THE USE OF RADIORESPIROMETRY FOR EVALUATION OF SUBSURFACE BIODEGRADATION/

by

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(ABSTRACT)

Current use of alcohols as neat automotive fuels or as inexpensive octane enhancers in gasoline-alcohol blends, in addition to their uses as solvents and starting materials in manufacturing, have created a concern due to the increased potential for groundwater contamination. Adsorption and water solubility are primarily responsible for separating gasoline-alcohol blend components in soils and would allow alcohols to move ahead of the remaining gasoline components (e.g., benzene). The presence of alcohols would be difficult to detect, and levels hazardous to humans or animals could be reached readily.

The primary objective of this study was to investigate the use of a ^{14}C-tracer technique for evaluation of subsurface biodegradation of groundwater contaminants. A modification of the heterotrophic activity assay, the radiorespirometric method, was employed as the ^{14}C-tracer technique. The microorganisms used were those present in soil sampled aseptically at locations in Pennsylvania and Virginia. Both saturated and unsaturated zone soils were used. The alcohols used were methanol and tertiary-butanol.

Methanol was easily degraded under both aerobic and anoxic conditions up to approximately 3000 mg/L. Tertiary-butanol was degraded very slowly under both aerobic and anoxic/anaerobic conditions, and an inhibitory concentration was not readily apparent. Tertiary-butanol was degraded at rates approximately 10^2 slower than methanol. The data generated in this study compare favorably with data obtained by oxygen-uptake and static-microcosm methodologies.

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I. INTRODUCTION

Current use of alcohols as neat automotive fuels or as inexpensive octane enhancers in gasoline-alcohol blends, in addition to their uses as industrial solvents and starting materials in the chemical industry, have created a concern due to the increased potential for groundwater contamination. This contamination may arise because of either storage tank or pipeline leakage or because of spillage during manufacturing and processing operations.

Concern focuses on the changes in component concentrations which may occur as a gasoline-alcohol blend travels through a groundwater aquifer. These changes may be due to dispersion, adsorption and biological degradation mechanisms. Adsorption is responsible for separating gasoline-alcohol blend components and for producing a component wave effect as groundwater flows through the aquifer. Thus, due to their greater solubility in water, alcohols would move ahead of the remaining gasoline components, which are more easily detected by taste and odor (e.g., benzene and toluene). Because the presence of alcohols would be difficult to detect by taste, odor or appearance, levels hazardous to the health of humans or animals consuming contaminated water could be reached readily. Additionally, most gasoline constituents are readily adsorbed by soil particles and have low solubilities in water. Thus, one could not rely on the taste, odor or appearance of these compounds for an indication of the presence of alcohols.

Alcohols, because of their greater solubilities in water and low adsorption affinities, would likely be removed from groundwater by the mechanism of biodegradation. Little information on the biodegradation of these types of compounds in subsurface environments is available. That which is available has been primarily concerned with biodegradation by pure cultures or by activated sludge systems, not by naturally occurring mixed microbial populations found in subsurface environments. This study will provide a detailed, site-specific investigation into the biodegradation of methanol and tertiary butyl alcohol (TBA).

The primary objective of this thesis was to investigate the use of a ^{14}C-tracer technique as a means of assessing initial rates of biodegradation of chemicals of environmental concern, of estimating inhibitory concentrations of such substances, and of determining the potential degradability of such substances. This radiolabeled substrate technique has the requirements of: simplicity in both mechanics and data analysis, minimized incubation periods, low cost for laboratory materials, and sensitivity to low levels of biodegradation. Additionally, due to the high cost associated with aseptic subsurface sampling and because the quantity of material is limited at a particular location, the technique used must rely on use of small quantities of subsurface material.

II. BACKGROUND

The Radiorespirometric Technique

Radiolabeled substrate studies have been used during the past 20 years to estimate substrate utilization rates or heterotrophic activity in a variety of natural and pure culture systems. More recently, such studies have also been used to characterize bacterial growth and the response to toxins. The heterotrophic activity assay, introduced by Parsons and Strickland [30] in 1962 was first used to quantify the amount of microbial assimilation of heterotrophic carbon. The assay was further developed by Wright & Hobbie [37] and by Hobbie and Crawford [22] as a means for greater experimental flexibility, to enable the maximum uptake velocity, v, to be found, and to incorporate $^{14}CO_2$ production during incubation. This further development, the radiorespirometric technique, has found application in freshwater, marine, estuarine, sediment and soil analyses. The basic technique consists of the addition of a compound (or substrate), which has been labeled with carbon-14 (^{14}C), to a soil, water or sediment sample, followed by incubation in a sealed reaction vessel. Microbial action is terminated, usually by acidification. Acidification also serves to release ^{14}C-labeled carbon dioxide ($^{14}CO_2$) from solution. Substrate which has been respired by the microorganisms present in the sample is "trapped" by a CO_2-trapping agent (typically a strong base or an amine) that is

absorbed onto a filter paper support. The filter paper support is suspended in the air space above the sample in the sealed reaction vessel. At the end of the incubation period, the $^{14}CO_2$ trapping filter is carefully removed, and the sample is gently filtered to remove microorganisms which have incorporated ^{14}C-labeled substrate into their biomass. Both filters are analyzed by liquid scintillation methods. The total of the incorporated and respired carbon is taken to represent total microbial uptake. Alternative $^{14}CO_2$ trapping systems that use gas flow through the sample container and subsequent $^{14}CO_2$ scrubbing of the purging gas stream are documented [1]. These alternative methods have largely been used in studies of recalitrant substances (e.g., pesticides) where incubation times of months or longer are used.

By necessity, only the respired, radiolabeled carbon is measured when working with sediment or soil samples [12,18,26]. Measuring only the respired radioactivity in aquatic ecosystems research simplifies the assay, thus reducing analytical time, and also eliminates concern regarding losses and imprecisions caused by filtration error [5,34].

Most frequently, the data obtained by the heterotrophic activity assay is analyzed using the Michaelis-Menten enzyme-substrate model [35,36,38-40]. Typically, these analyses require the addition of only trace quantities of radiolabeled substrates. Some researchers have reported an inability to generate a Michaelis-Menten response [24]. Other researchers have discussed difficulties of the assay

including the validity of the kinetic model used [34,38] and Hobbie and Crawford [22] concluded that the extent of $^{14}CO_2$ respired varies with type of substrate, location of the label, and time of year that sampling of the aquatic ecosystem was conducted.

Assay Use in Toxicological Research

Few researchers have employed the heterotrophic activity assay as a toxicological bioassay, even though it has been in general use for about two decades. The method has been used to detect effects of metallic salts on the relative heterotrophic response of natural microbial communities, and, in all cases, it successfully detected perturbations [2,3,7,41].

Less information is available on the use of this assay for detection of the effects of toxic organics. A modification of the heterotropic activity assay was used by Pfaender and Bartholomew [31] to estimate rates of organic pollutant biodegradation. Pfaender and Bartholomew [31] focused their research on providing a rapid and simple technique for estimating organic pollutant biodegradation rates under environmental conditions of low pollutant concentration.

Although studies have been conducted on the effects of low concentrations of toxics on microbial populations [20,31], little information has been found on use of the assay for estimating the effects of elevated concentrations of toxic pollutants on natural microbial populations.

Chemicals of Concern: Methanol and Tertiary-Butanol

Both methanol (methyl alcohol) and tertiary-butanol (t-butyl alcohol, TBA), have been widely used as solvents in industry and are currently being investigated for use as octane enhancers in gasoline and as raw materials in the production of other octane extenders (e.g., methyl tert-butyl ether, MTBE, production from methanol).

The potential for and occurrence of groundwater contamination by leakage or spillage from storage or transport facilities is of great concern in the chemical and petroleum industries. Specific interest lies with gasoline and alcohol mixtures such as Atlantic Richfield Company's Oxinol 50 blending component, which contains 50 percent methanol and 50 percent gasoline-grade TBA. The Oxinol 50 component, when added to unleaded gasoline, yields a final mixture which contains five percent methanol and five percent gasoline-grade TBA [6].

Both methanol and TBA have been suggested as being substances amenable to anaerobic biotechnology [11,33], and methanol metabolism, both aerobic and anaerobic, is widely documented [13,16,21]. It is also well known that methanol is an intermediate in the overall pathway of methane oxidation to CO_2 [9,13,16,21]. This pathway is shown in Figure 1. The pathway, in which methane is used as a sole carbon and energy source, is believed to be applicable to a number of bacteria and a few strains of yeast (Colby et al. [13] citing Hanson [17]).

Little information has been found on either the metabolism of TBA or on possible metabolic pathways for its occurrence. Horn et al.

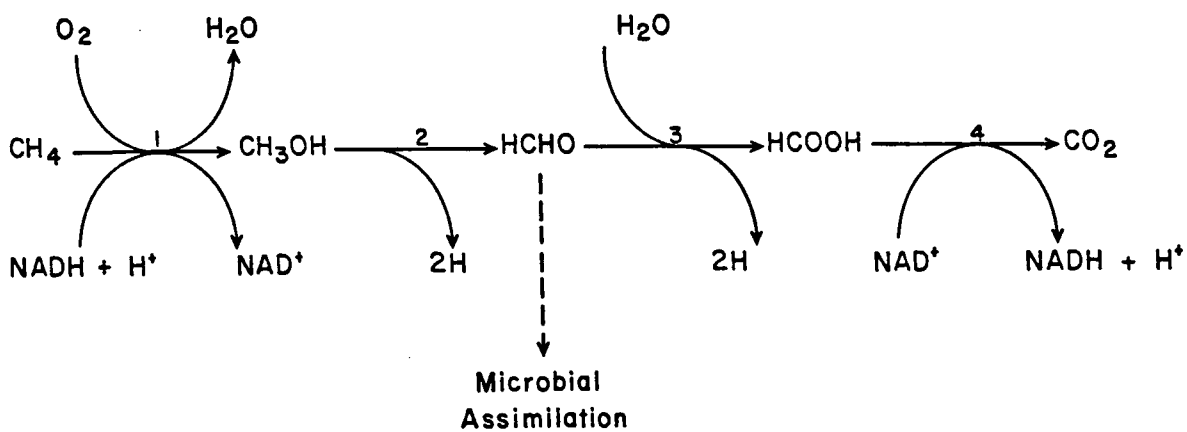

Figure 1. Pathway for the bacterial oxidation of methane and methanol. 1) Methane monooxygenase; 2) methanol dehydrogenase; 3) formaldehyde dehydrogenase; 4) formate dehydrogenase (after Colby et al., reference 13).

[23] concluded that a microbial culture can be developed to degrade TBA aerobically and that the presence of other carbon sources, more readily degradable than TBA, have no detrimental effects on TBA biodegradation. These conclusions were reached following continuous-flow, biotreatment studies with completely mixed reactors. Theoretical detention times ranged between 4.3 and 1.0 days and substrate concentrations ranged from 350 to 1800 mg/L (as carbon). It is also concluded "that at least 99 percent of the TBA removal occurring was due to biodegradation rather than air stripping." It has been reported (Horn et al. [23] citing Buzzell et al. [10]) that TBA is air stripped at a fairly rapid rate.

Marion and Malaney [27], using Warburg studies of Alcaligenes faecalis, indicated that TBA degradation was minimal in comparison to endogenous respiration. McKinney and Jeris [28], using Warburg studies of an acclimated activated sludge, concluded that TBA was not metabolized to any appreciable extent. The TBA concentration in both of these studies was 500 mg/L.

Goldsmith [15] has suggested that it may be feasible for enzymes to convert TBA to the corresponding aldehyde, ketone or acid.

U.S. Federal Groundwater Protection Legislation

Lehr et al. [25] have reviewed Federal Environmental Protection Acts and have concluded that the massive, national, pollution clean-up efforts associated with the 1970's landmark environmental legislation largely ignored groundwater and, ironically, hastened the

contamination of groundwater supplies by strictly regulating surface and air discharges and by the encouragement of land disposal and underground injection of wastes. Lehr et al. [25] discussed eight major pieces of Federal environmental legislation that either directly or indirectly contributed to the cause of groundwater pollution in the United States. Because of the fragmented groundwater protection policy, evidenced by these acts, a concerted effort is required to bring about a useful national program for groundwater protection.

Legislation in the future may emphasize protection rather than clean-up requirements. Spill clean-up has typically been shown as a "band-aid" approach and is most likely the more expensive of the two alternatives.

Studies such as the one reported in this thesis should be expected to be used for estimating the amount of low-level clean-up required and may serve as indicators of the degree of hazard that could be expected from a pollution event.

III. METHODS AND MATERIALS

General Description

The heterotrophic activity assay modification, which is based on the measurement of respired $^{14}CO_2$ as an index of heterotrophic activity, was investigated as a rapid and relatively simple method for determining the potential for biological degradation of toxic chemicals in subsurface systems and for estimating initial biodegradation rates. The microbial populations were obtained from soil cores taken at sites in Pennsylvania and Virginia. The procurement of the soil cores is detailed in a following subsection.

