBA.

If the specific degrading organism is an oligotroph, the amount of organic addition may be crucial. Addition of low levels of carbon, within the oligotrophic range, may increase rather than inhibit the oligotrophs' growth. Oligotrophs are less selective and are not well protected from by-products so the composition and degrability of the organic may be very important, too. Ideally, an added substrate should be easily utilized by a wide variety of organisms.

This study was concerned with enhancement of the biodegradation of TBA at low concentrations. Low levels of a contaminant may persist because the energy derived from the compound as a substrate is not sufficient to support the metabolic requirements of the degrader population. A second substrate may allow the population to completely utilize the contaminant or may stimulate the production of non-specific enzymes which will transform both substrates.

Because of the complicated needs and restrictions of the microbial population, three different organic compounds were introduced to sets of microcosms. Two added substrates, pyruvic acid and citric acid, are easily utilized and common organics which take part in several metabolic pathways. The third, Bacto-peptone, is a heterogeneous source of energy in the form of carbon and also contains other nutrients, such as nitrogenous compounds. The carbon concentrations in the microcosms upon dosing ranged from less than 1 mg/L to about 200 mg/L.

One set of TBA-dosed microcosms was not dosed with additional substrate, this "reference" or "original condition" set is a standard for comparison of the other treatments. Figure 1 is a graph of TBA concentration versus time in the reference microcosms with two initial concentrations, approximately 1 and 2 mg/L TBA. The degradation rate in this set was similar to results from previous research with these concentrations of TBA in Virginia subsurface soil microcosms (23,25).

The variation in degradation rates observed in replicate microcosms is a reflection of the lack of homogeneity in the subsurface soils. Despite efforts to standardize soil amount and type in replicate tubes, no two microcosms can be identical.

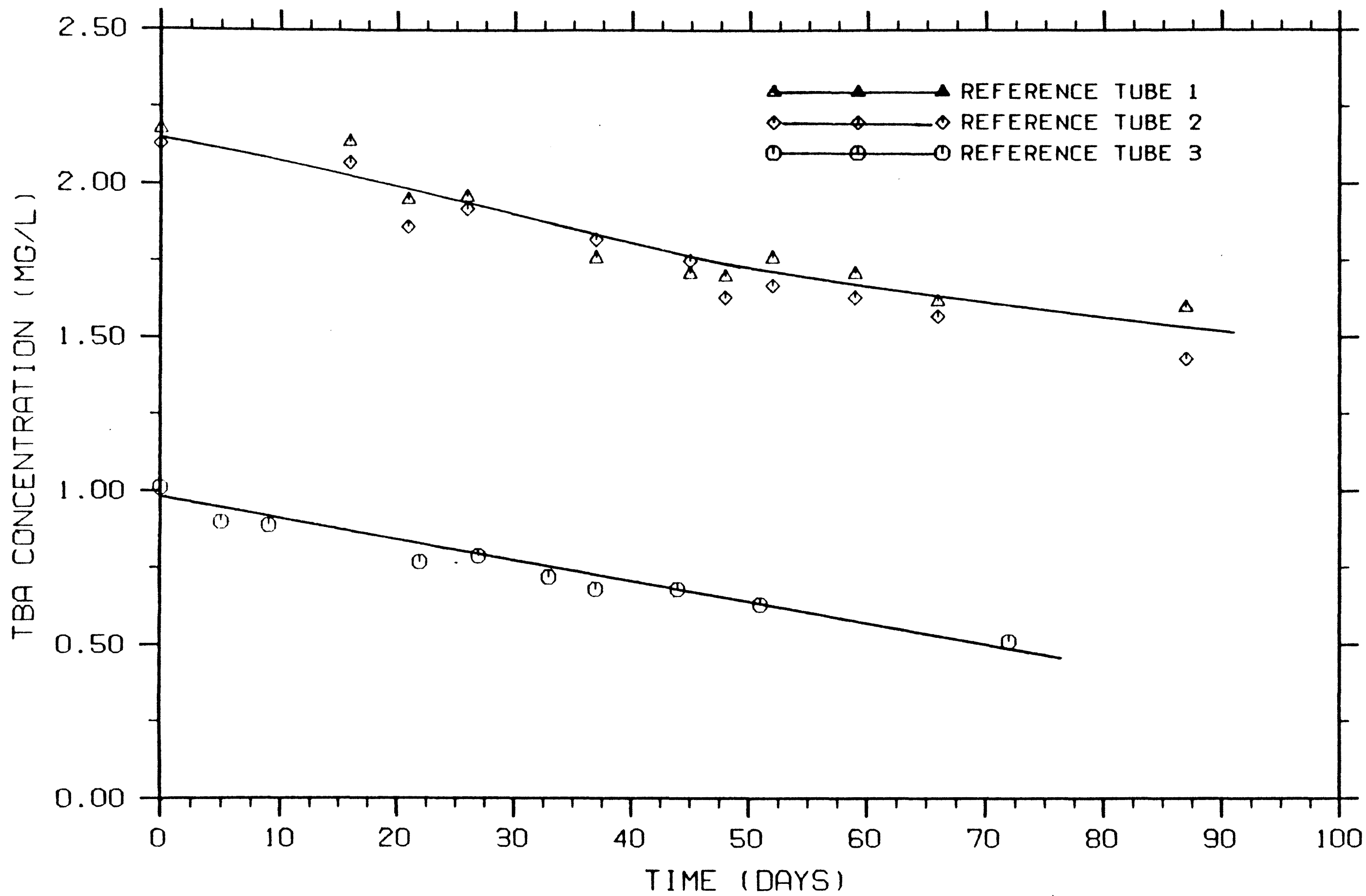

Figure 1. TBA degradation in reference microcosms: Two initial concentrations.

CITRATE/ CITRIC ACID: Addition of ferric or sodium citrate to the microcosms yields citric acid, a compound which is an intermediate in the Kreb's (TCA) cycle. Citric acid is a six-carbon tricarboxylic acid which may serve as a metabolic intermediate for biosynthesis. By providing a readily available energy and carbon source, citric acid can promote the growth and activity of many types of bacteria. Iron is an essential element and may affect biological activity itself, so two sets of microcosms were prepared for each citrate treatment, one with ferric citrate and one with sodium citrate. This should allow distinction of effects due to iron from effects due to citric acid. Figure 2 shows the averaged results of both ferric citrate and sodium citrate treatments at 1 and 5 mg/L citrate. This experimental subset was meant to investigate the effects of organic compound addition within the oligotrophic range. It was thought that strict oligotrophic bacteria would not be inhibited by these levels, especially the 1 mg/L treatment.

Degradation of TBA in these microcosms was slow and was similar to that seen in reference microcosms. Neither the associated cation (Fe or Na) nor the citrate concentration had an apparent effect on biodegradation. Lack of enhancement suggests that the citric acid either did not stimulate microbial activity at all or it did not stimulate bacteria capable of directly or indirectly degrading TBA. Perhaps the citric acid had no effect because its use was blocked by

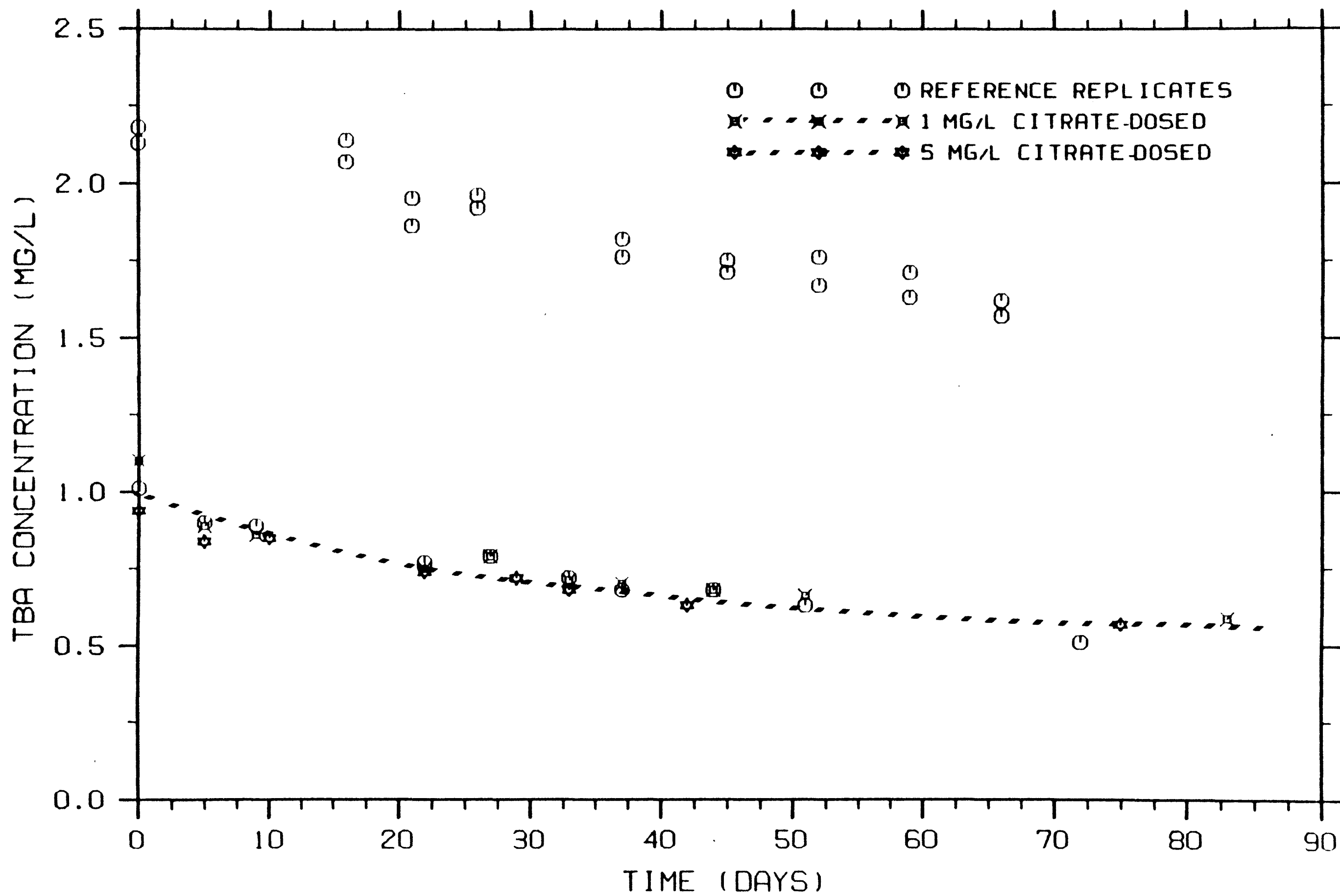

Figure 2. TBA degradation in citrate-dosed microcosms: Ferric and sodium citrate at 1 and 5 mg/L. Reference replicates included for comparison.

other limitations, such as oxygen, nitrate, sulfate, or nutrient deficiencies. Otherwise, the citric acid would provide energy to at least some of the bacterial species via the TCA cycle.

PYRUVATE/ PYRUVIC ACID: Pyruvic acid, a three-carbon carboxylic acid, is a common intermediate in metabolism. Referred to as the "crossroads compound", pyruvic acid is a key component of many biochemical pathways, both aerobic and anaerobic. Enzymatic decarboxylation and oxidation of pyruvic acid yields the important intermediate acetyl coenzyme A (Acetyl Co-A), which enters the TCA cycle (18). This substrate was added in the concentrations of 100 and 500 mg/L as pyruvate to determine the effects of relatively high concentrations of organics on biotransformation of TBA. Although strict oligotrophs should be inhibited by these organics levels, some facultative oligotrophs and other species may adapt to this condition. Less specialized subsurface bacteria may be stimulated a great deal by the addition of these concentrations of pyruvic acid.