Two ^{14}C-labeled organics, methanol and TBA, were used in these studies, and results of studies were compared to determine the relative usefulness of the method for evaluating biodegradation of a relatively easily degraded substance (e.g., methanol) and a relatively recalcitrant compound (e.g., TBA).

Experiments with ^{14}C-labeled sodium bicarbonate ($NaH^{14}CO_3$) in distilled water were carried out to determine physical and chemical aspects of the technique not related to those caused by microbial action in test samples.

Sampling Site Selection and Site Descriptions

Potential soil sampling sites were limited to Atlantic Richfield Company (ARCO) storage terminals in Pennsylvania, New York and Virginia. Basic geologic conditions were obtained from general documents by Groundwater Technology, Inc. to aid in the selection of

a site with principally sandy soils and a relatively high water table. Preliminary drilling was done under the supervision of Mr. James H. O'Brien of ARCO Petroleum Products by local drillers who used a small diameter, split spoon sampler. The number of potential sites was narrowed from the preliminary drilling results and from discussions between ARCO personnel and Dr. G. W. Clough of VPI. The final selection was made by ARCO personnel based on the available information. The four most suitable sites, where sampling operations were expected to be successful, were Williamsport, PA; Corning and Wayland, NY; and Dumfries, VA (Figure 2).

The first study location was Williamsport, PA. The terminal was located on level terrain at the base of a mountain range, and drilling was conducted within 100 yards (91 meters, m) of the Susquehanna River. The water table was found to be at approximately 14 feet (4.3 m) and roughly corresponded to the level of the river surface. Subsoils consisted of a loamy silt to a depth of 12 feet (3.7 m) followed by a medium-dense, clean sand from 15 to 16 feet (4.6 to 4.9 m). Dense gravelly sand was found to 30 feet (9.1 m). At 36 to 38 feet (11.0 to 11.6 m) a relatively clean dense sand was present, underlain by rock.

The Corning, NY site proved to be an unsatisfactory sampling location due to the presence of large cobbles throughout the subsoil which prevented efficient drilling and sample collection.

Drilling at the Wayland, NY site was marginally more successful than at the Corning site, although only a small quantity of material was collected because of problems in collecting samples from a

Figure 2. Soil sampling locations.

heavily cobbled area. Drilling was conducted within 400 yards (366 m) of a small lake on flat terrain. The upper two feet (0.6 m) of soil was dark and interspersed with marl. Below this, a continuous layer of marl of approximately six inches (15 centimeters, cm) thickness was found. The water table was located at three to four feet (0.9 to 1.2 m).

The fourth boring location was at the ARCO Cockpit Point Terminal, located on Route 783 in Dumfries, VA. The subsurface investigation was performed on a grassed, nearly level area. The generalized soil strata encountered in the two borings were: Stratum A, from the ground surface to depths of three to nine feet (0.9 to 2.7 m), brown fine sandy silty clay which is believed to represent fill materials placed during construction of the terminal; Stratum B, from below Stratum A to depths of 42 to 43 feet (12.8 to 13.1 m), brown to gray, fine to coarse sand, some clayey silt to silty clay with gravel, fine to coarse sandy gravel and fine sandy silty clay; Stratum C, from below Stratum B to the maximum depth of penetration, 102 feet (31.1 m), brown, gray to green, fine to coarse silty clayey to clayey silty sand with gravel and feldspar and silty clay to clay, trace fine sand.

Groundwater occurred at multiple horizons beneath the site, generally within granular strata underlain by clayey strata of lower permeability. Perched groundwater was encountered during drillings in Borings B-1 and B-2 at depths of 15 and 12 feet (4.6 and 3.7 m), respectively, in the Stratum B soils. A 24 hour water level reading indicated water at a depth of two feet (0.6 m) in Boring B-1. This

was not considered to be the true water table because drilling mud was still present in the boring. A deeper water table was measured at a depth of 42.8 feet (13.0 m) in the terminal well. Construction details were not available for this well, but it was believed that this well is screened in granular Stratum C materials.

All four sampling sites were considered to be free of chemical contamination from the gasoline terminals.

Sampling

Samples of subsurface material were obtained from the unsaturated zone (above the primary water table) and the saturated zone (below the water table) for bacteriological measurements and biodegradation studies.

Primary objectives of the sample work were to:

- Apply minimum shock to the soil.
- Avoid the introduction of foreign materials or organisms into the soil being sampled.
- Extrude the samples on-site rapidly using a procedure which would ensure that only sterile surfaces would contact the soil.
- Capture the samples in sterilized storage and transportation containers which could be sealed immediately.

A conventional sampling procedure was adopted to fit the project requirements. The Warren George, Inc. drilling firm of Jersey City, NJ offered four sampling equipment alternatives: (1) an Osterberg

hydraulic sampler; (2) a Denison sampler; (3) a Pitcher Barrel sampler, and, (4) a Dames and Moore sampler. Shelby tubes made of brass or stainless steel were employed. The sample tube is pushed into place by the Osterberg sampler, both the Denison and Pitcher Barrel samplers use rotary drilling to place the sample tube, and the Dames and Moore sampler is impact driven. The Dames and Moore sampler, though available, was not used.

Drilling mud is commonly used to maintain the stability of the walls of the hole during drilling operations, especially in sandy soil below the water table. It is also required for rotary drilling operations to bring cuttings to the surface. While the drilling mud serves to enhance the basic drilling process, a primary concern was that the mud might contaminate the soil cores. The mud is very viscous and does not flow freely, thus contamination was considered to be a relatively remote possiblilty [15].

Observations were made for drilling fluid penetration into the soil cores during extrusion, and lithium chloride was added to the mud as a tracer. No mud was observed in any soil cores, and no contamination by the fluid was evident, based on lithium analysis.

The soil cores were extruded using a device constructed by Warren George, Inc. The device consisted of a hydraulically driven piston, a dual clamp bed for holding Shelby tubes and a removable stainless steel paring device (Figure 3). As the soil core was extruded from the sample tube the first several centimeters were cut away with a flame sterilized spatula to eliminate any possibly contaminated material. The core was then pushed through the flame sterilized

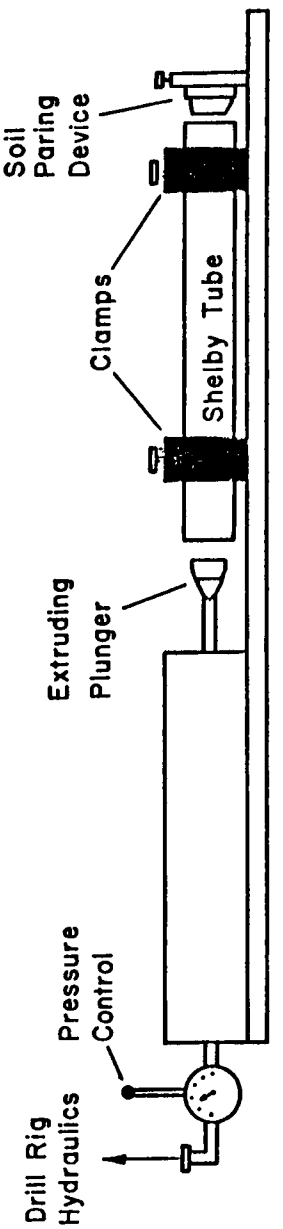

Figure 3. Soil extrusion device.

paring device which trimmed away the outer one centimeter of the original sample core. The final few centimeters of the core were also discarded. The extruded samples were captured in acid washed, sterilized one-quart or half-gallon, glass containers fitted with sterile, Teflon-lined lids. The containers were transported on ice to the VPI laboratories for storage until use.

Bacterial Enumeration

Microbial populations in the soil samples were quantified by the acridine-orange, epiflourescence, direct count (A-O) method and by dilute-media MPN enumeration by Dr. Robert Benoit and his assistant, Mr. George Allen. Plating media used included soil extract, methyl amine, formate, actinomycete, acid agar and rose bengal. Microbial populations for all sites, based on soil extract media and A-O, are presented in Appendix A. The other MPN media enumerations yielded values comparable to the soil extract media.

Toxins

The radiolabeled organics used in the assay as microbial substrates were carbon-14 labeled methanol [specific activity of 45 millicuries per millimole (mCi/mM), (lot 782, code CMM-87, Research Products International Corp., Mount Prospect, Illinois)] and (Methyl-$^{14}C_{1/3}$) tert-butanol [specific activity of 9.6 mCi/mM,

total activity of 11.2 mCi, (lot 82-7-21, Midwest Research Institute, Kansas City, Missouri)]. The ^{14}C-labeled methanol was aseptically diluted with 5.0 milliliters (mL) of commercially obtained, unlabeled methanol (Pesticide quality, Matheson, Coleman & Bell, Norwood, Ohio) to yield a final ^{14}C concentration of 0.2 microcuries per microliter (μCi/μL). The ^{14}C-TBA, which originally had been prepared for ARCO in early 1982 for toxicological research, also was aseptically diluted from its initial volume of 110 μL to a final volume of 3.2 mL with commercially obtained, unlabeled TBA (Fisher Scientific Company, Fairlawn, New Jersey) to yield a final ^{14}C concentration of 3.5 μCi/μL. A 0.50 mL aliquot of the TBA solution was diluted further with sterile distilled water to yield a final ^{14}C concentration of 0.35 μCi/μL in a final 5.0 mL volume.

Radiolabeled toxins were transferred from storage vials to reaction vessels using Hamilton microliter syringes, and the toxins were stored in acid washed, sterile, glass vials fitted with septum caps. The compounds were stored in a freezer at approximately -10°C (14°F) to minimize possible loss by volatilization.

Glassware Preparation

All glassware was acid washed prior to initial use to remove manufacturing residues or residues left on the glassware by previous users. Following each use, reaction vessels were soaked in a two percent solution of Count-off (New England Nuclear Corp., Boston, MA) in water at ambient temperatures for a minimum of 24 hours. Reaction

vessels were scrubbed to remove soil particles adhering to the inner surface and sequentially rinsed from four to six times with hot tap water and then distilled water.

Glassware sterilization was in accordance with Part 904, Washing and Sterilization, in Standard Methods for the Examination of Water and Wastewater [4], by either autoclave sterilization for 30 minutes at 103 KPa (15 psi) and 121°C (250°F), or dry-heat sterilization for two hours at 170°C (338°F).

All reaction vessels were washed, capped with aluminum foil and sterilized between uses.

Dilution Water

Because these assays were to determine biodegradation potential, a dilution water that would supply needed nutrients for the microbial communities was desired. Therefore, Biochemical Oxygen Demand dilution water was prepared in accordance with Part 50, Oxygen Demand (Biochemical) in Standard Methods for The Examination of Water and Wastewater [4]. Standard solutions were prepared and stored in acid washed, sealed, glass containers and were replaced as required during the course of the study.

Total Organic Carbon (TOC) levels in the dilution water after sterilization were determined in random samples, on a Dohrmann/ Envirotech (Santa Clara, California) Model DC-50A/52A Ultra Low Level TOC Analyzer. The TOC in the dilution water was approximately 0.6 mg/L.

Incubation Procedures

All experiments using ^{14}C-sodium bicarbonate ($NaH^{14}CO_3$) in distilled water, ^{14}C-methanol and ^{14}C-TBA were carried out in the same manner. Erlenmeyer flasks (25 mL), fitted with rubber stoppers and plastic centerwells (Kontes, Vineland, New Jersey), served as reaction vessels during incubation. Experiments were performed using triplicate flasks. The filter support, which was inserted in the centerwell, was a 2.1 cm Whatman, glass-microfiber, filter disc (GF/B, catalog number 146002, Research Products International Corp., Mount Prospect, Illinois). Radiolabeled materials on a glass-fiber disc can be analyzed in a suspension by using vigorous agitation to break the disc in the liquid scintillation counting vial [19]. Less quenching of the liquid scintillation sample will occur because of the easy fragmentation of the glass-fiber filters, and the liquid scintillation analysis will be more efficient. The assembled incubation equipment is illustrated in Figure 4.

Control flasks, which provided measurements of the background radioactivity due to compound volatility and spontaneous decomposition, were prepared in the same manner as the test flasks except that either no soil was added to the flask, or the soil was added and poisoned with two percent mercuric chloride ($HgCl_2$).

Flasks were incubated in the dark at approximately 20°C (68°F), and were neither agitated, nor their contents mixed, during the incubation period.

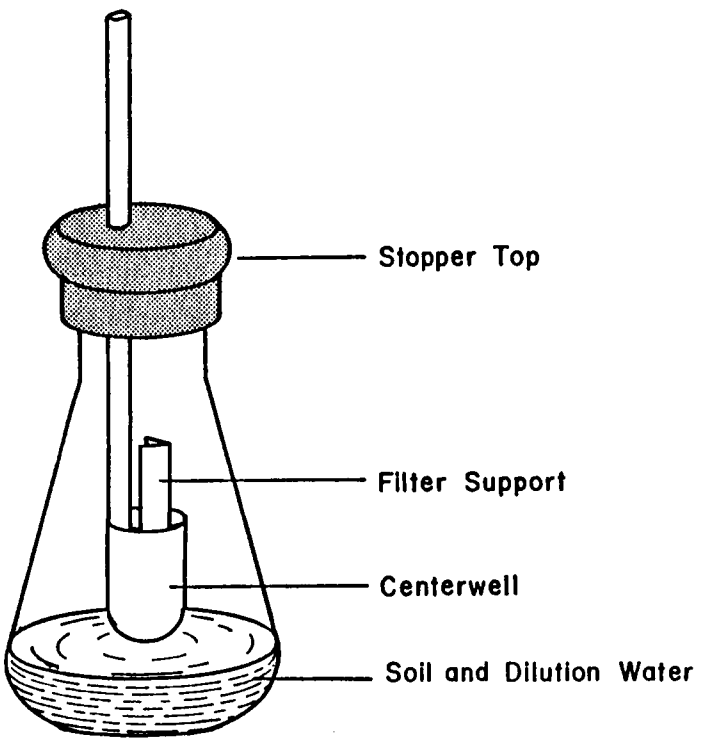

Figure 4. Flask fitted with a rubber stopper and centerwell apparatus used for trapping $^{14}CO_2$.

Quantities of soil were aseptically transferred directly from the storage containers to the sterile 25 mL flasks using flame sterilized, stainless steel spatulas. The flasks were continuously monitored for their weight on an analytical balance (Mettler Instrument Corp., Model H34). The approximate soil weights used in the preliminary optimization experiments were 0.10 grams (ranging from 0.0985 to 0.1035 grams), 1.0 grams (ranging from 0.9978 to 1.0037 grams) and 5.0 grams (ranging from 4.9479 to 5.0377 grams). The optimum soil weight for the assay, based on these experiments, was chosen as 5.0 grams. The soil samples were not slurried, mixed or modified in any way prior to use to prevent possible contamination of the sample.

After the soil was placed in the flasks, the soil was flooded with 10.0 mL of sterile dilution water, and the flasks were sealed with the centerwell stopper assemblies. To promote anoxic conditions, that is, to remove molecular oxygen from the test flasks, the flasks were flushed with prepurified nitrogen to displace the air in the flask headspace. The measured nitrogen flow rate was 450 mL/min for a minimum of 25 seconds. Molecular oxygen in solution was assumed to be depleted by the end of the first 24 hours of incubation [32]. Radiolabeled compounds were then added to the flask contents using a microliter syringe, and the flasks were placed in an incubator.

Flasks were removed from the incubator at 24 hour intervals and $^{14}CO_2$ evolution was determined. Ethanolamine (Research Products International Corp., Mount Prospect, Illinois), the CO_2 trapping

agent, was injected from a syringe through the stopper onto the filter disc in the centerwell of each flask, 0.20 mL per flask. Following the ethanolamine addition, the flask contents were acidified with 0.1 mL of 2N sulfuric acid (H_2SO_4) which was also injected through the stopper. The pH of the solution was randomly checked following acid addition, and was consistently at or below 2.0. The flasks were then placed on a shaker table for 30 minutes. The shaker table was operated at a low speed to eliminate splashing flask contents onto the filter disc. A preliminary experiment had shown that addition of ethanolamine at the start of incubation, or shaking the acidified flasks for greater than 30 minutes, resulted in background radioactivity levels high enough to possibly mask $^{14}CO_2$ evolution.

At the end of 30 minutes, the flasks were removed from the shaker table, the centerwell stopper assemblies were removed and the filter discs were transferred, using tweezers, to prepared 22 millimeter (mm), glass scintillation vials (Catalog number 121053, Research Products International Corp., Mount Prospect, Illinois) containing 10.0 mL of Bio-Count scintillation cocktail (Research Products International Corp.). A blank vial was also prepared by placing a glass-microfiber, filter disc into a vial containing 10.0 mL of Bio-Count. After the vials were closed, each was vigorously shaken to break the filter disc to insure sample homogeneity.

Scintillation Counting

Analyses for $^{14}CO_2$ evolution were done using a Beckman LS-230 Liquid Scintillation System (Beckman Instruments, Inc., Fullerton, CA), a Beta Spectrometer. Scintillation vials were normally analyzed 21 to 27 hours after cocktail preparation, following recommendations by Henry [20]. The vials were wiped with an ethanol moistened tissue prior to analysis to remove fingerprints and other residues, and to prevent the buildup of static electricity. Channel A of the scintillation counter was set to monitor the ^{14}C above tritium (^{3}H) energy window while Channel B was set to monitor the wide ^{14}C energy window. The scintillation counter was set to calculate the External Standard-Channels Ratio (ESR). A quench curve relating counting efficiency and ESR was prepared using a set of commercially obtained, sealed, quenched carbon-14 standards (Order No. 566322, Beckman Instruments, Inc.). From the quench curve, counting efficiency could be estimated for each sample vial. The corrected disintegrations per minute (DPM) were calculated by subtracting the counts per minute (CPM) of the prepared blank from the CPM of each sample, and dividing by the counting efficiency expressed in decimal form. The correction from CPM to DPM is shown as an equation below:

$$DPM = \frac{(\text{Sample CPM} - \text{Blank CPM})}{\text{counting efficiency}}$$

Apparent loss of activity with time from prepared scintillation vials, a phenomenon reported by other researchers using this assay [20], was not observed in any of the experiments of this project.

^{14}C-Bicarbonate Experiments

Experiments using ^{14}C-labeled $NaHCO_3$ in distilled water were carried out to evaluate the percent recovery, or trapping efficiency, of evolved $^{14}CO_2$.

A stock solution of $NaHCO_3$ was prepared by diluting 0.688 grams $NaHCO_3$ (unlabeled) in 105 mL of distilled water with 5 µCi of ^{14}C-labeled $NaHCO_3$ (lot 670-065, New England Nuclear, Boston, MA). The final solution, with a specific activity of 48 µCi/L, was prepared immediately prior to use. Flasks, without soil, were filled with distilled water and the stock solution to give final volumes of 10.0 mL. The HCO_3^- concentrations were 100, 500, 1000, 3000 and 5000 milligrams per liter (mg/L). Three flasks were prepared at each HCO_3^- concentration. The flasks were acidified to pH 2.0 or lower and received ethanolamine in the same manner as the normal test flasks.

Rate Analyses

Data were subjected to rate analysis where multiple concentrations were used in a single experiment. The analysis used is similar to that employed for kinetic analysis of the

Michaelis-Menten type. That is, a $^{14}CO_2$ evolution velocity was determined by use of the equation:

$$v = f\ C/t$$

where:

v = $^{14}CO_2$ evolution velocity, mg/L/day

f = the fraction of organic evolved as $^{14}CO_2$

= (sample DPM - background DPM) / (DPM added to sample)

C = organic concentration, mg/L

t = time of incubation, days.

Statistical Analyses

Data from these experiments were subjected to limited statistical analysis which consisted of computation of arithmetic means for each set of data points. Individual data points which appeared to be inconsistent with others of the same set were normally eliminated from the calculation of the mean. Means were used for rate analyses and graphical observations for overall trends of $^{14}CO_2$ evolution versus time and of $^{14}CO_2$ evolution rate versus toxin concentrations.

IV. RESULTS AND DISCUSSION

This section is divided into five subsections. In the first, the results of preliminary investigations and experiments are presented and briefly discussed. The preliminary work presented and discussed includes development of a quench curve for estimating scintillation counting efficiency, estimation of $^{14}CO_2$ trapping efficiency for the range of initial concentrations used in the study and background radioactivity level determinations for both methanol and TBA.

The second subsection is concerned with technique development and optimization. Variability in $^{14}CO_2$ evolution rates and the effects of concentration and the presence of molecular oxygen are discussed.

The third and fourth subsections are, respectively, methanol degradation variations with depth and concentration, and TBA degradation variations with depth and concentration. Both subsections include results and discussion of the effect of aerobic and anoxic conditions on degradation of these organics by soil microorganisms.

The final subsection is concerned with a variety of subjects, including bacterial enumeration and groundwater characteristics for each site, limitations of this assay in similar research investigations, and comparison of the results of this study with those of other researchers using different techniques.

The results of the experiments performed during this study are presented as figures in this section. The raw data and the calculated values used in the preparation of these figures are tabulated in Appendix B.

Preliminary Investigations

Quench Curve Development

The liquid scintillation process involves conversion of part of the kinetic energy of an ionizing particle into light photons as the particle is slowed or stopped in the scintillation detector material. The light photons are detected by the photomultiplier tubes of the liquid scintillation counter. Any process that reduces the photon output of the scintillation liquid, and therefore, the count rate reported for the sample under investigation, is defined as quench. Some degree of quenching occurs in every sample prepared for liquid scintillation counting.

Three of the more common methods of quench correction are; internal standardization, sample channels ratio and external standardization. The external standardization technique was used in this project to develop a correction curve for quench and counting efficiency because of its advantages over the other two methods [14]. The sample channels ratio is limited in accuracy to a relatively narrow range and decreases at low count rates while the internal standards techniques require additional sample manipulation

and precise addition of an accurately labeled standard of the same material as that of the sample. More extensive discussions of quench and Liquid Scintillation Counting can be found in Hendee [19] and in Elliott [14].

Trapping Efficiency

The experiments with ^{14}C-labeled sodium bicarbonate ($NaH^{14}CO_3$) were conducted to estimate the trapping efficiency over a range of concentrations. These data on $^{14}CO_2$ recovery are not used as corrections in the data presented in the following subsections of this study, but they are presented here so that readers may, if desired, estimate actual $^{14}CO_2$ evolution from the experimental results of this study.

The experiments are based on the fact that a known quantity of bicarbonate in solution can be converted to CO_2 by acidification. Triplicate flasks containing HCO_3^- concentrations of 100, 500, 1000, 3000 and 5000 mg/L were acidified following ethanolamine addition and shaken gently for 30 minutes. The filter supports were analyzed in the same manner set forth in Methods and Materials.

The results (Figure 5) generally show decreasing $^{14}CO_2$ recovery with increasing HCO_3^- concentration. The average $^{14}CO_2$ recovery of about 78 percent at the HCO_3^- concentration of 500 mg/L was unexpected and may not be a true representation of recovery at that concentration. The high value may be a result of

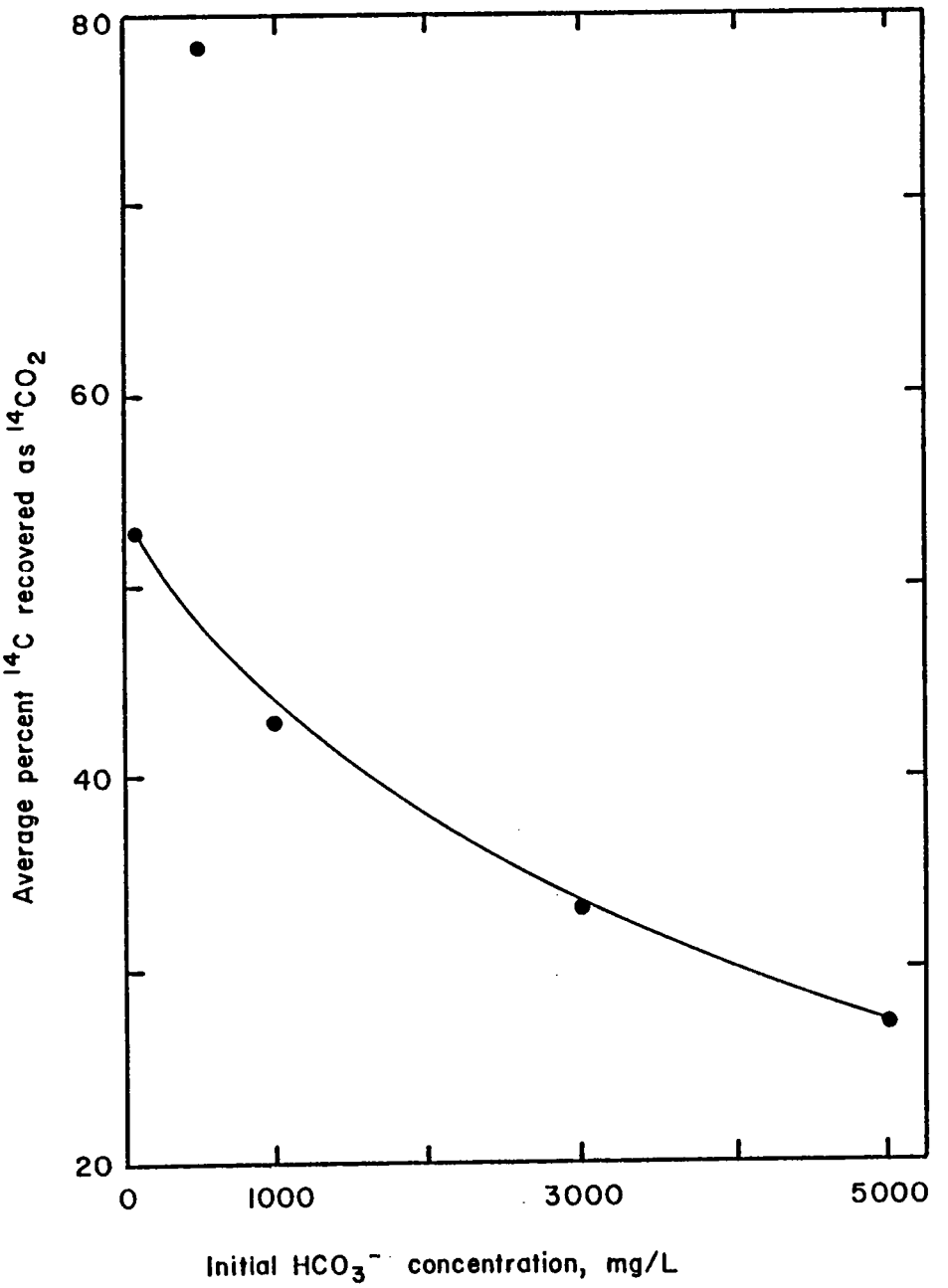

Figure 5. Percent recovery of $^{14}CO_2$ from $NaHCO_3$ solution acidified to pH 2.0. Data points plotted are averages of values obtained from triplicate flasks.

splashing of $NaHCO_3$ solution onto the filter support during the 30 minute shaking period.