The results, shown in Figure 3, indicate little change in TBA degradation rate from pyruvic acid addition. The high levels of substrate may have inhibited oligotrophic degraders, but the previous low-carbon treatment did not have a positive effect either. These results indicate that the addition of these organics at any level does not enhance de-

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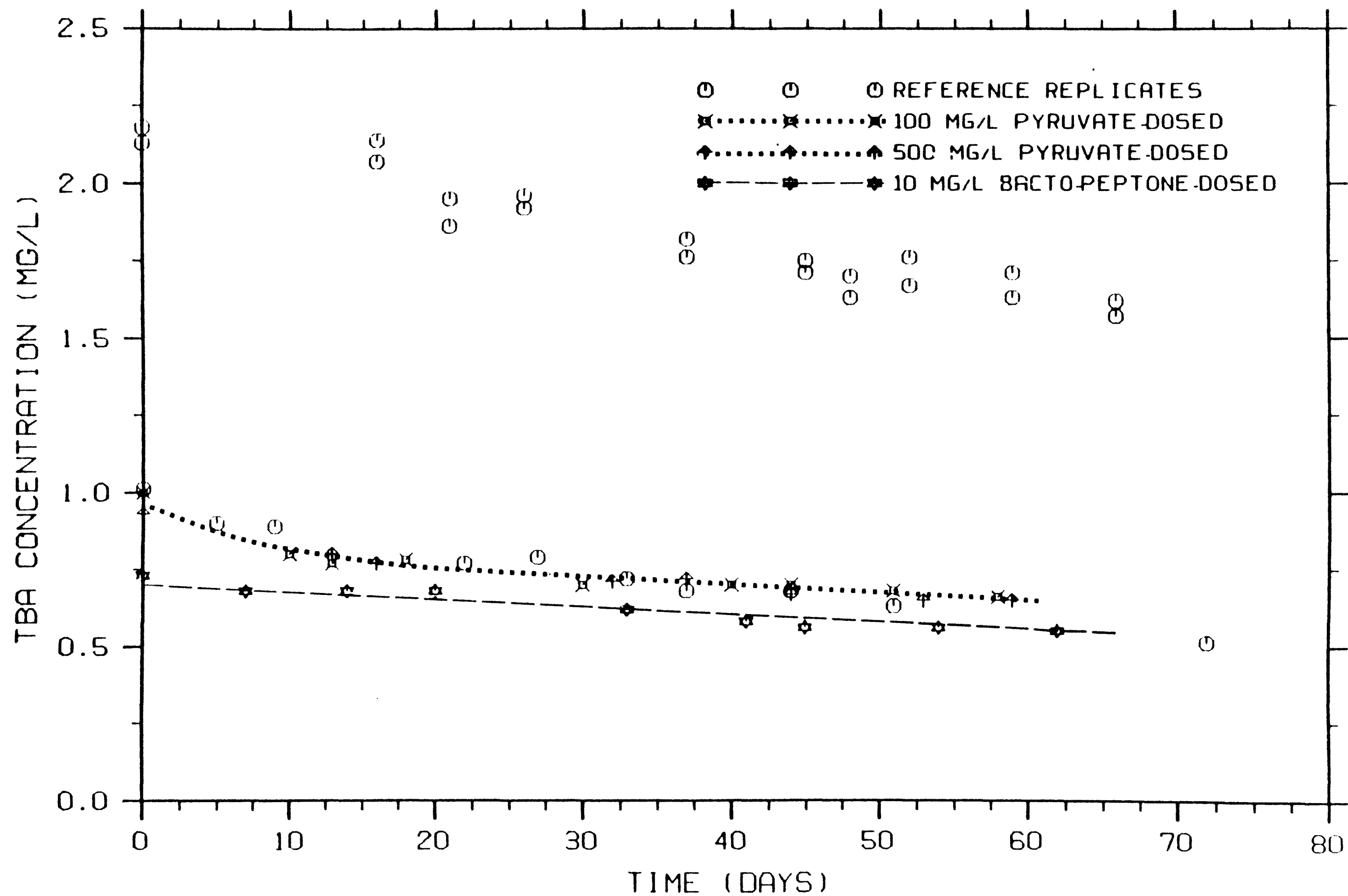

Figure 3. TBA degradation in pyruvate- and Bacto-peptone-dosed microcosms: Concentrations are averages of replicates, reference results provided for comparison.

at all it must be limited by conditions other than carbon and nutrient deficiencies.

Even if the degradation of TBA is purely incidental to the microbes in the microcosms, as in co-metabolism, the rate of removal should increase with a larger and more active population. However, if there are only a few species of bacteria which can degrade the recalcitrant TBA molecule, only those microbes must be stimulated to effect enhancement of TBA degradation. Because enhancement was not observed, the conclusion reached is that the addition of these substrates does not stimulate the growth of TBA degraders (if present), and may not stimulate the activity of any bacteria. The reasons for the lack of stimulation could include low pH, absence of dissolved oxygen, lack of alternative electron acceptors, or nutrient availability.

These results are in agreement with previous research findings which have demonstrated a direct relationship between the TBA concentration in microcosms and the TBA biodegradation rate (23). Very low TBA concentrations were shown to be particularly recalcitrant.

ALTERNATIVE ELECTRON ACCEPTORS/ VARIED PH

A significant limitation in these systems is the lack of oxygen to complete the electron transport system. It was postulated that the addition of alternative electron

acceptors might allow a higher level of microbial growth and more efficient use of carbon substrates, including methanol and TBA. Addition of oxygen would constitute a similar approach, but that technique is more difficult to implement because of the relatively low solubility of oxygen in water and the difficulties encountered in continuously supplying air or oxygen to the microcosms without introducing contamination.

A study by Mulheren found evidence of incomplete nitrate reduction in nitrate-dosed microcosms (25). After an initially rapid degradation period, the biodegradation of methanol slowed abruptly. Analysis of the microcosm water showed the accumulation of nitrite in the microcosms. Addition of sulfate was slightly beneficial for methanol removal, but not appreciably different from the non-amended treatment in rate of biodegradation. These findings are presented in Figure 4. The upper graph shows the buildup of nitrite over time in the nitrate-dosed tubes. There is an obvious inverse relationship between nitrite concentration and methanol degradation rate, suggesting nitrite inhibition. It was hypothesized that the low pH of the Virginia subsurface contributed to the nitrite toxicity.

In this study, an attempt was made to expand on Mulheren's work by varying the pH in microcosms dosed with 100 mg/L of methanol and 5 mg/L TBA and either nitrate or sulfate or neither alternative electron acceptor (non-amended). The

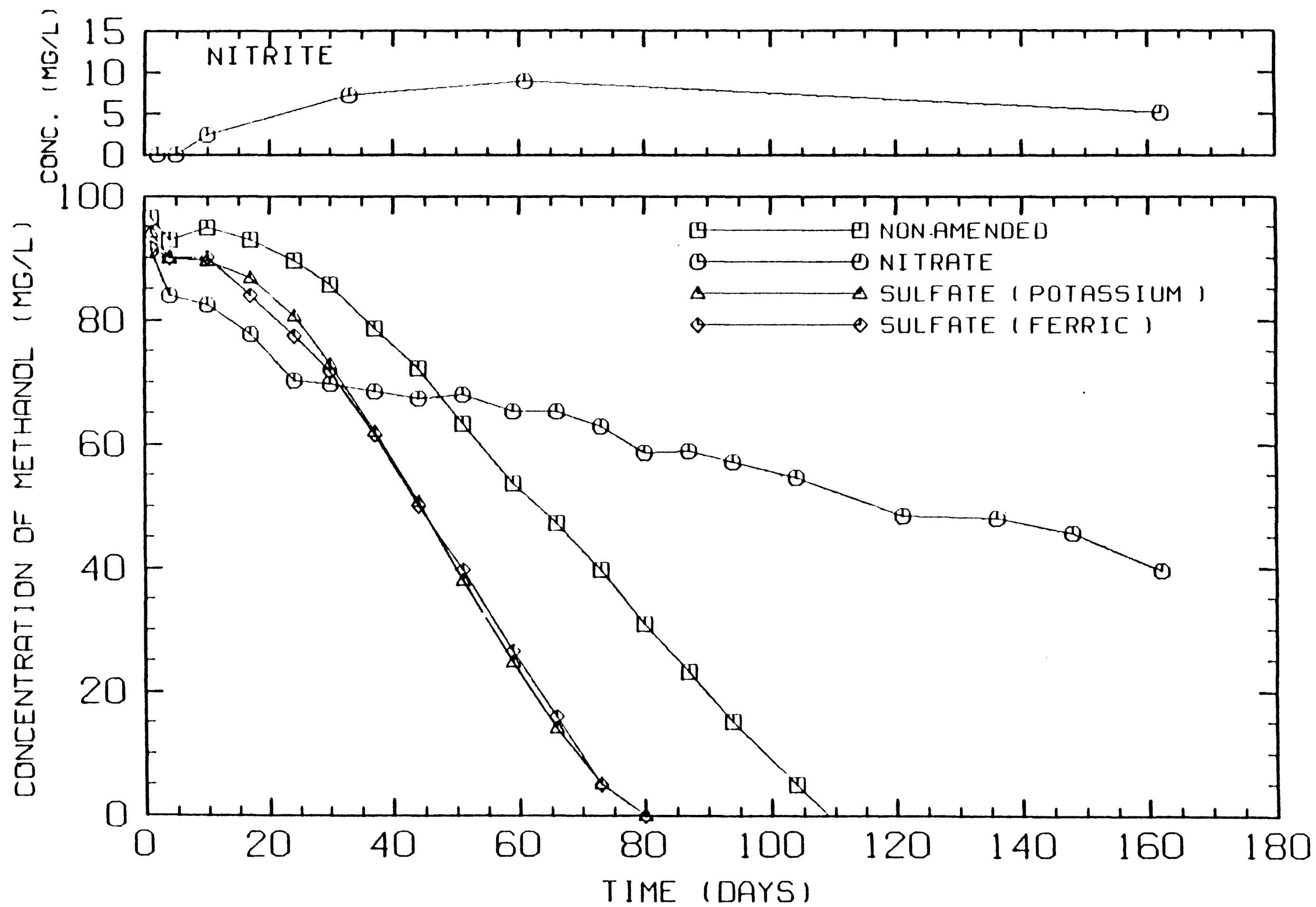

Figure 4. Degradation of methanol with and without electron acceptors (25): Dumfries, VA soil, 100 mg/L nitrate and sulfate, note nitrite.

microcosms which had no base added had a pH of 5.1 upon assembly. The microcosms which were dosed with base had initial pH levels of 6.0, 7.1, and 8.8, corresponding to NaOH additions of 4, 10, and 20 meq/L, respectively. These pH values indicate only the initial conditions in the tubes. No buffers or other agents were added to maintain these pH levels. Bacterial activity probably caused some changes in the microcosm pH over time. Varied pH was expected to affect nitrate-dosed microcosms most because of the pH effect on nitrite toxicity, but sulfate-dosed and non- electron acceptor-dosed microcosms were also dosed with base to raise their pH levels. If the biodegradation process is a result of co-metabolism or otherwise not directly influenced by microbial activity, the fastest rate should be expected to occur at the optimum pH for the degradative enzyme.

The horizontal sterile control lines on the concentration versus time graphs are evidence that the mechanisms of methanol and TBA removal are biological. Both alcohols are very soluble and do not sorb to microcosm soil particles to any apparent degree. The controls suggest that no abiotic transformations occur.

NON-AMENDED

Non-amended microcosms (no nitrate or sulfate added) which had no base added to raise the pH above the original

5.1 were the standard or reference set. These microcosms should most closely model the natural Dumfries, Virginia groundwater system because only methanol and TBA were added. The degradation rates of methanol and TBA for this set ,shown in Figures 5 and 6, were similar to those previously reported for subsurface microcosms dosed with similar concentrations (25).

Non-dosed microcosms with an initial pH of 6.0 or 7.1 exhibited similar methanol degradation to this reference set. The highest level of base addition resulted in diminished biodegradation. Microcosms with 20 meq/L of base (pH 8.8) had a considerably slower methanol degradation rate. The methanol degradation for the higher pH, non-amended microcosms is graphed in Figure 7. TBA degradation in the pH 6.0, 7.1, and 8.8 microcosms is shown in Figure 8. It seems that the highest pH level was either inhibitory to the microbial population, or to the enzymes which attack methanol and TBA, or both. TBA degradation was also incomplete in some replicates of the lower pH treatments. Overall, the pH 6.0 and 7.1 microcosms had TBA degradation rates which were similar to the reference group's.

Except for the 20 meq/L NaOH addition, varying the pH of these microcosms seems to have little effect on degradation.

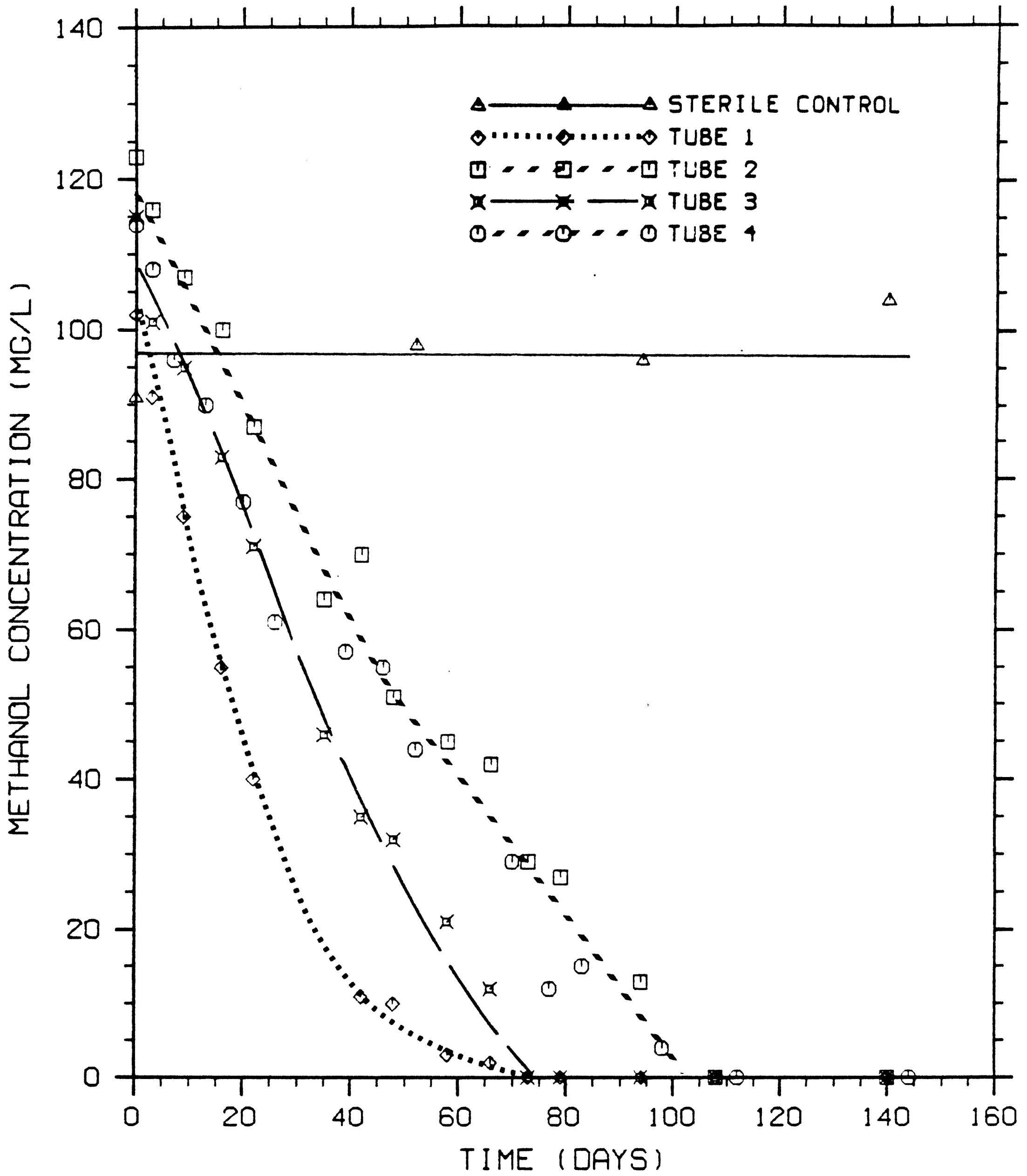

Figure 5. Methanol degradation in pH 5.1 (reference) microcosms: No nitrate or sulfate or base added.

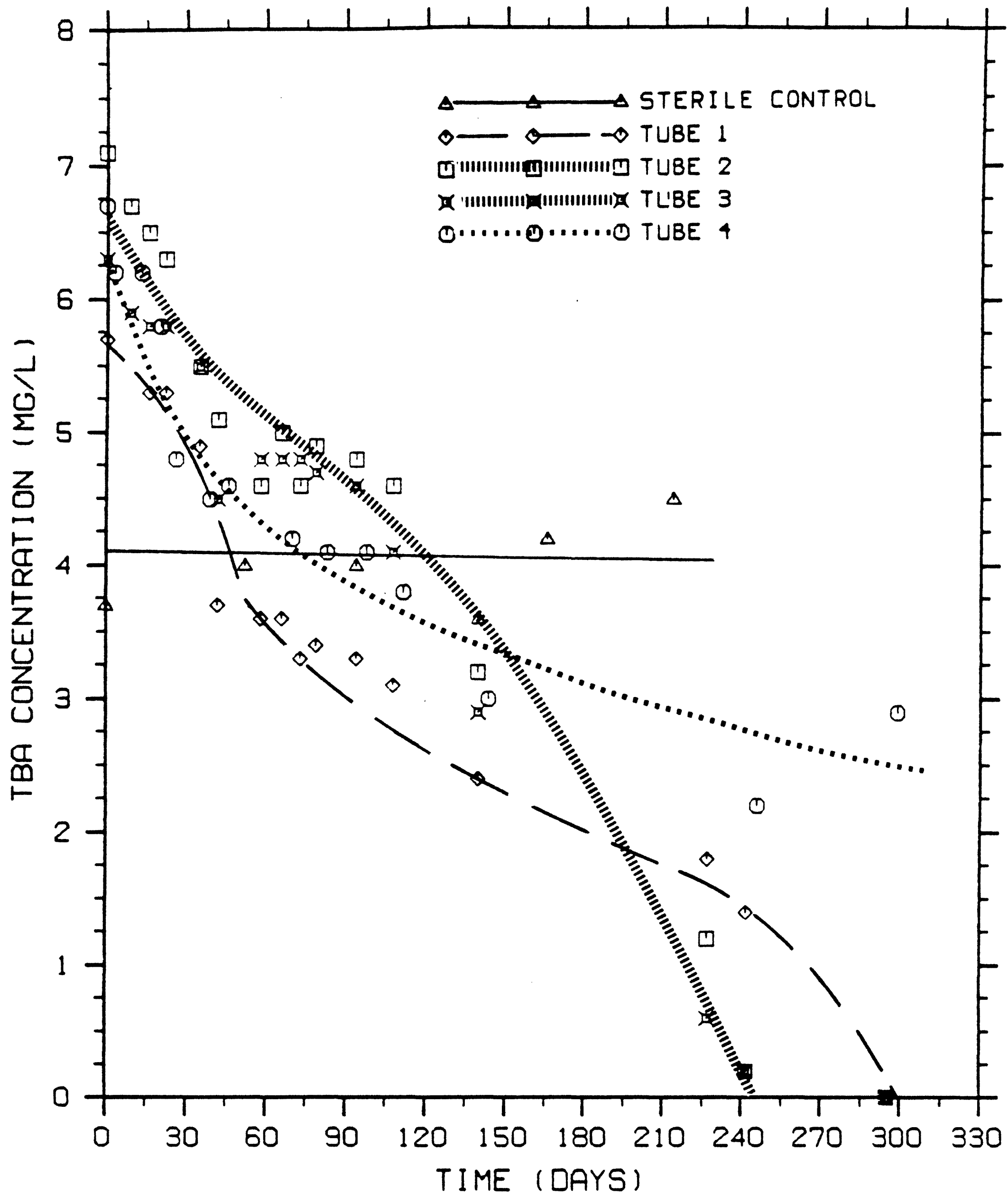

Figure 6. TBA degradation in pH 5.1 (reference) microcosms: No nitrate or sulfate or base added.

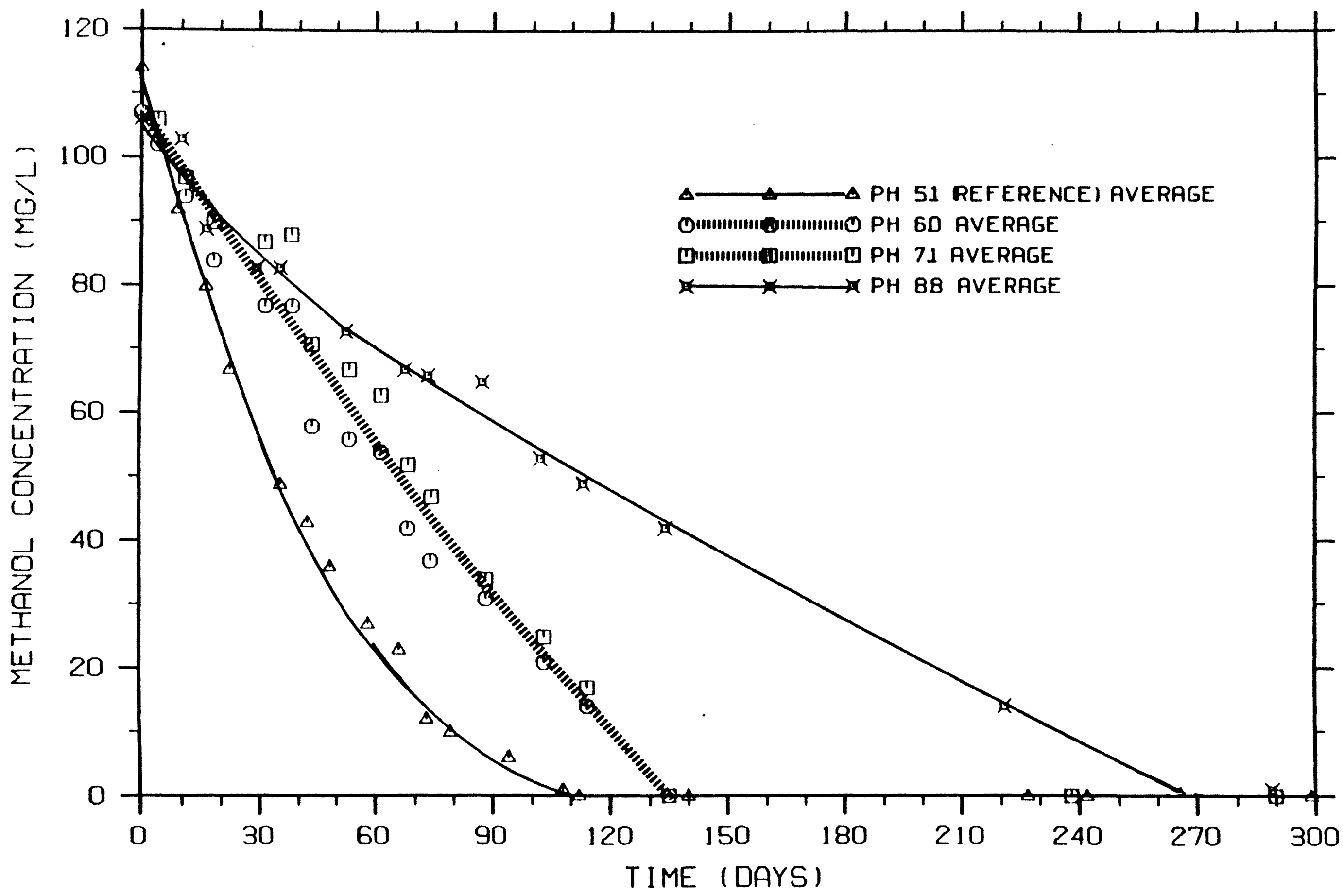

Figure 7. Methanol degradation in base-dosed microcosms and reference set: No nitrate or sulfate added; Concentrations shown are averages of replicates.

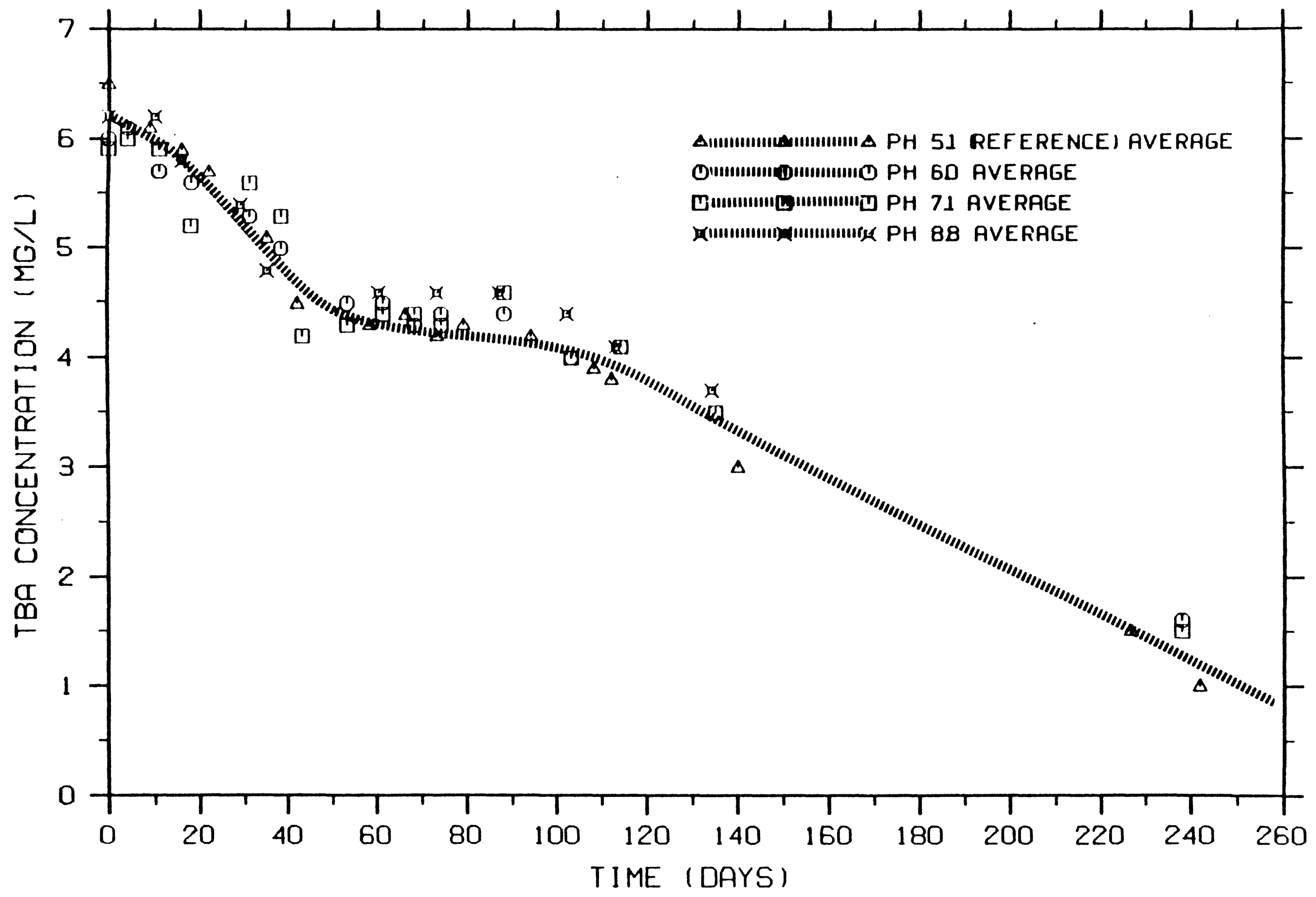

Figure 8. TBA degradation in base-dosed microcosms and reference set: No nitrate or sulfate added; Concentrations shown are averages of replicates.