At least one flask from each concentration group was checked for stopper leakage by using a technique suggested by Henry [20]. It was found that the flask stoppers at both the 3000 and 5000 mg/L HCO_3^- concentration leaked at a very high rate, probably due to the increased pressure caused directly by addition of acid and ethanolamine to the flasks, and indirectly by the complete conversion of HCO_3^- to CO_2. Because of this leakage, the data for these two concentrations would seem to represent lower than actual $^{14}CO_2$ recovery. Also, it must be emphasized that the HCO_3^- in solution was completely converted to CO_2 at all five concentrations. As discussed in the following subsections, complete conversion of methanol or TBA, at any concentration, did not occur during this study and as a result, the data for 3000 and 5000 mg/L HCO_3^- were not required.

Background Radioactivity Level Determinations

A concern about absorption of the volatile organic substrates by the CO_2 trapping system led to the investigation of background radioactivity levels versus both time and concentration. The first of these experiments undertaken was the investigation of ^{14}C-methanol absorption with time for an initial methanol concentration of 100 mg/L. This experiment was performed in the same manner as normal degradation experiments, except that no soil was

placed in the flasks and ethanolamine was added at the start of the incubation period. Triplicate flasks were prepared so that one set of three flasks could be sacrified on each of seven consecutive days. The experiment was conducted using aerobic conditions.

The results (Figure 6) show increased ^{14}C-methanol adsorption with increased incubation time. These results indicate that masking of slow $^{14}CO_2$ evolution rates would be likely. Ethanolamine addition at the begining of incubation was eliminated in favor of the addition of the CO_2 trapping agent at the end of incubation, just prior to acidification of the flask contents, even though a slight decrease in trapping efficiency and a slight increase in variability would be likely results of the change.

The two other background radioactivity level experiments were performed in the same manner as normal degradation experiments, except that no soil was used in the methanol experiment and soil poisoned with two percent $HgCl_2$ was used in the TBA experiment. Additionally, the TBA experiment used soil from two depths to determine any differences caused by different water contents of the soils. The results of these experiments are summarized in Table 1, along with the total activity of ^{14}C-methanol or ^{14}C-TBA added. The average values of ^{14}C evolved were used as corrections for background in the related concentration effects experiments presented later in this section, and the data from the 100 mg/L methanol and 10 mg/L TBA concentrations were used for corrections as background in the respective single concentration degradation experiments.

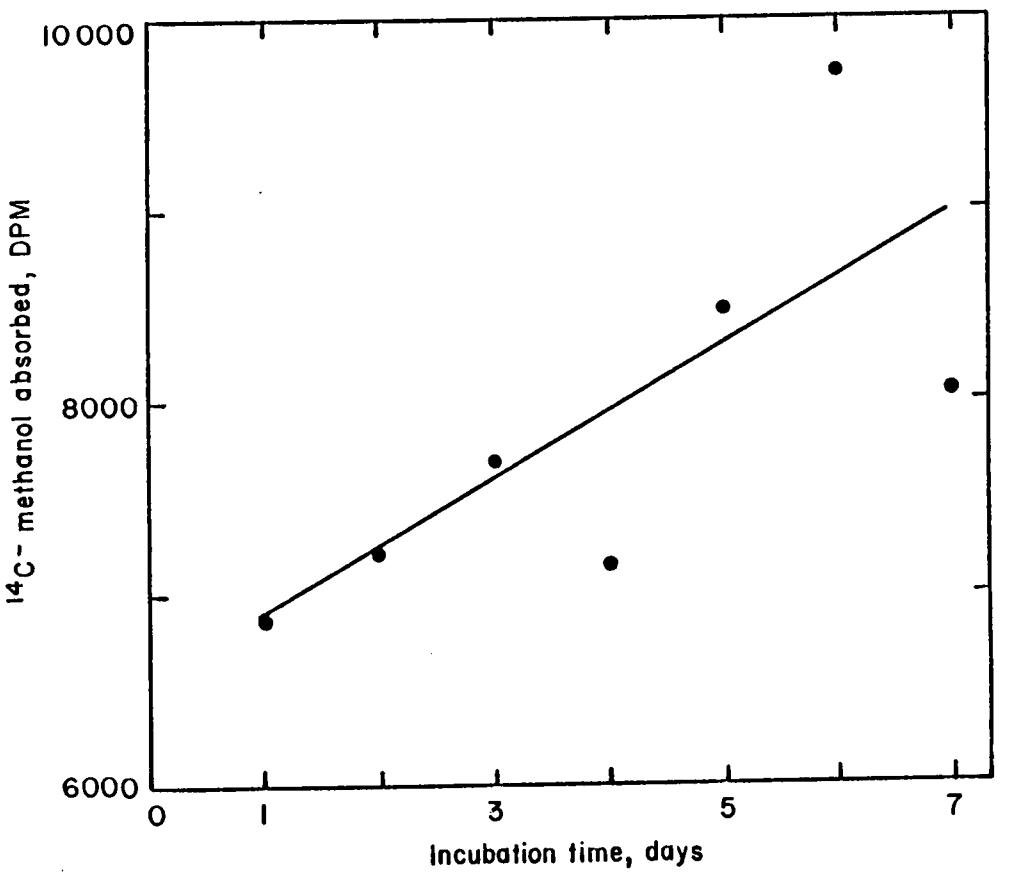

Figure 6. ^{14}C-methanol absorbed by ethanolamine with time. Data points plotted are averages of values obtained from triplicate flasks.

TABLE 1. Background radioactivity (^{14}C) evolution and initial radioactivity (^{14}C) addition in concentration effects experiments. Values are averages obtained from triplicate flasks.

Methanol (without soil)			t-Butanol			
					^{14}C evolved, DPM	
Concentration, mg/L	^{14}C added DPM	^{14}C evolved DPM	Concentration, mg/L	^{14}C added DPM	12′ soil w/$HgCl_2$	55′ soil w/$HgCl_2$
100	456,000	2,940	10	821,400	6,662	5,416
500	228,000	1,761	50	4,107,000	31,267	26,487
1000	456,000	2,689	100	8,214,000	54,947	48,659
3000	1,368,000	6,732	300	24,642,000	169,377	160,123
5000	2,280,000	12,459	500	41,070,000	290,669	248,269

The values presented in Table 1 represent averages of 0.6, 0.7 and 0.6 percent of the initial ^{14}C addition for all concentrations for methanol without soil, TBA with Dumfries, VA soil from 12 ft (3.7 m) and TBA with Dumfries, VA soil from 55 ft (16.8 m), respectively. It should be noted that the soil with the higher moisture content (i.e., the soil from the greater depth) had lower ^{14}C background levels than the soil containing less moisture. The difference between the two soils was between 5.4 percent and 18.7 percent.

Technique Development and Optimization

Degradation of Methanol Under Aerobic Conditions

The technique development and optimization experiments were originally performed to observe any differences in $^{14}CO_2$ evolution and evolution rates related to the use of various masses of soil (and therefore, various initial microbial populations) and related to the presence or absence of molecular oxygen. These experiments also provided an opportunity to become familiar and comfortable with the technique, thereby reducing the potential for human error in the degradation experiments which followed those conducted in the optimization phase.

The first of the technique optimization experiments investigated the use of three soil weights, 0.1, 1.0 and 5.0 g, from the Williamsport, PA site. Triplicate flasks were used and the soil was incubated under aerobic conditions. The initial methanol

concentration used in these experiments was 100 mg/L. Further experimental details are presented in the Methods and Materials chapter. Aerobic conditions were used because greater CO_2 evolution was expected to occur with oxygen present than without oxygen.

The results of these experiments (Figure 7) show apparent differences in the average percent $^{14}CO_2$ evolved. The average $^{14}CO_2$ evolution in the 5.0 g experiment appeared to be approaching a maximum value within ten days of incubation, while the $^{14}CO_2$ evolution in the other two experiments did not appear to be doing so. The initial rate of $^{14}CO_2$ evolution also appeared to increase as the soil weight increased. Figure 8 illustrates the differences between the initial (three-day) $^{14}CO_2$ evolution rates. The rates were obtained by dividing the average $^{14}CO_2$ evolution data from the third day of incubation for each experiment by the time of incubation (i.e., three days). Because the use of more than 5.0 g of soil per flask would have required larger reaction vessels and thus changed the experimental conditions, even though the change might have been slight, and because use of greater weights of soil would have rapidly depleted the available soil, the decision was made to use 5.0 g of moist soil in all further experiments. Additionally, the 5.0 g experiment had suggested that the maximum amount of CO_2 had been generated within a ten-day incubation period, a time constraint which was judged to be reasonable given that the sealed flask system could be subject to small losses of CO_2. These small losses could, over a longer period of incubation,

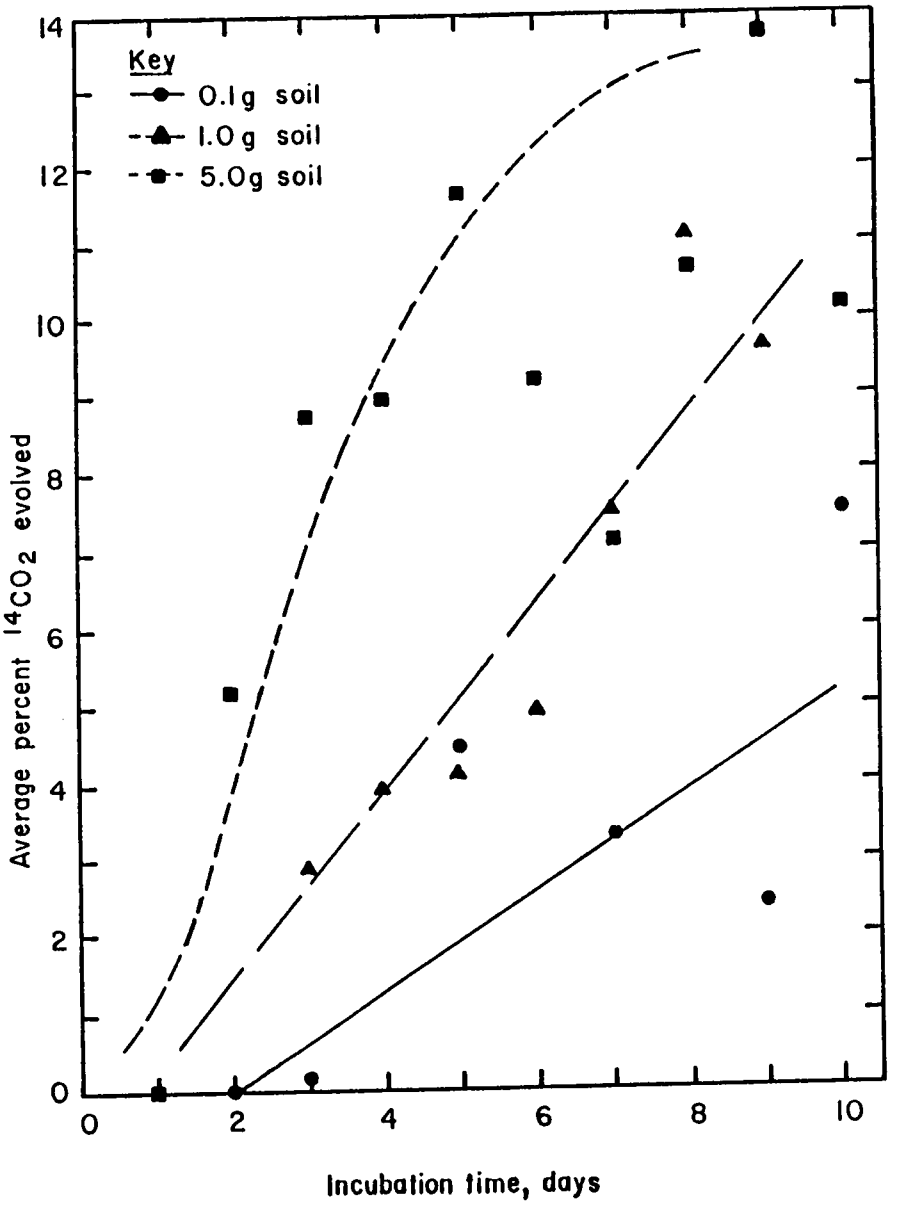

Figure 7. Average percent $^{14}CO_2$ evolved by 0.1 g, 1.0 g and 5.0 g of moist soil from Pennsylvania with time, under aerobic conditions. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

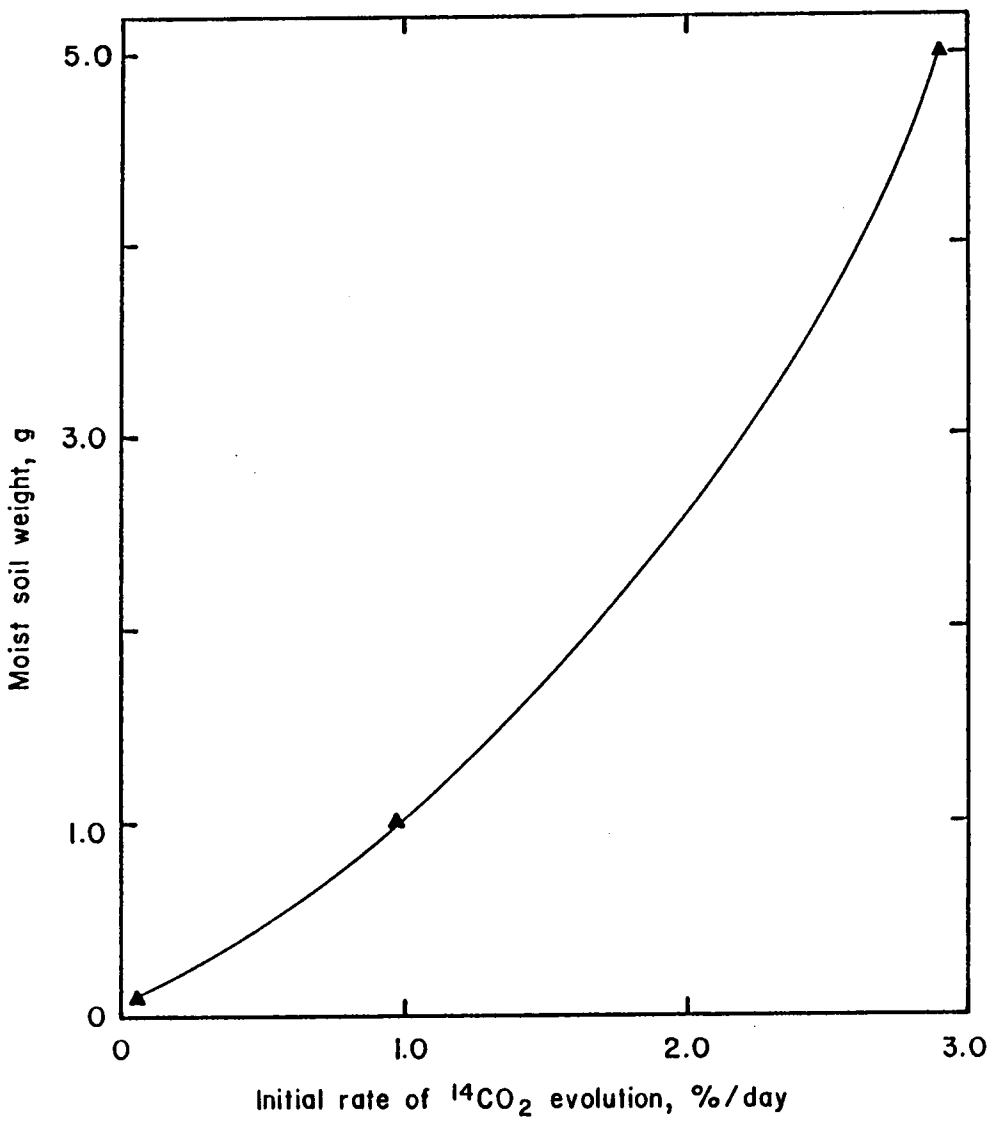

Figure 8. Changes in the initial rate of $^{14}CO_2$ evolution with increasing weight of soil from Pennsylvania, under aerobic conditions. Data obtained from third day of incubation experimental results tabulated in Tables B1-B3.

become serious sources of error and cause variability in the experimental data. It was also believed that the 5.0 g soil sample would contain a more homogenous microbial population than smaller soil samples. A final concern was that greater amounts of soil, or larger incubation periods, could cause the atmosphere of the sealed system to become oxygen depleted.

Degradation of Methanol Under Anoxic Conditions

The effect of anoxic conditions on the evolution of CO_2 by the microorganisms present in 5.0 g soil samples was investigated to determine if the potential for degradation of methanol under these conditions existed for the soil sampling sites. Anoxic conditions were promoted by flushing the flask headspace with nitrogen gas. Because of low concentrations of combined oxygen (about 16 mg/L PO_4 and 4 mg/L SO_4), it seems likely that the combined oxygen present in the anoxic condition experiments was depleted prior to the end of the incubation period. Thus, the conditions probably were anoxic followed by anaerobic. A methanol concentration of 100 mg/L was used as in the aerobic conditions experiments discussed previously.

Comparison of the results (Figure 9) with the results of 5.0 g experiment shown in Figure 7 (note the differences in scale) appears to indicate that $^{14}CO_2$ evolution was greatly reduced under anoxic conditions and that the rate of such evolution also decreased. The high dissolved oxygen level found at the Williamsport site (6.7 mg/L) may have caused a reduction in the portion of the microbial

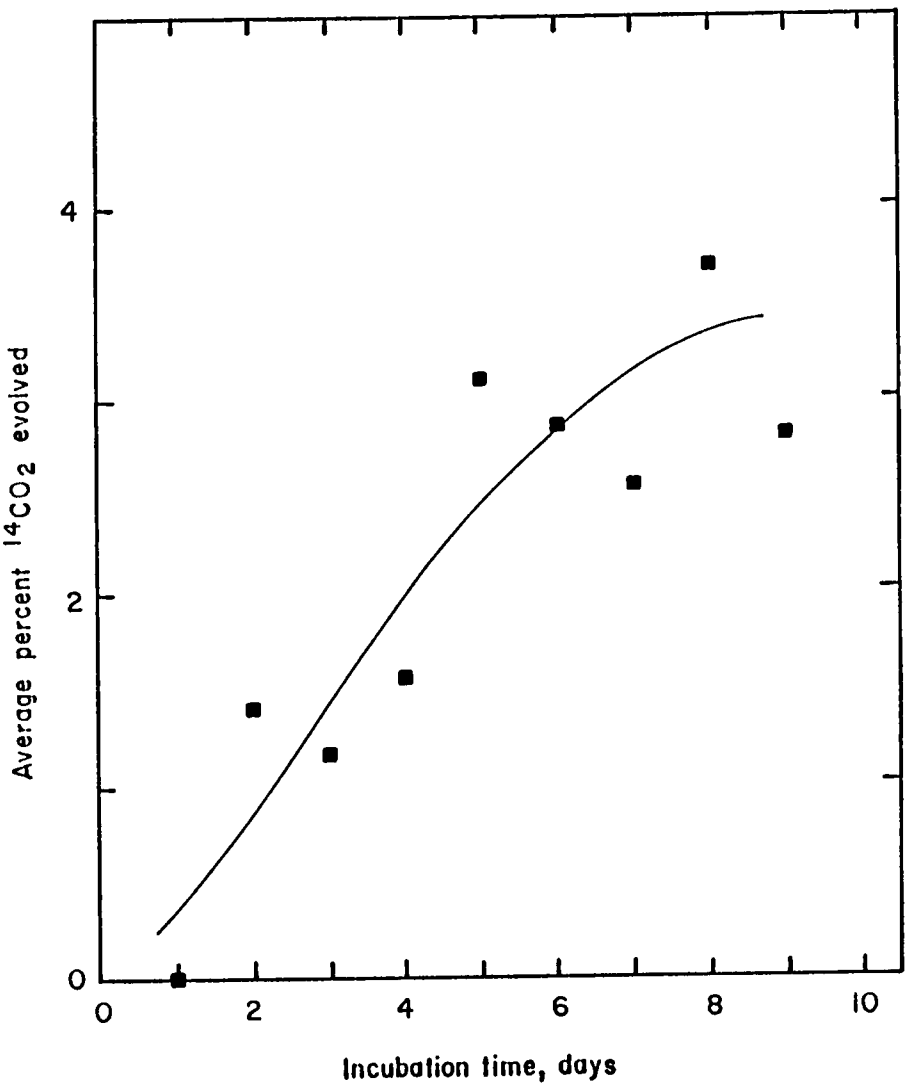

Figure 9. Average percent $^{14}CO_2$ evolved by 5.0 g of moist Pennsylvania soil with time, under anoxic conditions. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

population capable of methanol utilization under oxygen limited conditions. Of importance, however, is the observation that $^{14}CO_2$ was evolved under oxygen deficient conditions, albeit more slowly than aerobically.

Concentration Effects on Methanol Degradation at Williamsport, PA

The concentration effects experiments were conducted to estimate an inhibitory methanol concentration. The experiments were based on the presumption that data analysis by a method similar to that used in kinetics evaluation of the Michaelis-Menten type would provide rates of CO_2 evolution which would correspond to initial methanol concentrations. Observation of changes in these rates could then be seen as the effect of an inhibitory, or a stimulatory, methanol concentration. Data from different sites or depths, or both, could then be compared to establish whether or not differences in CO_2 evolution existed. Both aerobic and anoxic conditions were used for the purposes of determining the dominant type of microorgansims present and to determine the reaction of each type to the various methanol concentrations.

The results shown in Figure 10 indicate that a decrease in $^{14}CO_2$ evolution occurred at initial methanol concentration above about 3000 mg/L (incubation time of seven days). This inhibitory level was seen clearly at one, three and seven days of incubation for the aerobic conditions experiment (Figure 10a) and at three days of incubation for the anoxic conditions experiment (Figure 10b). One

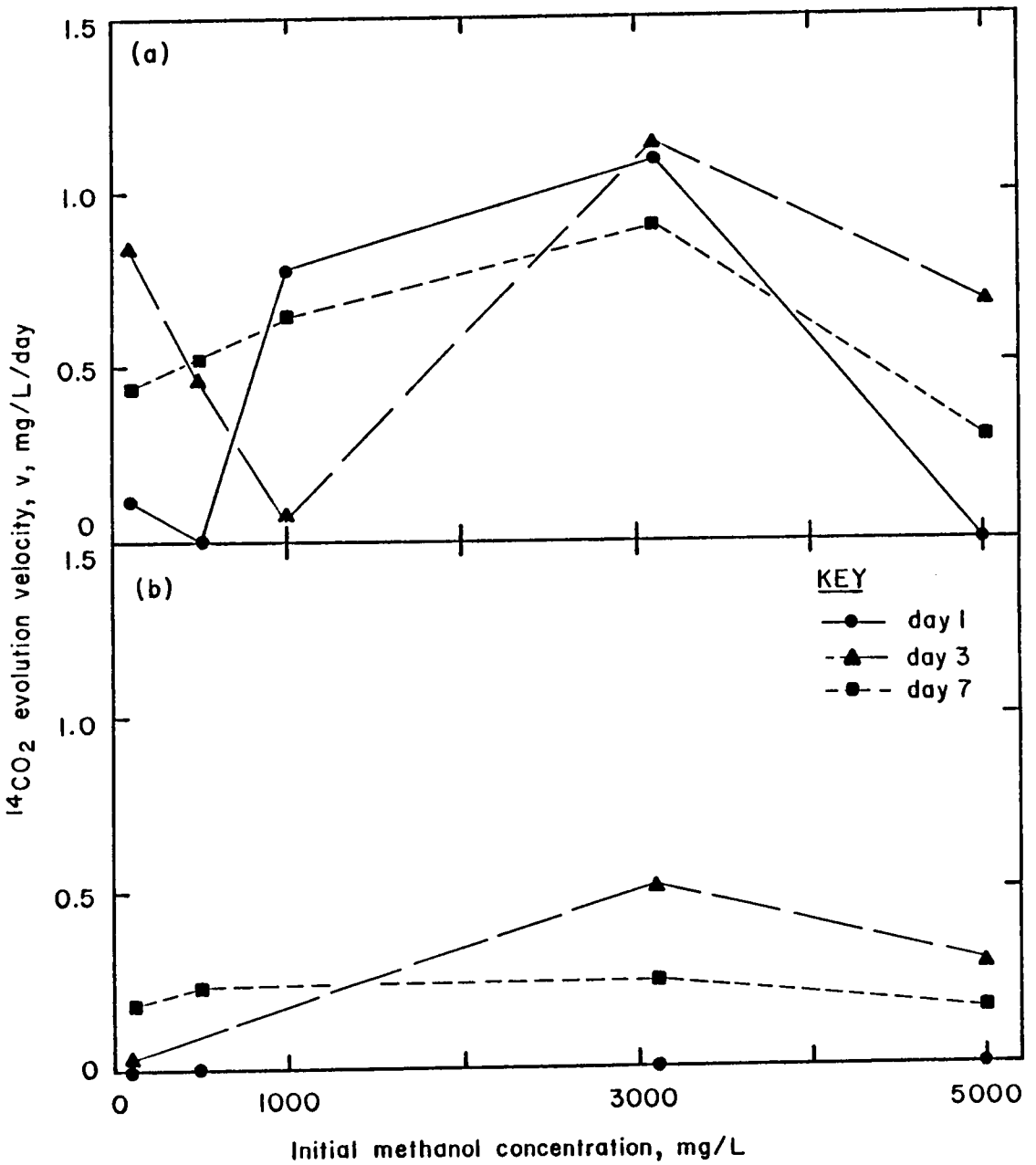

Figure 10. Changes in $^{14}CO_2$ evolution velocity, v, with changes in methanol concentration using Pennsylvania soil: (a) aerobic conditions; (b) anoxic conditions. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