SULFATE-DOSED

Previous work with 100 mg/L sulfate-dosed microcosms showed slight enhancement of methanol degradation and mixed results with TBA (25). Sulfate-dosed sets in this most recent experiment generally had slower degradation rates for both alcohols than seen in the reference set. There was quite a bit of variability in the results from some of these tubes. This has been noted throughout these experiments in microcosms which have displayed inhibited biodegradation. There is usually more "scatter" in the data when degradation is slowed, possibly because of the variable responses to toxicity in each microcosm.

The pH 7.1 subset exhibited biodegradation rates which were similar to the reference rates for both methanol and TBA utilization. Degradation in all other subsets was inhibited, as shown in Figure 9 and Figure 10.

The highest pH, sulfate-dosed treatment exhibited two distinct methanol degradation rates (see Figure 11). Methanol degradation in all three tubes was similar to the reference rate for the first 60 days, then slowed abruptly so that the time to complete methanol degradation was approximately twice that for reference, non-dosed microcosms. Interestingly, the TBA degradation rate in these pH 8.8 microcosms also changed with time. Figure 11 shows a TBA rate similar to the reference rate for 60 days, then almost

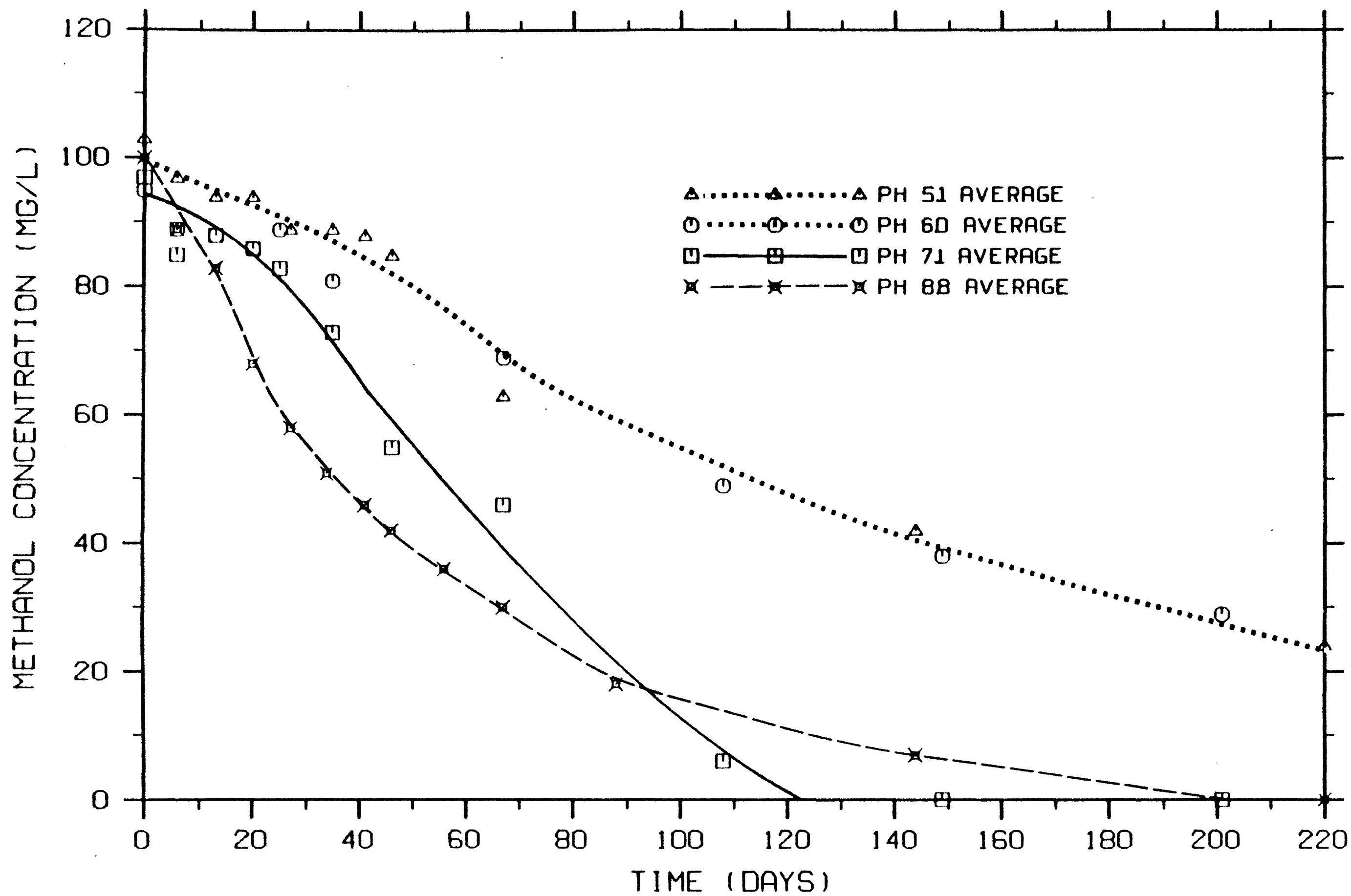

Figure 9. Methanol degradation in sulfate-dosed microcosms, varied pH.: Sulfate concentration is 100 mg/L.

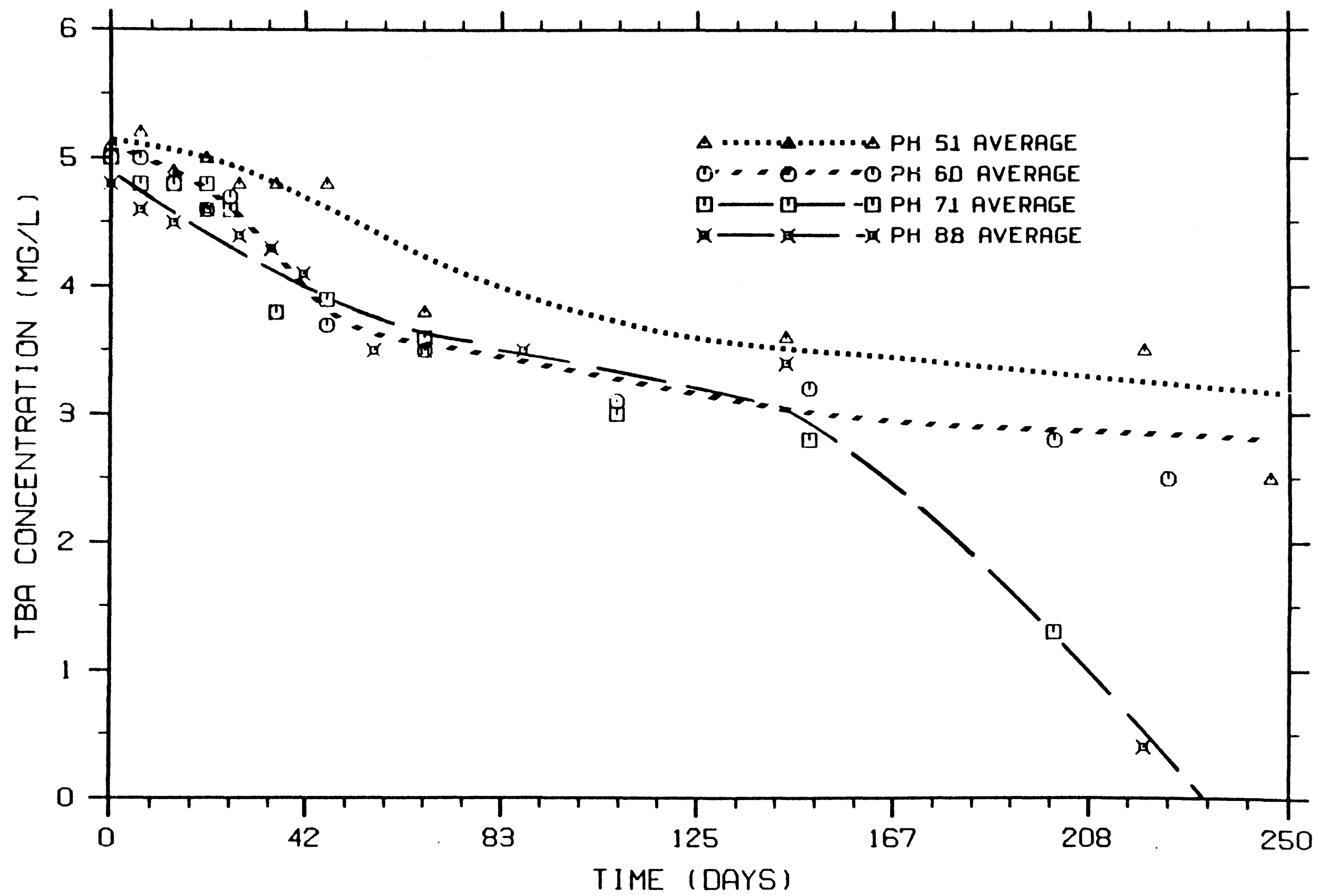

Figure 10. TBA degradation in sulfate-dosed microcosms: Sulfate concentration is 100 mg/L.

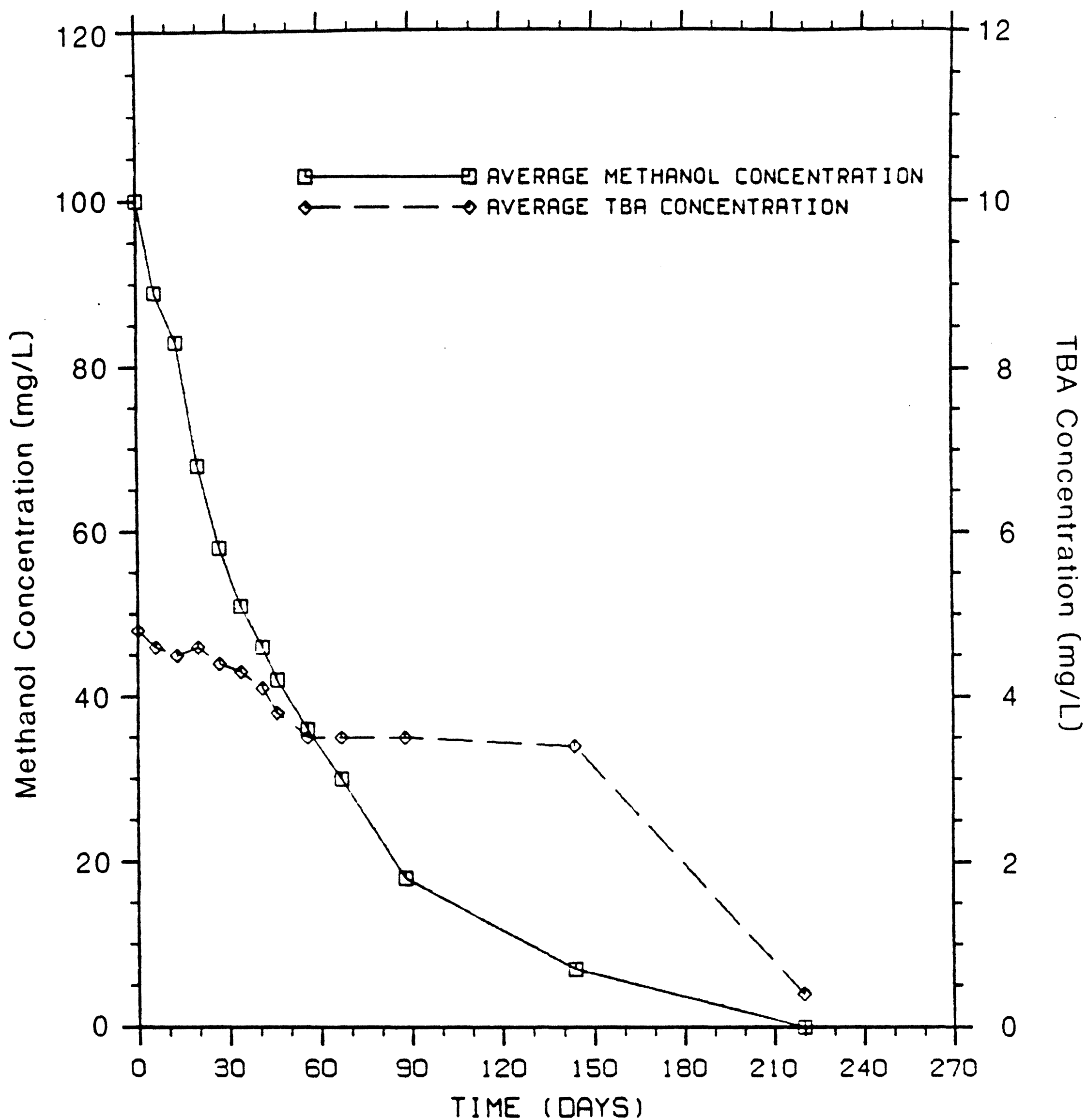

Figure 11. Methanol and TBA in pH 8.8, sulfate-dosed microcosms: Sulfate concentration is 100 mg/L, base is 20 meq/L. Diauxic growth effect apparent at 150 days.

no degradation for 100 days, then finally a high level of biodegradation beginning at approximately the same time that the methanol was completely degraded. This sequential degradation of first methanol, then TBA corresponds well with the diauxie growth theory. It is not known why this is seen in this particular set of microcosms, but Goldsmith also found isolated examples of diauxie growth (23).