day of incubation under anoxic conditions yielded no observable $^{14}CO_2$ evolution and seven days of incubation without molecular oxygen yielded rates of about 0.2 mg/L/day for all four concentrations tested. The data from the initial methanol concentration of 1000 mg/L under anoxic conditions were omitted from the experimental results because the flasks were believed to have received no ^{14}C-labeled methanol. This decision was made when counts much less than background were obtained for all of the flasks in question. It should also be noted, as with the 100 mg/L methanol experiments previously discussed, that $^{14}CO_2$ was evolved under both aerobic and anoxic conditions and that $^{14}CO_2$ evolution under anoxic conditions was less than that under aerobic conditions.

Methanol Degradation Variations at Dumfries, VA

Methanol Degradation at 100 mg/L Initial Concentration

The data from the experiments on methanol degradation at the Dumfries, VA site, based on an initial concentration of 100 mg/L, indicate that degradation occurs at both depths tested and under both aerobic and anoxic conditions.

Figure 11a illustrates the results of the experiment which used soil from 80 ft (24.4 m), incubated under aerobic conditions. It is apparent that the $^{14}CO_2$ evolution reaches a maximum at five days of incubation, decreasing to zero percent evolved by ten days. Because of the sacrifical nature of the assay experiments, it is believed that a loss of $^{14}CO_2$ occurred. This loss, evidenced by

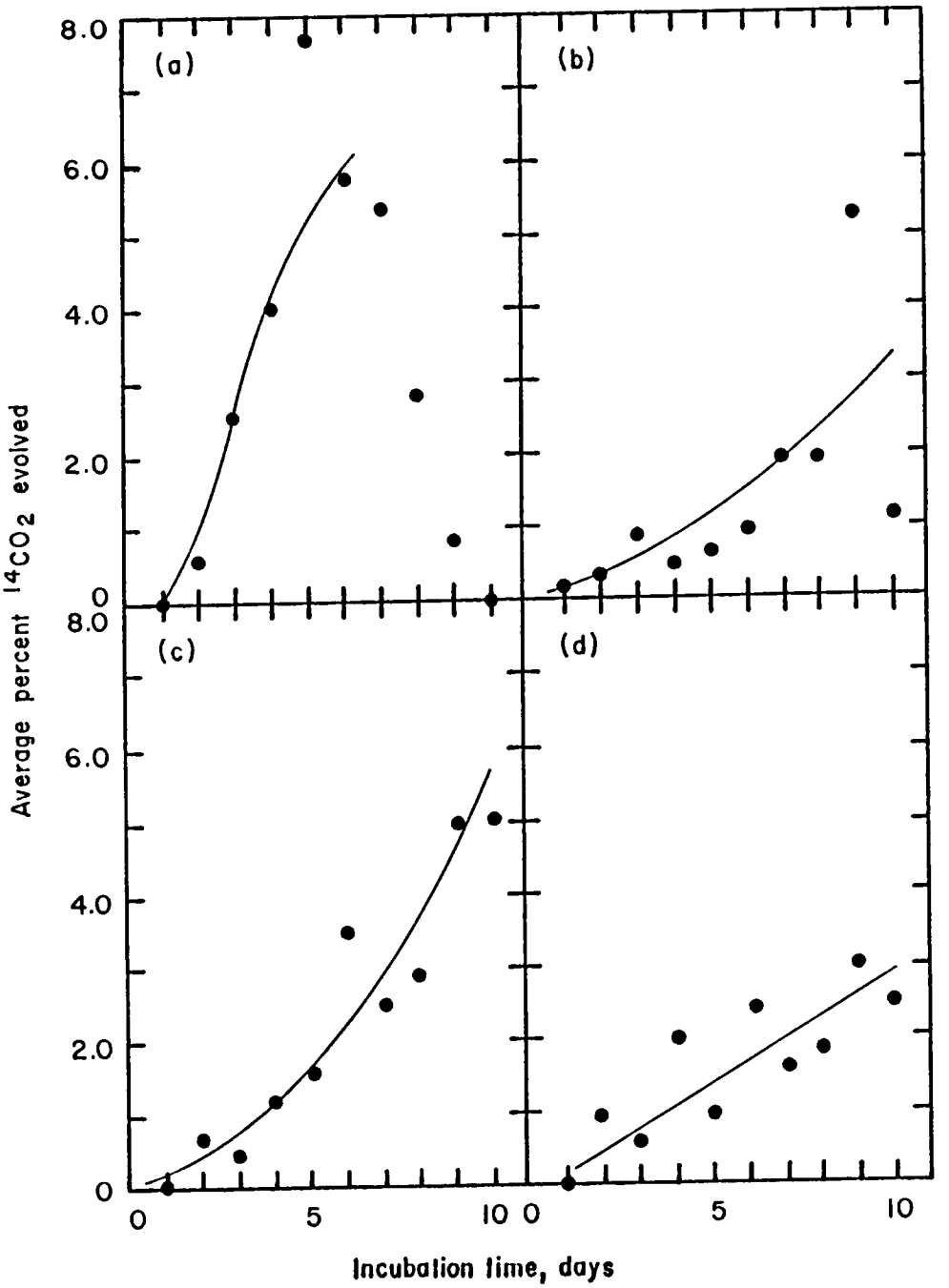

Figure 11. Average percent $^{14}CO_2$ evolved by 5.0 g of moist Virginia soil with time; initial methanol concentration of 100 mg/L: (a) aerobic conditions, 80 ft; (b) anoxic conditions, 80 ft; (c) aerobic conditions, 9 ft; (d) anoxic conditions, 9 ft. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

the decrease in $^{14}CO_2$ evolved past day five of the experiment, was most likely caused by one of the following: (1) increased CO_2 fixation by an increased population of lithotrophic bacteria; (2) leakage of CO_2 through the rubber stoppers; (3) both leakage and increased CO_2 fixation. Lithotrophs are suggested because their ability to use CO_2 as their only carbon source and because they do not require sunlight as their energy source. It seems likely that leakage was primarily responsible for the lowered $^{14}CO_2$ evolution at the later incubation times in these experiments.

The same soil from 80 ft (24.4 m), when tested under anoxic conditions yielded low $^{14}CO_2$ evolution values (Figure 11b), requiring about seven days of incubation to exceed the one percent level. Most of the methanol degraded under these conditions was probably converted into cellular biomass, CO_2 and methane (CH_4), while under an oxygen atmosphere methane is not produced. Because the assay has not been designed to do so, methane produced by microorganisms has not been included in the results. If a simple and effective trapping system could be devised for the separation and capture of $^{14}CO_2$ and $^{14}CH_4$, the assay could provide much more data of greater value to researchers. It could be argued that a correction could be used to incorporate the production of CH_4, based on the following, or other, stoichiometry:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \qquad [9]$$

This is, however, a mathematical adjustment of the data and without some direct measure of the methane to CO_2 ratio, cannot be justified.

The data obtained from the incubation of soil from 9 ft (2.7 m) under aerobic conditions (Figure 11c) appears to indicate that degradation occurred at a slower rate than at the 80 ft (24.4 m) depth. Note that the $^{14}CO_2$ evolution curve did not diminish to a zero evolution level as did the curve in Figure 11a. Thus, if losses occurred, it appears that they were either offset by the $^{14}CO_2$ production throughout the ten-day incubation period or that they are inconsistent and unpredictable. Evolution of $^{14}CO_2$ under anoxic conditions (Figure 11d) appeared to have occurred at a similar rate as under the aerobic conditions for the 9 ft (2.7 m) soil (Figure 11c). The anoxic conditions $^{14}CO_2$ evolution rates for both the 9ft (2.7 m) depth and the 80 ft (24.4 m) depth do not appear to be substantially different.

The differences between the experimental results from the 9 ft (2.7 m) and the 80 ft (24.4 m) depths, under aerobic conditions, tend to be supported by the differences in the bacterial populations at the two soil depths, based on soil extract media MPN enumeration (see Table A4).

Methanol Degradation - Concentration Effects

The data from the concentration effects experiments appear to indicate that microbial degradation of methanol occurred at both depths tested and under both aerobic and anoxic conditions. The

results from the experiments using soil from 80 ft (24.4 m) (Figure 12a and 12b) show maximum $^{14}CO_2$ evolution rates at an initial methanol of about 3000 mg/L for one and seven days of incubation. At an incubation time of three days, $^{14}CO_2$ evolution was about the same for the initial methanol concentrations of 3000 and 5000 mg/L, under aerobic conditions. Evolution of $^{14}CO_2$ increased about one mg/L/day, under anoxic conditions, for the 3000 and 5000 mg/L methanol concentrations. Still, a leveling off of the rates seems evident, however, the difference was not very large. The rates of $^{14}CO_2$ evolution were noticeably faster in these two experiments than were the rates observed in the concentration effects experiments using the Williamsport soil (Figures 10a and 10b). Also, the $^{14}CO_2$ evolution rates observed under anoxic conditions generally appeared to be slower than those observed under aerobic conditions for the Dumfries soil experiments, although the differences between the rates appeared to be small.

The rates of $^{14}CO_2$ evolution for the experiments using soil from 9 ft (2.7 m) show marked differences when the results of the aerobic (Figure 12c) and anoxic (Figure 12d) experimental conditions are compared. As observed earlier, the rates under aerobic conditions were generally faster than those under anoxic conditions. The large difference in rates may be attributable to a large percentage of aerobes in the total bacterial population at this depth. The rates of $^{14}CO_2$ evolution shown in Figure 12d are also very similar in magnitude to those shown in Figure 10b. The reason for the slow rates for the day seven data of Figure 12c are unknown.