No black sulfide precipitate was seen in any of the sulfate-dosed microcosms. This suggests incomplete reduction of sulfates, possibly because the oxidation-reduction potential was too high or due to a lack of sulphate-reducing bacteria. Lack of visible sulfide does not necessarily indicate a complete absence of sulfide, and even low concentrations of toxic sulfide could inhibit bacterial growth and alcohols degradation. Variable concentrations of sulfide in different microcosms and differing responses to its toxicity could explain the lack of consistency between some replicates.

NITRATE-DOSED

In order to further explore the effects of alternative electron acceptors on biodegradation, the next set of microcosms was dosed with KNO_3 to produce a microcosm concentration of 100 mg/L nitrate. Use of nitrate as an electron acceptor results in a higher energy yield than use of other anaerobic respiration electron acceptors. Another

advantage to this method is that the microbes which can use nitrate in respiration are not all obligate anaerobes , and some denitrifier species have been identified in groundwater (11,12).

Results from the pH 5.1, 100 mg/L nitrate microcosms did not correspond completely with previous research. Although nitrite inhibition of methanol degradation had been reported for a similar treatment (25), the rates in two of the microcosms were faster than non-dosed rates. These results are presented in Figure 12. The third microcosm exhibited an initial rapid methanol degradation rate, identical to the other tubes, for about 30 days, then the rate decreased abruptly to a moderately slow level of biodegradation. This suggests partial nitrite toxicity in this tube. TBA biodegradation in these microcosms, shown in Figure 13, was clearly inhibited. The initial, normal degradation rate slowed dramatically at approximately 60 days, the same time that methanol disappeared from tubes 1 and 2. The TBA concentration was essentially static from 100 to more than 300 days.

In order to more definitively determine nitrite toxicity, water from two of the pH 5.1 microcosms was analyzed for the presence of nitrite and nitrate ions on the ion chromatograph after 331 days. Nitrite and nitrate were both present in these microcosms, suggesting both incomplete use of nitrate and toxic effects from nitrite. Other microcosms

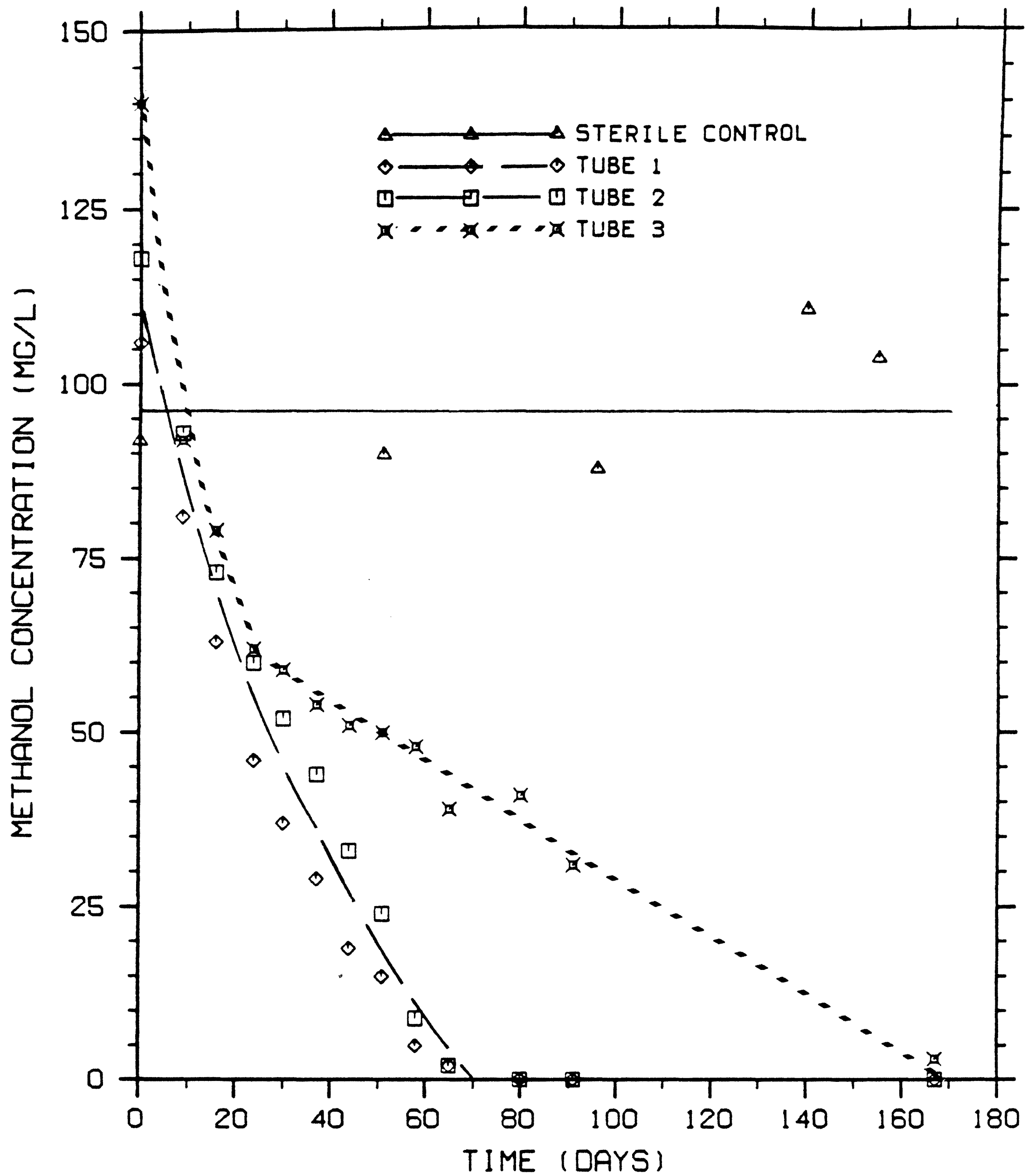

Figure 12. Methanol degradation in pH 5.1, nitrate-dosed microcosms: Nitrate concentration is 100 mg/L, no base added.

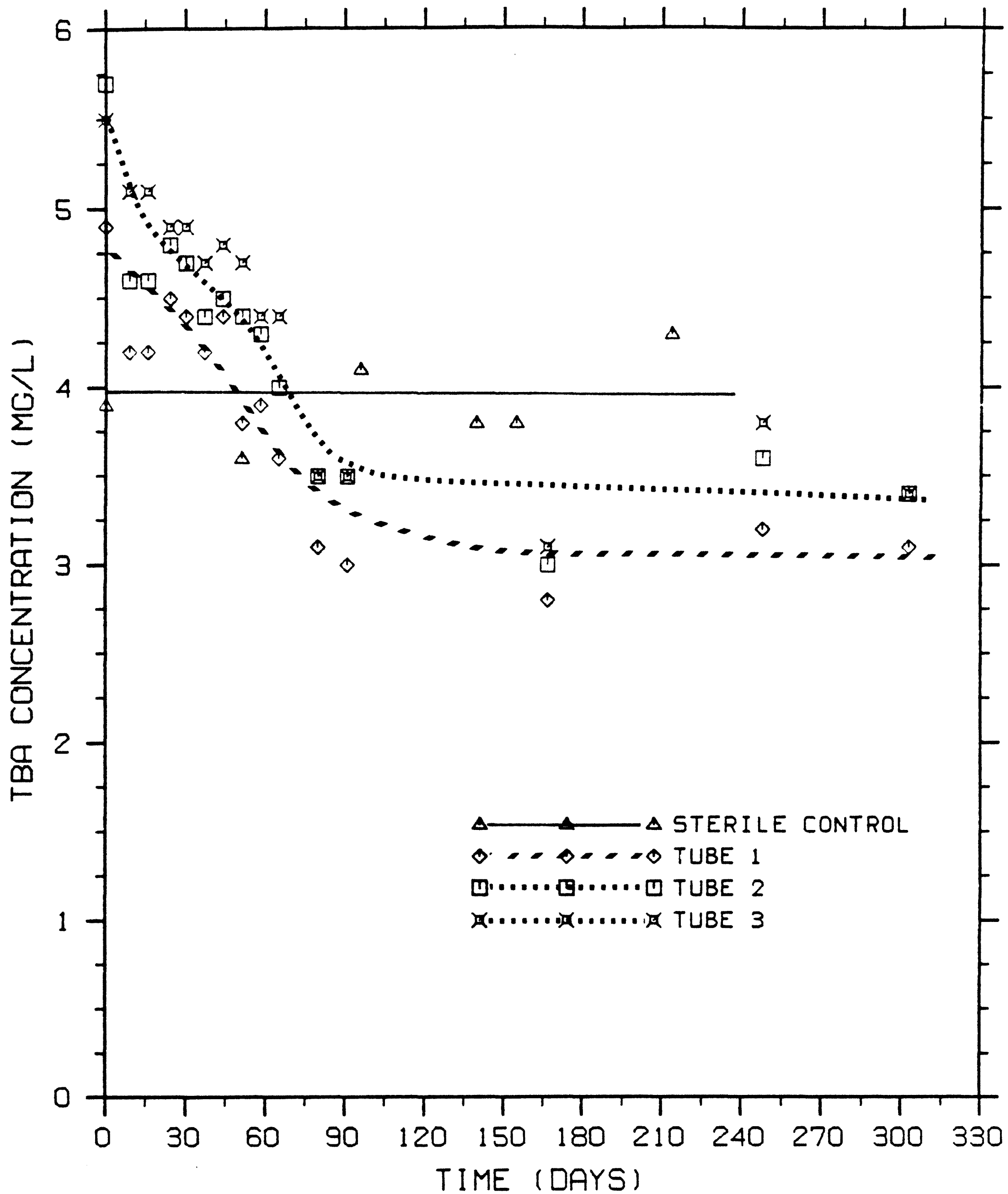

Figure 13. TBA degradation in pH 5.1, nitrate-dosed microcosms: Nitrate concentration is 100 mg/L, no base added.

with higher pH levels were also analyzed. None of the microcosms with an initial pH greater than 5.1 had detectable nitrite or nitrate. Results of analysis of the anions in the free water by ion chromatography are tabulated in Table 3.

Microcosms dosed with nitrate and base to adjust the initial pH to 6.0 exhibited enhanced rates of methanol degradation. The time to complete degradation of 90 mg/L methanol was about one half that seen in the reference set. Degradation of TBA was approximately the same as the reference in one microcosm, but much faster in the other two. This is quite a different effect than the previous treatment which differed only in pH. The potential nitrite toxicity would seem to be absent in this treatment, as supported by the lack of nitrite build-up and complete nitrate utilization shown in the ion analysis.

Methanol degradation was further enhanced in the pH 7.1 treatment. The degradation rate of methanol in these tubes was 3 to 5 times as fast as the reference (original condition) rates. TBA biodegradation did not follow suit, however. The TBA rates in all these tubes was similar to the reference rates. This further shows the lack of nitrite toxicity at higher pH levels, but seems anomalous when one considers the apparent enhancement of TBA rates at pH 6.0. One possible explanation could be the preferential use of methanol in these microcosms, but there seems to be no diauxie growth effect. These data are averaged and compared

Table 3. Aqueous nitrite and nitrate concentrations in microcosms

All microcosms initially dosed to 100 mg/L nitrate.

Initial pH	Day analyzed	Methanol (mg/L)	TBA (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)
5.1	331	0	3	8.6	15
5.1	331	0	3	6.2	32
6.0	291	0	0	<0.01	<0.01
7.1	291	0	0	<0.01	<0.01
8.8	342	0	0	<0.01	<0.01

to the non-amended reference microcosms' average in Figure 14 and Figure 15.

The inhibition seen in non-amended, pH 8.8 microcosms was absent in the nitrate-dosed, pH 8.8 set. Methanol degradation was once again enhanced, nearly as much as in the pH 7.1 set. A significant result was the high TBA biodegradation rate in each microcosm. The rate of TBA removal in these tubes was 2 to 3 times as fast as the reference rate. As seen before in enhanced conditions, the rate was uniform in the replicates, see Figure 16. Perhaps enhancement was only obvious in the highest pH treatment because the TBA degradation process is more sensitive to nitrite and the lower pH levels do not completely eliminate the toxic effects. It may be that the TBA degradative enzymes work best at a pH above 7.

As mentioned before, the pH of the microcosms is only known at the beginning of the experiment. Biological processes which produce acidic by-products may lower the pH of the basic microcosms. Since nitrite toxicity has been shown to be irreversible (39), perhaps the prevention of toxic effects for enough time will allow further reduction of the nitrite to less than toxic levels, even when the pH is lowered later. Since these microcosms are static, by-products will not be dispersed and may cause changes in the system. This may not be a serious defect since the same phenomenon

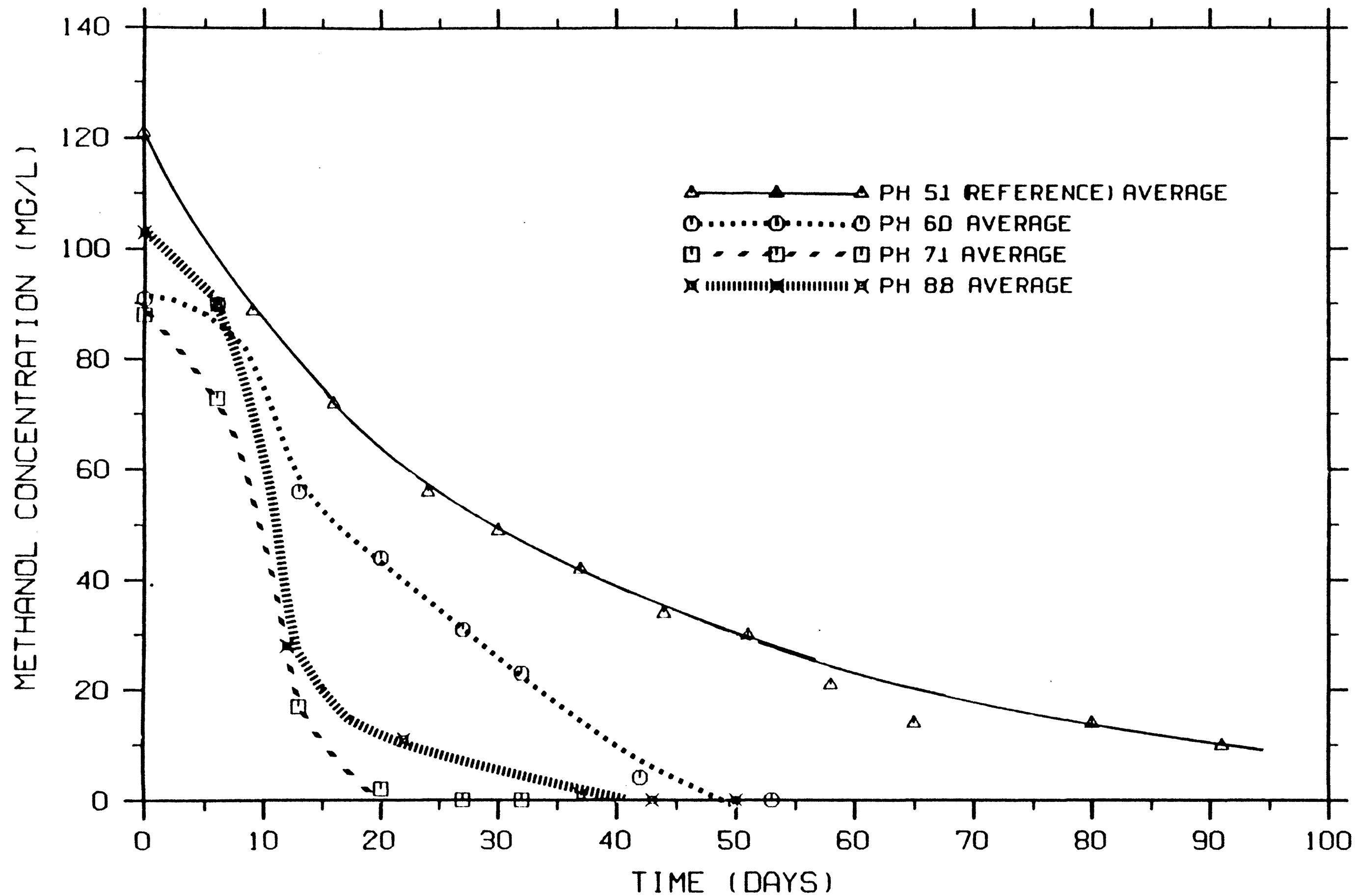

Figure 14. Methanol concentrations in base- and nitrate-dosed microcosms: Nitrate concentration is 100 mg/L. Reference (non-amended) results shown for comparison

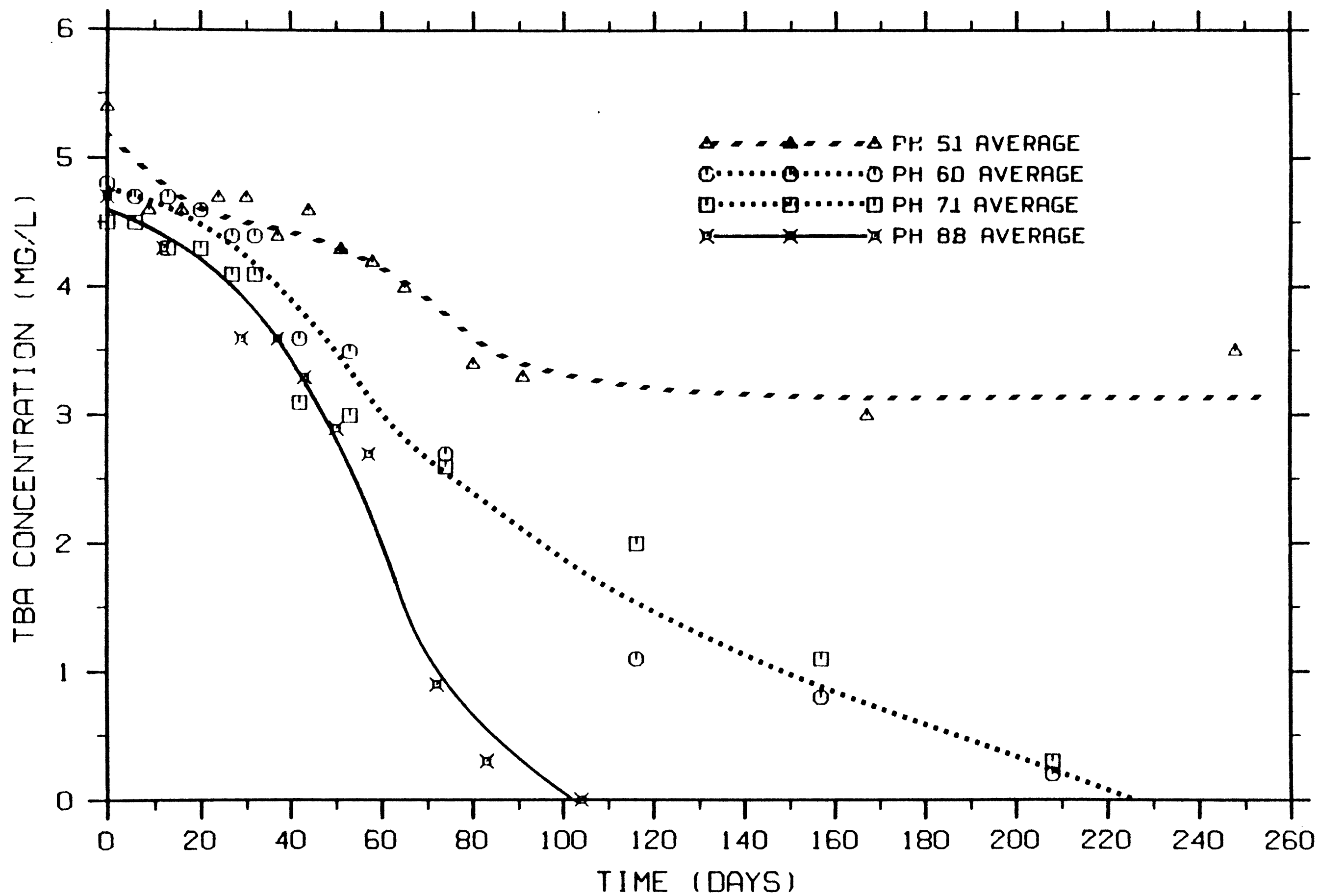

Figure 15. TBA concentrations in base- and nitrate-dosed microcosms: Nitrate concentration is 100 mg/L. Reference (non-amended) results shown for comparison.

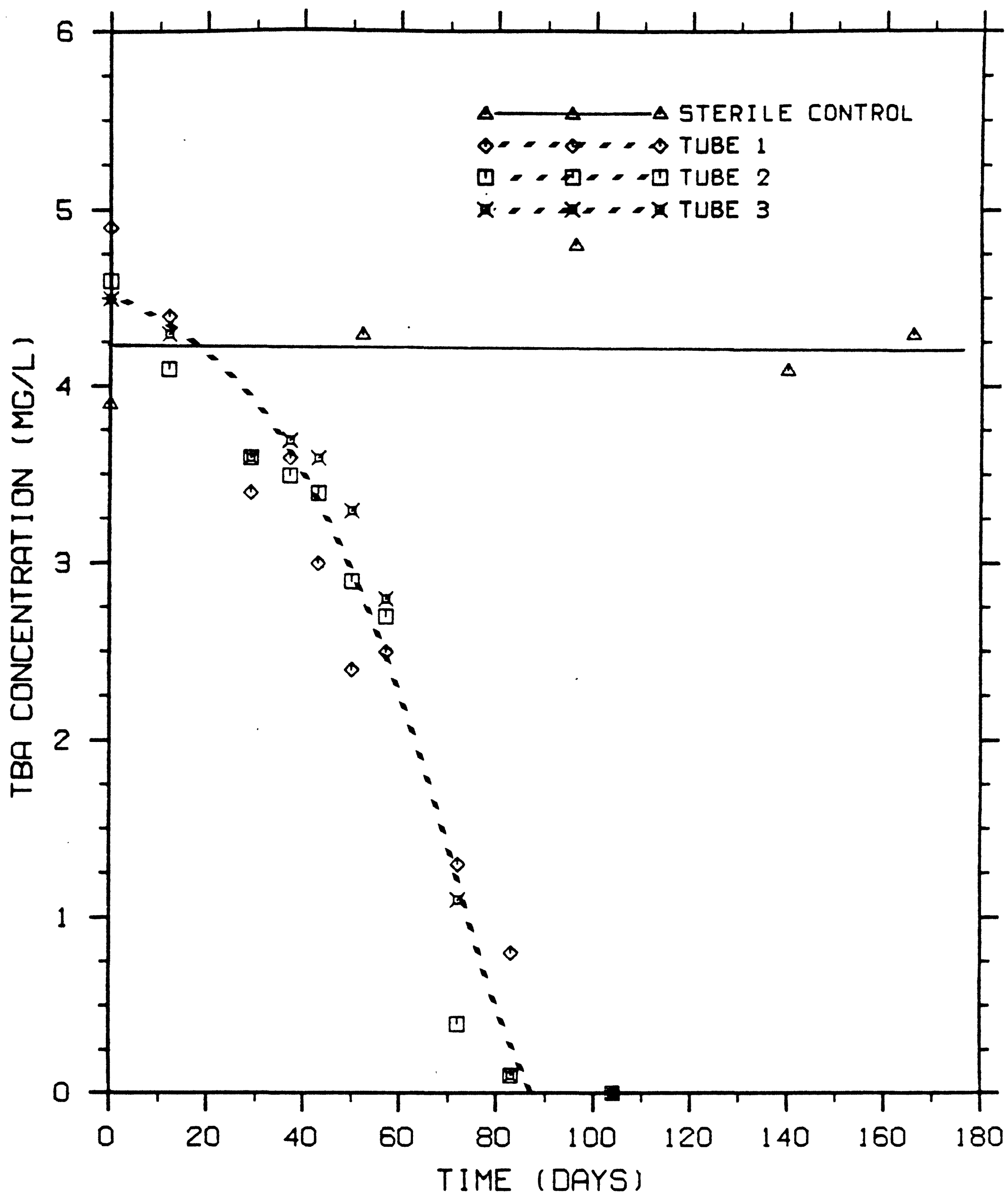

Figure 16. TBA concentrations in pH 8.8, nitrate-dosed microcosms: Nitrate concentration initially was 100 mg/L, base was 20 meq/L.

may occur *in situ* due to extremely slow groundwater flow in many aquifers.

OVERVIEW OF RESULTS

The addition of organic substrates at various concentrations did not enhance TBA degradation in subsurface microcosms. This may have been due to the minimum substrate concentration effect proposed by Bouwer and McCarty (36) since the TBA concentration was about 1 mg/L. Different organics were added in varying concentrations in order to monitor the effects of substrate addition both within and above the organic concentration range reported for oligotrophic conditions. Lack of response indicates that there are other limitations on the growth and activity of TBA degrading bacteria besides organic carbon supply. The addition of a heterogeneous carbon and nitrogen supply (Bacto-peptone) was as ineffectual as the addition of simple organics alone. This suggests that the conditions within the microcosms were not conducive to the use of these common substrates. Subsequent experiments explored the effects of pH and alternative electron acceptor addition on this low pH, oxygen deficient system.

Non-amended microcosms did not respond to increased pH with enhanced biodegradation. The highest pH treatment caused inhibition of both methanol and TBA degradation.

White has proposed that the mechanism of degradation of these alcohols in the subsurface may be a broadly-specific, extracellular enzyme (24). If this were the case it might be expected that an optimum pH would be evident in the pH range investigated in these experiments. Perhaps the enzyme is the prime mechanism, but its optimum pH is not within the range of pH 5.1 to 8.8.

Addition of sulfate was generally unsuccessful in stimulating alcohols degradation. The optimum pH for biodegradation in sulfate-dosed microcosms appeared to be near neutrality. All other pH treatments had lesser biodegradation rates than the non-amended sets.

Nitrite toxicity was evident in the nitrate-dosed microcosm set with a pH of 5.1. A higher pH allowed enhancement of methanol degradation to proceed, especially at pH 7 and above. Biodegradation of TBA was moderately stimulated in some tubes at pH 6.0 and all pH 8.8 microcosms. However, the TBA biodegradation rate in pH 7.1 microcosms was about the same as the reference rate. This uneven behavior is difficult to predict or explain.