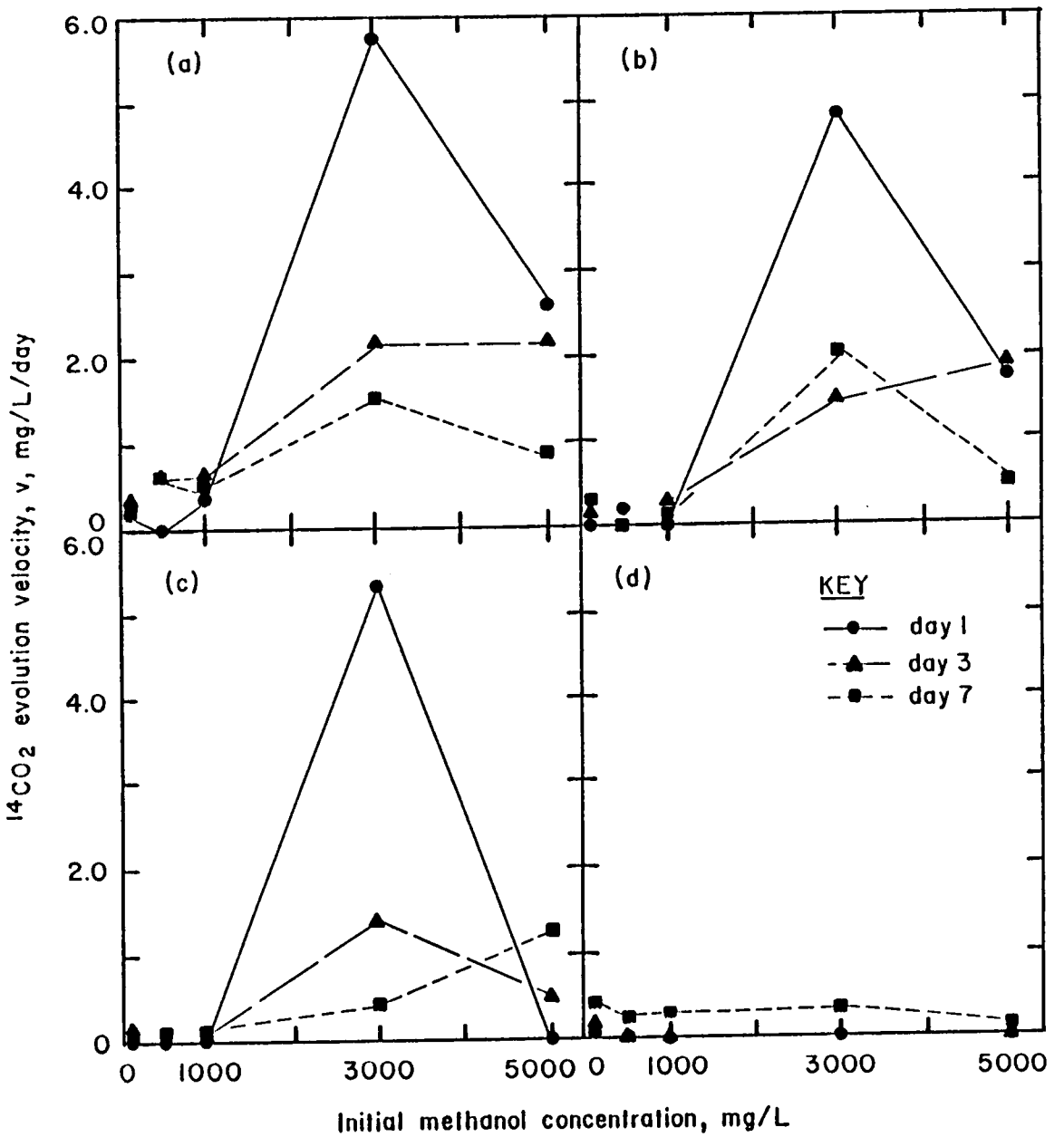

Figure 12. Changes in $^{14}CO_2$ evolution velocity, v, with changes in methanol concentration using Virginia soil: (a) aerobic conditions, 80 ft; (b) anoxic conditions, 80 ft; (c) aerobic conditions, 9 ft; (d) anoxic conditions, 9 ft. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

It seems reasonable that the low value at the 3000 mg/L methanol concentration may have been the result of an experimental deficiency. When the rates of $^{14}CO_2$ evolution for the two depths, under aerobic conditions, are compared, it can be seen that the rates observed at the 9 ft (2.7 m) depth (Figure 12c) appear to generally be slower than those at the 80 ft (24.4 m) depth (Figure 12a). This is in agreement with the comparison of the 100 mg/L initial concentration experimental results for the same soils under aerobic conditions.

When the rates of $^{14}CO_2$ evolution for these two depths, under anoxic conditions, are compared, initial observation would seem to contradict the results of the related 100 mg/L initial concentration experiments. On closer examination (see Tables B16 and B20) it can be seen that the rates at the 9 ft (2.7 m) soil depth are slower than those observed at the 80 ft (24.4 m) depth for the initial concentration of 100 mg/L at one, three, and seven days of incubation. Further comparison of the rates observed at higher concentrations show a reversal of the trend, that is, the rates at the greater soil depth were the slower of the two sets of $^{14}CO_2$ evolution rates. The reason for this reversal as concentration increased is unclear, however, one possibility is that a required nutrient was exhausted much sooner in the 80 ft (24.4 m) soil at high initial concentrations (1000-1000 mg/L) than in the 9 ft (2.7 m) soil, or than at the low initial concentrations. This explanation would also seem plausible when the bacterial populations of the two depths are compared and it is seen that the population at the greater

depth is almost three orders of magnitude higher than that at the 9 ft (2.7 m) depth. Another viable explanation, also based on the different sizes of bacterial populations, is that toxic microbial byproducts or secretions may have accumulated to inhibitory levels sooner at the higher initial methanol concentrations than at the 100-1000 mg/L initial concentrations.

TBA Degradation Variations at Dumfries, VA

TBA Degradation at 10 mg/L Initial Concentration

The data from the experiments on TBA degradation at the Dumfries, VA site are difficult to interpret. From the time-study experiments performed at an initial TBA concentration of 10 mg/L, data trends are difficult to observe. In Figure 13a data are presented for the aerobic conditions experiment using soil from a depth of 55 ft (16.8 m). It can be seen that only five of the ten days of incubation yielded positive data with the greatest average value for percent $^{14}CO_2$ evolved (0.15%) occurring on the final day of the experiment. Figure 13b, which presents data for the same soil under anoxic conditions, shows that from the fourth day of incubation to the end of the experiment positive values for $^{14}CO_2$ evolution were observed. These six consecutive days producing data suggests that TBA degradation may occur under anoxic conditions after a period of acclimation. This degradation, however, proceeded at a slow

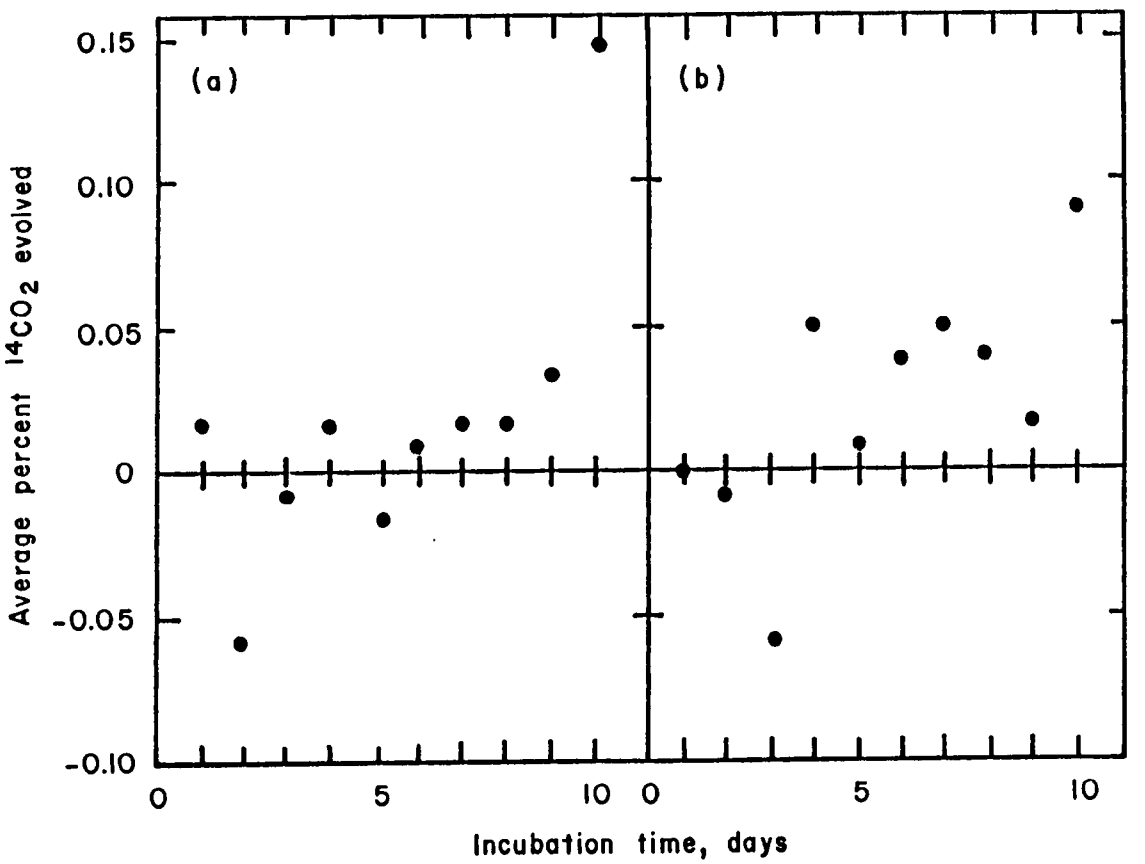

Figure 13. Average percent $^{14}CO_2$ evolved by 5.0 g of moist Virginia soil with time; initial TBA concentration of 10 mg/L: (a) aerobic conditions, 55 ft; (b) anoxic conditions, 55 ft. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

rate. The data from the aerobic conditions experiment (Figure 13a) indicate low $^{14}CO_2$ evolution (about 0.02%), starting at the first day of incubation. Because the values were so low in the aerobic conditions experiment, up to the tenth day, it cannot be assured that these values do not represent extremes of radioactivity background. The data from the anoxic conditions experiment were higher in value than those in the aerobic experiment and appear to be scattered less and are considered to represent the occurrence of biodegradation.

Figure 14a presents the data for TBA degradation under aerobic conditions using soil from a depth of 12 ft (3.7 m) and Figure 14b presents data for TBA degradation for the same soil under anoxic conditions. No trends in the data for these two experiments could be observed, however, the values from the anoxic conditions experiment generally appeared to be greater than those in the aerobic conditions experiment.

The data from these four experiments suggest that TBA degradation may occur at an initial concentration of 10 mg/L. This degradation, however, may occur only after a sizeable acclimation period and then only at a slow rate. More conclusive data may be obtainable by using flasks which can be sealed against leakage better and by extending the incubation period to three or four weeks. A longer incubation period may lead to a greater amount of $^{14}CO_2$ evolution and thus a better measure of slow degradation rates. A disadvantage to the use of longer incubation periods is the great increase in the quantity of soil required, assuming daily checks for $^{14}CO_2$ evolution. Thus, researchers with a limited budget for soil sampling may be hard

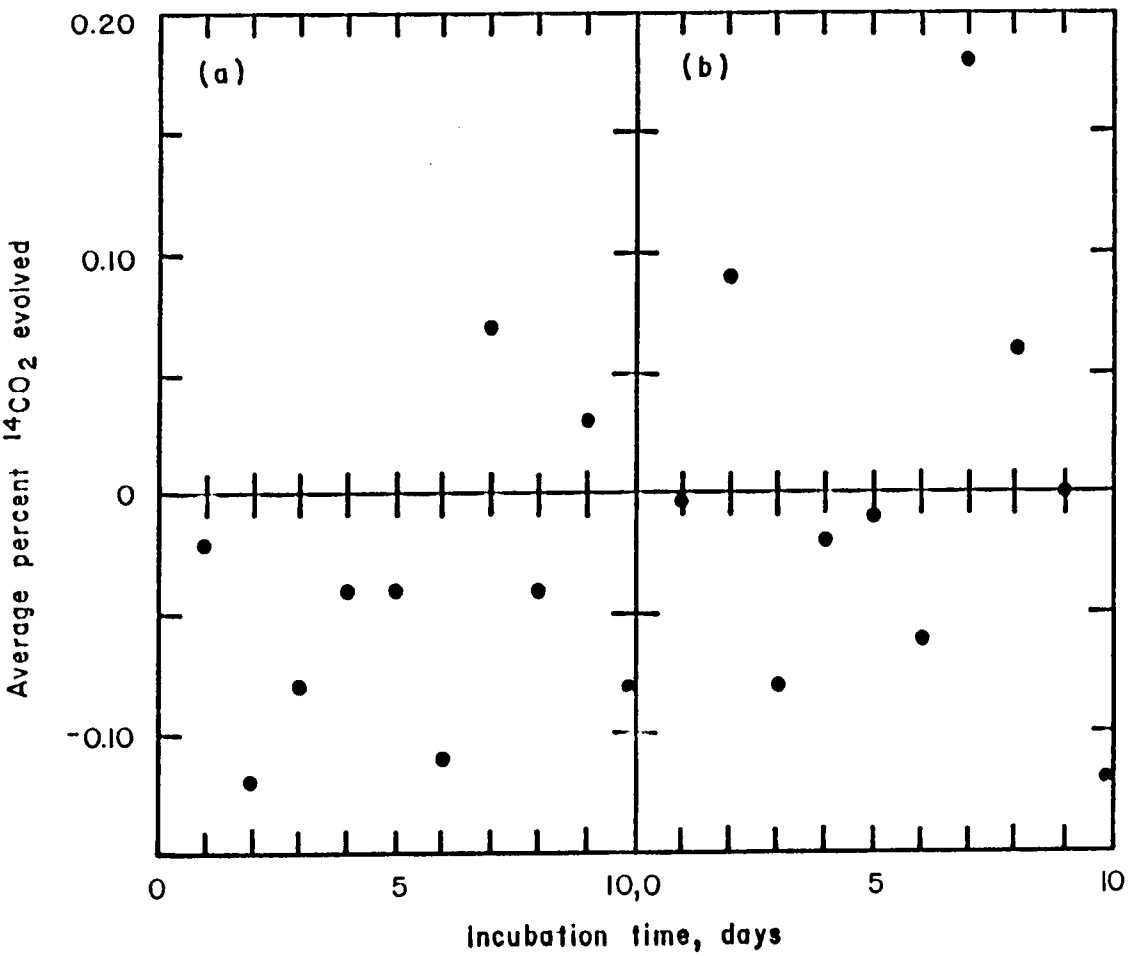

Figure 14. Average percent $^{14}CO_2$ evolved by 5.0 g of moist Virginia soil with time; initial TBA concentration of 10 mg/L: (a) aerobic conditions, 12 ft; (b) anoxic conditions, 12 ft. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

pressed to extend incubation and retain the required statistical significance of any results gained.