The uptake rates were calculated as one overall rate from the time of dosing to the time of complete removal of the alcohol in each microcosm. The rate for each microcosm tube is given to show the variability of the data. The same symbols were used for each tube in both graphs to allow comparisons of methanol and TBA rates. In case of incomplete

biodegradation this "zero concentration time" had to be estimated. The uptake rates were normalized on a soil mass basis to allow more accurate comparison between microcosms, although there was little variability in soil amounts. These results are presented graphically in Figure 17 for methanol, and Figure 18 for TBA.

Methanol rates are, overall, highest in nitrate-dosed microcosms and lowest in sulfate-dosed microcosms. The enhancement of methanol biodegradation with increasing pH in nitrate-dosed microcosms is readily apparent. The stimulatory effects of nitrate and the inhibitory effects of nitrite on biodegradation are evident in these results. The addition of base alone had little effect, and the addition of nitrate alone was inhibitory, but the combined addition of base and nitrate caused a marked increase in methanol utilization. This information, combined with the ion analyses, indicates that nitrate can increase degradative activity in oxygen-deficient groundwater systems as long as nitrite does not accumulate. The higher pH prevents or lessens the toxic effects of nitrite and allows complete reduction of the nitrate to proceed.

The TBA rate values are approximately two orders of magnitude lower than the methanol uptake rates. The TBA data

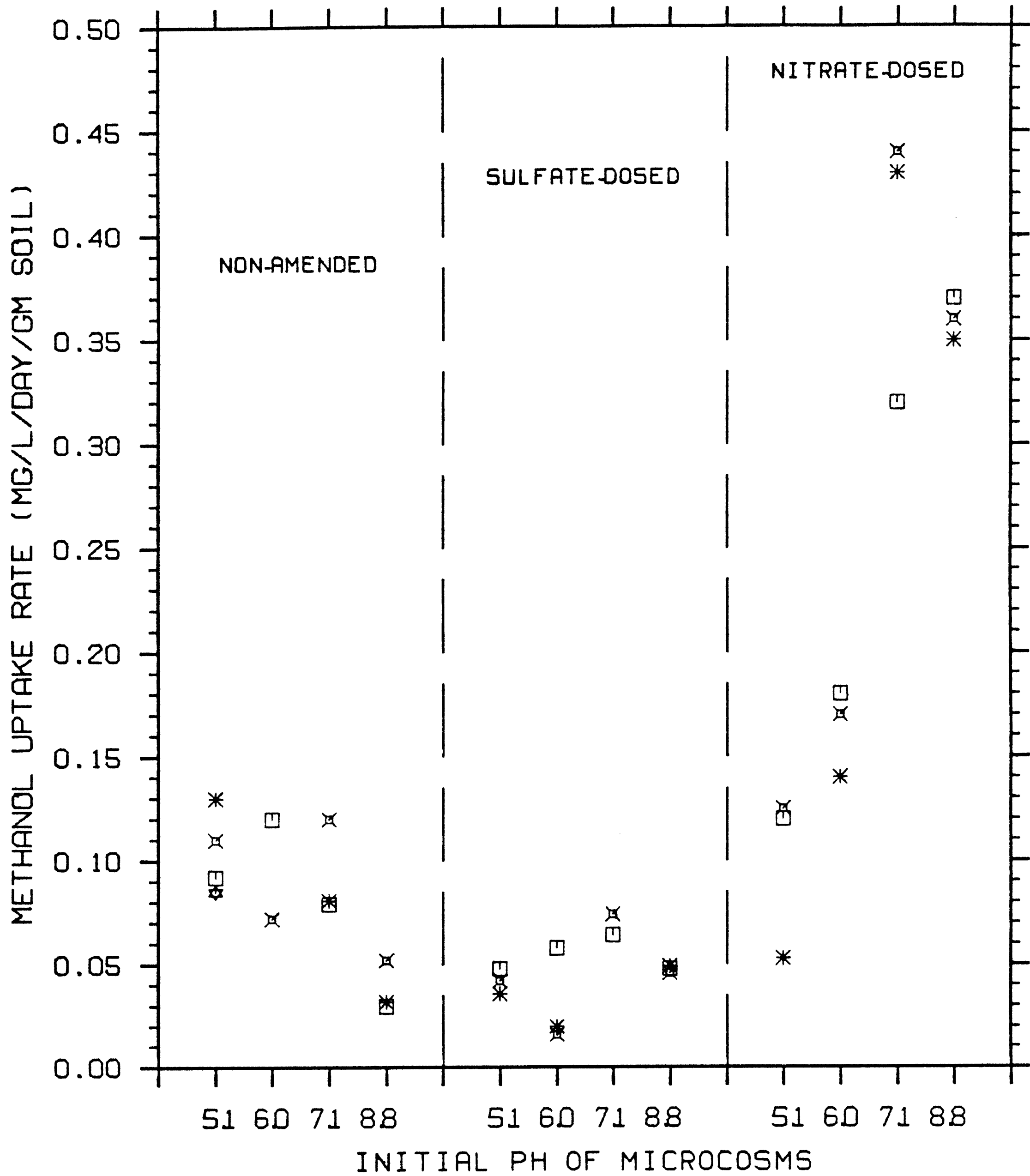

Figure 17. Methanol uptake rates in various microcosms: Overall rate of methanol utilization is expressed in units of mg/L per day per gram of soil in the microcosm. Each point represents the rate in a single replicate.

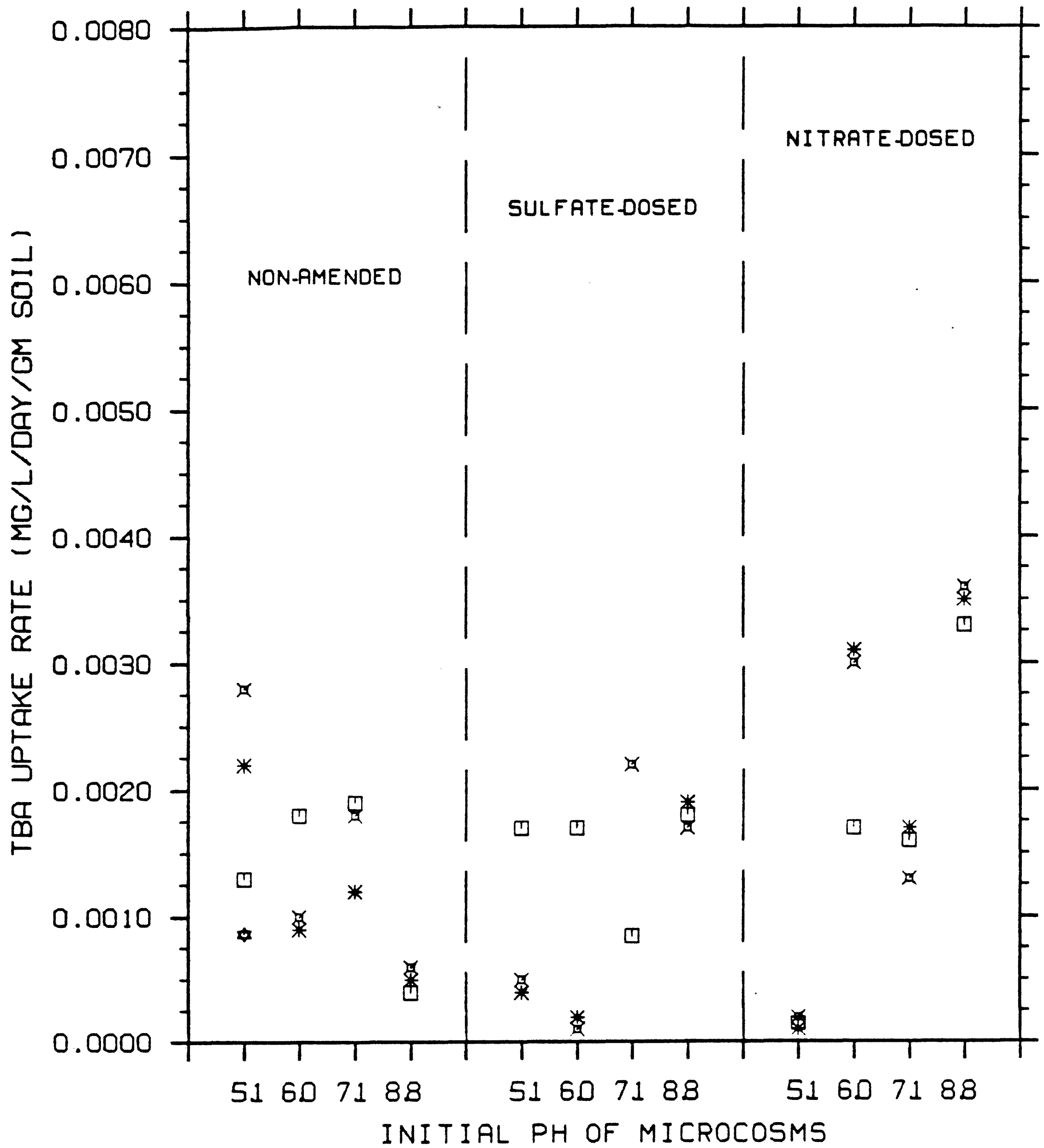

Figure 18. TBA uptake rates in various microcosms: Overall rate of TBA utilization is expressed in units of mg/L per day per gram of soil in the microcosm. Each point represents the rate in a single replicate.

exhibits much less order and tends to show the variability in response between replicates more than the effects of a specific treatment. A conclusion that may be reached using these data is that the natural biodegradation rate of TBA is difficult to increase.

These two alcohols are interesting to compare because they are very similar in most physical characteristics, but are quite different in their relative amenabilities to biodegradation. In this way they may be viewed as "model compounds", i.e., methanol being readily biodegradable and TBA being recalcitrant. Even though the degradation of the biodegradable compound was stimulated, removal rates of the recalcitrant compound were not affected by the same treatments in the same systems. This suggests the conclusion that the biodegradation of a compound is more readily enhanced if that compound is more easily biotransformed.

The TBA uptake rates were higher for some treatments, especially pH 8.8, nitrate-dosed, than others. Still, there seemed to be no obvious pattern as with methanol and even the highest rates were an order of magnitude less than the lowest methanol utilization rates. TBA biodegradation enhancement seems to be both marginal and unpredictable. In cases where the TBA biodegradation rate was elevated, methanol degradation was also enhanced. The reverse was not true. It seems that the mechanisms for enhancement do not vary so much in method as in magnitude.

CHAPTER V

CONCLUSIONS

The conclusions reached after analyzing the data from these experiments are:

- Both methanol and TBA are degraded in the subsurface microcosms. The removal process is a biologically-based degradative mechanism. Methanol biodegradation occurs at a much higher rate than TBA biodegradation in all treatments examined.

- Addition of organic substrates to these subsurface microcosms did not substantially affect TBA biodegradation. This was most probably not due to inhibition of oligotrophic bacteria or nutrient limitations. The very low TBA concentration, or the low pH, or the lack of dissolved oxygen, or a combination of these conditions could have been responsible for the apparent lack of response to these readily available carbon substrates.

- Variation of pH alone in these groundwater systems does not have a great effect on the degradation rates of the

two alcohols, except for inhibition of biodegradation at pH 8.8.

- Addition of sulfate resulted in inhibition of methanol and TBA degradation and the pH had little effect on this phenomenon. Optimum pH for sulfate addition to these microcosms was pH 7.1, but this treatment resulted in rates of removal similar to those seen in the non-amended sets.

- Nitrate addition will enhance biodegradation of methanol in these systems if the pH is adjusted above pH 6 to prevent the toxic effects of nitrite.

- TBA biodegradation is sensitive to nitrite toxicity and may be enhanced somewhat if the pH of nitrate-dosed microcosms is adjusted above neutral. Isolated instances of both enhancement and inhibition of TBA degradation in replicates of various treatments has been observed. This behavior cannot be explained, except to say that the degradation of TBA is quite variable but always quite slow relative to methanol.

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APPENDIX A

DATA TABLES

Table A-1. TBA concentrations (mg/L) in microcosms dosed only with TBA.

DAY	1	2	3
0	2.18	2.13	1.01
5			0.90
9			0.89
16	2.14	2.07	
21	1.95	1.86	
22			0.77
26	1.96	1.92	
27			0.79
33			0.72
37	1.76	1.82	0.68
44			0.68
45	1.71	1.75	
48	1.70	1.63	
51			0.63
52	1.76	1.67	
59	1.71	1.63	
66	1.62	1.57	
72			0.51
87	1.60	1.43	

Table A-2. TBA (mg/L) in 5 mg/L Bacto-peptone-dosed microcosms.

DAY	1	2	3	4	5
0	0.68	0.76	0.73	0.75	0.72
7	0.66	0.67	0.65	0.72	0.70
14	0.65	0.67	0.68	0.72	0.70
20	0.63	0.67	0.68	0.73	0.67
33	0.65	0.64	0.59	0.59	0.63
41	0.56	0.56	0.58	0.60	0.60
45	0.53	0.56	0.55	0.58	0.60
54	0.56	0.53	0.57	0.56	0.58
62	0.53	0.52	0.56	0.57	0.57

Table A-3. TBA concentrations (mg/L) in 100 mg/L pyruvate-dosed microcosms.

DAY	1	2	3	4	5
0	1.0	0.94	1.1	1.1	0.84
10	0.81	0.82	0.80	0.78	0.80
13	0.78	0.77	0.78	0.76	0.78
18	0.76	0.78	0.81	0.76	0.77
30	0.70	0.65	0.72	0.72	0.71
40	0.69	0.67	0.73	0.72	0.71
44	0.69	0.69	0.71	0.71	0.69
51	0.68	0.69	0.70	0.66	0.69
58	0.68	0.65	0.68	0.64	0.66

Table A-4. TBA concentrations (mg/L) in 500 mg/L pyruvate-dosed microcosms.

DAY	1	2	3	4	5
0	0.86	0.85	1.1	0.86	0.97
13	0.81	0.73	0.91	0.77	0.78
16	0.77	0.71	0.90	0.73	0.76
32	0.69	0.67	0.83	0.70	0.64
37	0.67	0.67	0.84	0.69	0.75
44	0.67	0.61	0.76	0.62	0.69
53	0.65	0.60	0.72	0.62	0.64
59	0.67	0.58	0.72	0.62	0.64
87	0.67	0.57	0.73	0.62	0.68

Table A-5. TBA concentrations (mg/L) in 5 mg/L citrate-dosed microcosms.

DAY	SODIUM CITRATE				FERRIC CITRATE		
	1	2	3	4	1	2	3
0	0.97	0.89	0.92	0.87	1.1	0.90	0.90
5	0.82	0.81	0.81	0.83	0.96	0.80	0.84
10	0.83	0.85	0.79	0.83	0.95	0.85	0.85
22	0.76	0.71	0.71	0.75	0.83	0.72	0.73
29	0.71	0.67	0.71	0.73	0.76	0.71	0.73
33	0.66	0.64	0.68	0.67	0.74	0.68	0.67
42	0.57	0.57	0.64	0.66	0.70	0.65	0.65
48	0.61	0.55	0.65	0.66	0.68	0.64	0.67
69	0.53						
72	0.45	0.38	0.58	0.60			
77		0.40			0.62	0.65	0.72

Table A-6. TBA concentrations (mg/L) in 1 mg/L citrate-dosed microcosms.

DAY	SODIUM CITRATE			FERRIC CITRATE	
	1	2	3	1	2
0	1.3	1.0	1.1	1.1	1.0
5	1.0	0.81	0.88	0.90	0.85
9	0.96	0.80	0.84	0.87	0.84
22	0.74	0.76	0.71	0.80	0.73
27	0.86	0.73	0.78	0.82	0.74
33	0.78	0.67	0.71	0.73	0.67
37	0.76	0.66	0.71	0.72	0.67
44	0.73	0.61	0.67	0.71	0.68
51	0.70	0.63	0.64	0.67	0.66
83	0.64	0.64	0.49		

Table A-7. Reference microcosms- pH 5.1, non-amended.

DAY	METHANOL CONCENTRATIONS					TBA CONCENTRATIONS				
	C	1	2	3	4	C	1	2	3	4
0	91	102	123	115	114	3.7	5.7	7.1	6.3	6.7
3		91	116	101	108					6.2
7					96					
9		75	107	95				6.7	5.9	
13					90					6.2
16		55	100	83			5.3	6.5	5.8	
20					77					5.8
22		40	87	71			5.3	6.3	5.8	
26					61					4.8
35			64	46			4.9	5.5	5.5	
39					57					4.5
42		11	70	35			3.7	5.1	4.5	
46					55					4.6
48		10	51	32						
52	98				44	4.0				
58		3	45	21			3.6	4.6	4.8	
66		2	42	12			3.6	5.0	4.8	
70					29					4.2
73		0	29	0			3.3	4.6	4.8	
77					12					
79		0	27	0			3.4	4.9	4.7	
83					15					4.1
94	96	0	13	0		4.0	3.3	4.8	4.6	
98					4					4.1
108		0	0	0			3.1	4.6	4.1	
112					0					3.8
140	104	0	0	0		3.6	2.4	3.2	2.9	
144					0					3.0
166	104					4.2				
214	100					4.5				
227		0	0	0			1.8	1.2	0.6	
242		0	0	0			1.4	0.2	0.2	
246					0					2.2
295		0	0	0			0	0	0	
299					0					2.9

Units are mg/L.

Table A-8. Non-amended microcosms, pH 6.0.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	88	106	110	106	4.0	5.8	6.1	6.1
4		100	106	101		6.1	6.2	5.9
11		87	100	94				5.7
18		75	92	86		5.4	5.9	5.5
31		60	88	83		5.1	5.5	5.3
38		57	89	84		4.9	5.3	4.8
43		41	70	64				
53	94	28	71	68	3.8	4.5	4.6	4.5
61		24	71	66		4.4	4.8	4.2
68		9	60	56		4.4	4.4	4.0
74		3	55	52		4.3	4.6	4.2
88		1	46	47			4.7	4.2
94	96				4.5			
103		0	31	32		3.9	4.2	3.8
114		0	22	20		4.0	4.3	4.1
135		0	0	0		3.5	3.8	3.3
140	111				4.2			
166	108				4.3			
214	100				4.6			
238		0	0	0		0.3	2.6	1.8
290		0	0	0		0	3.0	2.1

Units are mg/L.

Table A-9. Non-amended microcosms, pH 7.1.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	92	138	148	144	3.9	5.7	6.1	5.8
4		103	105	110		5.9	6.1	6.1
11		100	91	100		5.8	5.8	6.2
18		95	81	93		5.8	4.9	4.8
31		93	76	92		5.4	5.5	5.8
38		93	77	95		5.0	5.3	5.5
43			57	71		4.0	4.1	4.4
53	96	74	54	72	4.1	4.1	4.5	4.4
61		76	43	71		4.2	4.5	4.5
68		65	31	60		4.1	4.6	4.6
74		60	26	56		4.2	4.3	4.5
88		52	0	51		4.3	4.6	4.9
94	97				4.4			
103		39	0	37		3.7	3.9	4.3
114		26	0	25		3.7	4.2	4.3
135		0	0	0		3.3	3.4	3.9
140	111				4.1			
166	109				4.4			
214	100				4.7			
238		0	0	0		0.5	1.7	2.2
290		0	0	0		0	0	1.9

Units are mg/L.

Table A-10. Non-amended microcosms, pH 8.8.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	93	105	111	101	4.1	6.2	6.8	5.5
10		108	106	96		6.3	6.2	6.1
16		93	98	76		6.1	5.9	5.5
29		86	85			5.8	5.5	4.9
35		86	85	78		4.4	5.1	5.0
52	95	85	71	64	4.1	4.4		
60						4.6	4.9	4.2
67		80	62	59				
73		78	60	60		4.6	4.9	4.3
87		81	54	59		4.6	4.9	4.1
96	99				3.8			
102		68	40	51		4.4	4.7	4.1
113		69	31	47		4.2	4.5	3.7
134		60	25	41		3.8	3.8	3.4
140	107				4.1			
166	110				4.4			
214	98				4.7			
221		32	0	9		3.0	3.1	2.9
289		3	0	0		3.6	3.6	3.5
313		0	0	0		3.8	3.8	3.4

Units are mg/L.

Table A-11. Nitrate-dosed microcosms, pH 5.1.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	92	106	118	140	3.9	4.9	5.7	5.5
9		81	93	92		4.2	4.6	5.1
16		63	73	79		4.2	4.6	5.1
24		46	60	62		4.5	4.8	4.9
30		37	52	59		4.4	4.7	4.9
37		29	44	54		4.2	4.4	4.7
44		19	33	51		4.4	4.5	4.8
51	90	15	24	50	3.6	3.8	4.4	4.7
58		5	9	48		3.9	4.3	4.4
65		2	2	39		3.6	4.0	4.4
80		0	0	41		3.1	3.5	3.5
91		0	0	31		3.0	3.5	3.5
96	88				4.1			
140	111				3.8			
155	104				3.8			
167		0	0	3		2.8	3.0	3.1
214	95				4.3			
248		0	0	0		3.2	3.6	3.8
303		0	0	0		3.1	3.4	3.4

Units are mg/L.

Table A-12. Nitrate-dosed microcosms, pH 6.0.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	94	90	93	90	4.6	4.7	4.9	4.8
6		86	93	90		4.7	4.6	4.7
13		48	64	56		4.5	4.7	4.8
20		27	54	50		4.6	4.7	4.6
27		16	43	34		4.4	4.4	4.4
32		10	36	23		4.5		4.5
40	113				4.4			
42		0	12	0		3.6	3.6	3.5
53		0	0	0		3.5	3.4	3.5
63	111				4.4			
74		0	0	0		3.1	2.5	2.4
87	116				4.3			
116		0	0	0		2.7	0.4	0.2
122	100				4.7			
157		0	0	0		2.5	0	0
208		0				0.5		

Units are mg/L.

Table A-13. Nitrate-dosed microcosms, pH 7.1.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	91	85	91	88	3.9	4.3	4.6	4.7
6		80	71	67		4.3	4.6	4.5
13		26	15	11		4.1	4.3	4.3
20		2	1	1		4.0	4.4	4.5
27		0	0	0		3.9	4.2	4.1
32		0	0	0		3.9	4.3	4.2
42		0	0	0		3.0	3.4	2.9
53	95	0	0	0	4.3	2.8	3.3	2.8
74		0	0	0		2.5	3.0	2.4
96	98				4.6			
116		0	0	0		1.9	2.4	1.6
140	107				4.2			
157		0	0	0		1.1	1.6	0.7
166	108				4.1			
208		0	0	0		0	0.8	0
214	97				4.6			

Units are mg/L.

Table A-14. Nitrate-dosed microcosms, pH 8.8.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	93	105	103	102	3.9	4.9	4.6	4.5
6		90	101	80				
12		20	31	32		4.4	4.1	4.3
22		10	11	12				
29						3.4	3.6	3.6
37		1	1	1		3.6	3.5	3.7
43		0	0	0		3.0	3.4	3.6
50		0	0	0		2.4	2.9	3.3
52	94	0	0	0	4.3			
57		0	0	0		2.5	2.7	2.8
72		0	0	0		1.3	0.4	1.1
83		0	0	0		0.8	0.1	0.1
96	98	0	0	0	4.8			
104		0	0	0		0	0	0
140	109				4.1			
166	102				4.3			

Units are mg/L.

Table A-15. Sulfate-dosed microcosms, pH 5.1.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	100	102	106	102	4.9	5.1	5.1	5.0
6		95	98	98		5.3	5.2	5.1
13		93	95	94		4.9	4.9	5.0
20		94	95	93		4.9	5.0	5.0
27		89	89	88		4.8	4.8	4.8
35		88	91	88		4.7	4.8	4.9
41	111	89	87	88	4.2			
46		87	83	85		4.8	4.8	4.9
63	110				4.2			
67		63	63			3.8	3.9	3.8
87	116				4.4			
122	102				4.6			
144		16	39	72		3.6	3.4	3.7
212		0	0	74		2.7	3.9	4.2
220		0	0	72		2.6	4.0	3.8
246		0	0	67		0.4	3.2	3.9

Units are mg/L.

Table A-16. Sulfate-dosed microcosms, pH 6.0.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	90	95	93	98	4.6	4.9	5.0	5.2
6		87	88	92		5.0	4.8	5.1
13		89	88	87		4.8	4.8	4.9
20		83	86	89		4.6	4.3	4.8
25		85	90	93		4.8	4.5	4.8
35		76	83	83		3.8	3.7	3.9
40	116				4.5			
46						3.7	3.6	3.7
63	109				4.3			
67		57	76	73		3.5	3.4	3.5
87	113				4.4			
108		19	67	62		3.1	3.1	3.2
122	110				4.7			
149		0	59	56		2.7	3.3	3.5
201		0	55	32		1.7	3.2	3.4
225		0	57	41		0.5	3.4	3.5

Units are mg/L.

Table A-17. Sulfate-dosed microcosms, pH 7.1.

DAY	METHANOL CONCENTRATIONS			TBA CONCENTRATIONS		
	C	1	2	C	1	2
0	102	96	98	4.3	5.0	5.0
6		87	83		4.7	4.8
13		86	90		4.8	4.9
20		88	83		4.9	4.7
25		86	80		4.5	4.7
35		74	71		3.8	3.8
40	107			4.3		
46		58	51		4.0	3.7
63	110			4.3		
67		49	43		3.6	3.5
87	111			4.4		
108		11	0		3.0	3.0
122	102			4.7		
149		0	0		3.1	2.5
201		0	0		2.5	0

Units are mg/L.

Table A-18. Sulfate-dosed microcosms, pH 8.8.

DAY	METHANOL CONCENTRATIONS				TBA CONCENTRATIONS			
	C	1	2	3	C	1	2	3
0	104	96	101	103	5.0	4.8	4.8	4.9
6		86	92	90		4.5	4.8	4.6
13		80	85	83		4.4	4.6	4.4
20		66	72	66		4.6	4.7	
27		58	60	56		4.3	4.4	4.5
34		47	54	53		4.2	4.4	4.3
40					4.4			
41		46	51	42		3.9	4.2	4.1
46		45	46	36		3.8		
56		39	37	33		3.4	3.5	3.6
63	117				4.4			
67		31	32	26		3.3	3.7	3.5
87	113				4.4			
88		18	18	16		3.3	3.6	3.5
122	100				4.7			
144		5	10	5		3.4	3.4	3.3
220		0	0	0		0.4	0.4	0.4

Units are mg/L.

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