TBA Degradation - Concentration Effects

Figure 15 presents the experimental data for TBA degradation using soil from a depth of 55 ft (16.8 m) and 12 ft (3.7 m). In these experiments, degradation rate variations with depth and under aerobic and anoxic conditions were measured. The TBA concentrations used in the experiments were 10, 50, 100, 300 and 500 mg/L.

Generally, the $^{14}CO_2$ evolution rates were slow, ranging from undetectable to 0.43 mg/L/day. Most of the rates for the 10 mg/l initial concentration were zero or undetectable, suggesting that the assay may not be responsive enough at this low concentration for recalcitrant compounds such as TBA. The alternative suggestion would be that the concentrations chosen for this study may have been higher than the actual inhibitory concentration. That is, faster rates of $^{14}CO_2$ evolution and, therefore, biodegradation may occur at initial TBA concentrations lower than 10 mg/L. It was assumed that further increases in the initial concentrations of TBA above 500 mg/L would not yield faster $^{14}CO_2$ evolution rates than the rates calculated for the concentration used.

In Figure 15a, the results from the first day of incubation show a general increase in the $^{14}CO_2$ evolution rates with increased TBA concentration. The highest rate of 0.43 mg/L/day was observed at 500 mg/L TBA. The results from the third and seventh days of

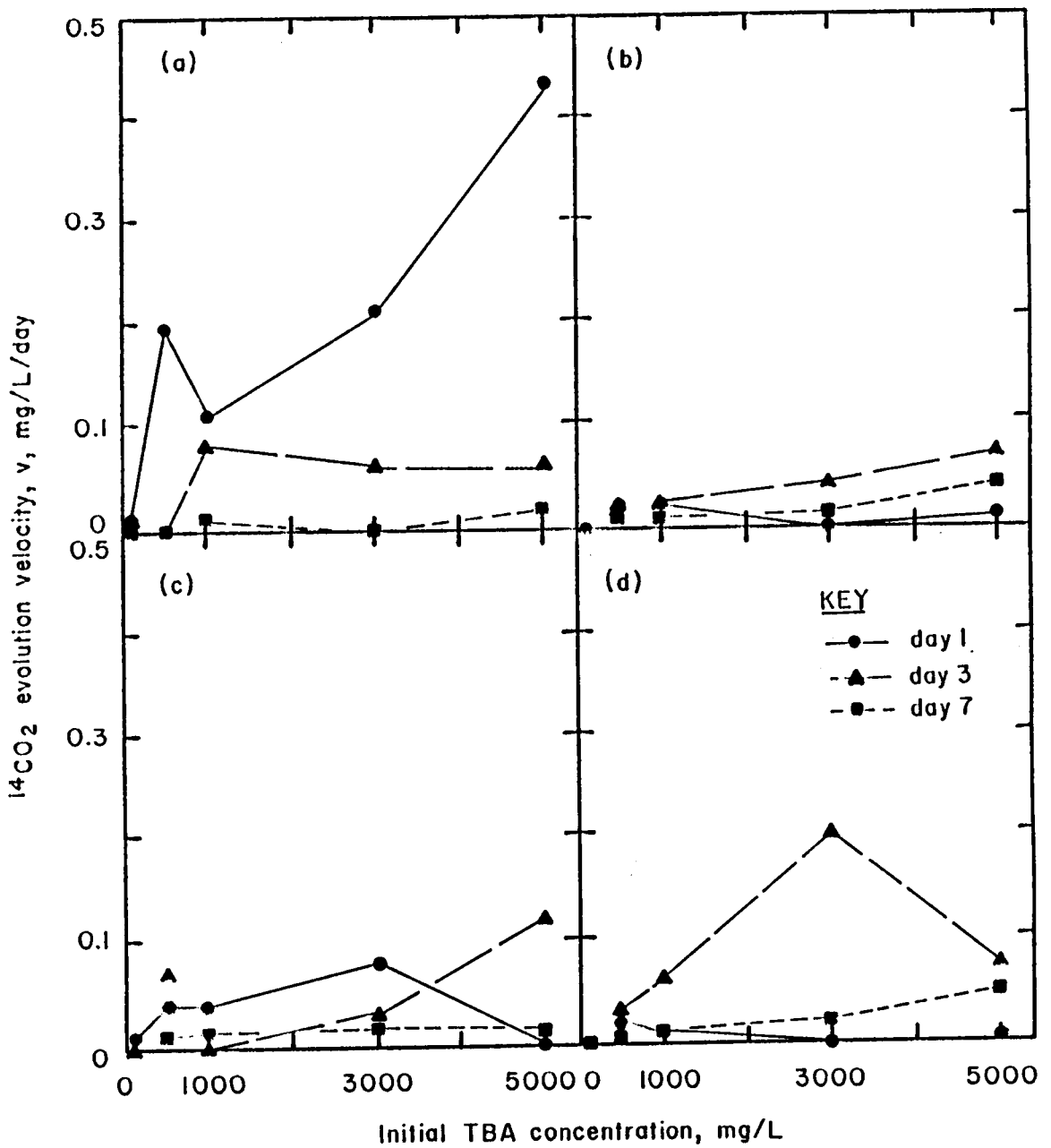

Figure 15. Changes in $^{14}CO_2$ evolution velocity, v, with changes in TBA concentration using Virginia soil: (a) aerobic conditions, 80 ft; (b) anoxic conditions, 80 ft; (c) aerobic conditions, 9 ft; (d) anoxic conditions, 9 ft. Data points plotted are averages of values obtained from triplicate flasks incubated at 20°C (68°F).

incubation did not exceed 0.08 mg/L/day. In Figure 15b, the rates from all incubation times did not exceed 0.07 mg/L/day, but the rates did exhibit a general increase in value with increased TBA concentration. The data from day three of the aerobic conditions experiment (Figure 15a) remained at about 0.07 mg/L/day for the TBA concentration range of 100 to 500 mg/L while the day three data from the anoxic conditions experiment (Figure 15b) gradually increased to approximately the same level. The day seven data for both experiments were similar, 0.04 mg/L/day or less. This seems to indicate that at higher TBA concentrations (100-500 mg/L), faster rates may occur under aerobic conditions than under anoxic conditions at the 55 ft (16.8 m) soil depth.

The results of the 12 ft (3.7 m) soil depth experiments (Figure 15c and 15d) generally consisted of rates less than 0.10 mg/L/day. Comparison of the data from the two experiments failed to identify an inhibitory TBA concentration regardless of the presence or absence of molecular oxygen. Under aerobic conditions (Figure 15c), no TBA concentration was observed to inhibit $^{14}CO_2$ evolution. The day one and day seven data generally follow the same pattern in which both yielded their fastest $^{14}CO_2$ evolution rate at 300 mg/L TBA. The day three data from the anoxic conditions experiment (Figure 15d) yielded the fastest rates of the experiment (0.20 mg/L/day) at 300 mg/L TBA. The other data, from days one and seven, did not exhibit any single inhibitory TBA concentration.

Assay Limitations and Recommendations

Briefly, the heterotrophic activity assay used in these experiments shows promise as a tool for investigating the biodegradation of environmental contaminants. For this assay modification to be more valuable, several limitations need to be eliminated.

The potential exists for leakage of $^{14}CO_2$ from the closed system used. This may be eliminated by using screw-cap vials or flasks where the caps have been fitted with self-sealing septa lined with, or made from, an inert material such as Teflon. Hypo vials with this type of septa might also be used. The centerwell could be permanently attached to either the septum or the reaction vessel with a non-toxic adhesive. The size of the reaction vessel could also be larger than those used in this study (e.g., 200 ml hypo vials) in order to minimize buildup of pressure caused by gas production or material addition. Large reaction vessel volumes, relative to soil weight and time of incubation, would also allow aerobic conditions to exist for long incubation periods [32].

Isotopic discrimination by soil microorganisms, a factor not taken into account in this study, should be investigated. The correction factor of 1.06 has been used by other researchers. The indiscriminant use of this value, or others, is to be avoided.

Development of a methane trap, which could be used in conjunction with, or separate from, a carbon dioxide trap, is recommended. This development could allow for the acquisition of much valuable data from anoxic and anaerobic conditions experiments.

Wherever possible, the use of chromotography is encouraged. Its use may provide data on microbial uptake of carbon for biomass or development of a relation between total uptake of toxin and $^{14}CO_2$ evolution. The use of this assay may be preferred where the research involves highly volatile compounds or compounds with high adsorption affinities (e.g., benzene).

Finally, the researcher is limited to the use of soils which do not contain marl, or other carbonate materials, in large amounts. These materials, which were present in the Wayland, NY site soils, could cause pressure buildup within the closed system after acidification, exhaustion of the CO_2 trapping material prior to complete $^{14}CO_2$ recovery, erroneous results due to preferential use of the carbonate material over the toxin being tested, or the pH of the soil-liquid mixture being acidified may not reach the level required for complete conversion to CO_2.

Groundwater Parameters and Bacterial Populations

Groundwater Parameters

The groundwater parameters for the Williamsport, PA, Wayland, NY and Dumfries, VA sites are presented in Table A1 (Appendix A). The sites were quite different in many respects.

The Williamsport site was characterized by aerobic subsurface waters and high nitrate levels. The groundwater from the Wayland site was anoxic, containing sulfate but little nitrate or oxygen.

The New York site groundwater also contained high alkalinity and the pH was about three units higher than the pH of the other sites. The groundwater at the Dumfries site was poorly mineralized and contained little oxygen.

Bacterial Enumeration

The results of soil extract agar MPN counts and acridine orange slide counts are presented in Tables A2-A4 (Appendix A). These tables contain the counts from Williamsport, PA, Wayland, NY and Dumfries, VA, respectively. Substantial bacterial populations were found to be present in the subsurface system of each site.

At Williamsport, PA, surface and saturated zone soils yielded higher counts than the unsaturated zone soils by both counting methods. Both soil extract MPN counts and acridine orange slide counts on saturated soils which had been dosed with 100 mg/L methanol and 10 mg/L TBA exhibited a ten-fold increase in bacterial numbers indicating that the microorganisms were viable and that the substrates were being utilized [15].

Bacterial populations at the Wayland site compared favorably with those at the Williamsport site with respect to changes in numbers with depth [15].

Acridine orange slide counts at the Dumfries site were consistent with the other sites and varied little with depth. Counts on soil extract agar varied considerably. However, bacteria were plentiful in the soils. Although the 14.8 ft (4.5 m) soil sample was above the primary water table, it did contain free water which separated from the soil sample [29].

Comparison With Other Research

Results of preliminary experiments by Benoit [8] have shown that the methanol concentration experiments discussed previously may be providing correct results with regard to the inhibitory methanol concentration. The work by Benoit involved use of a pure culture, isolated from the Williamsport site, in a Clark cell. The culture used was described as the best methanol degrader, but has not been identified. Oxygen uptake was observed to reach a maximum level between the final methanol concentration of 1100 and 11000 mg/L.

Data obtained by Goldsmith [15] also lend support to this assay as a quick and valid method of estimating biodegradation rates and of observing biodegradation. Utilization rates for both methanol and TBA, for the Dumfries site, are presented in Table 2. Goldsmith's work involved the use of the static microcosms where substrate utilization was determined by gas chromatographic analyses. By comparing the data in Table 2 with the results shown in Table 3, it can be observed that the utilization rates of methanol and TBA compare favorably with the rates of $^{14}CO_2$ evolution from methanol and TBA. Methanol, by both methods, appears to be utilized at rates approximately two orders of magnitude faster than TBA.

It is clearly evident that few advantages of the ^{14}C tracer method investigated in this study over the microcosm approach, used by Goldsmith, exist. The ^{14}C-labeled substrate method, with appropriate minor modifications, would apparently provide data comparable to that which could be obtained from microcosm studies.

TABLE 2. Utilization rates (mg/L/day) for Dumfries, VA: from microcosm studies after Goldsmith [15]. Incubation temperature of 10°C (50°F).

Initial Concentration, mg/L	Soil Depth, ft	
	11	80
Methanol		
95.1	0.93	
724.0	1.89	
87.5		2.36
996.0		7.89
TBA		
1.0	4.0×10^{-3}	
6.4	8.0×10^{-3}	
1.0		1.50×10^{-3}
8.2		2.45×10^{-2}

TABLE 3. Maximum $^{14}CO_2$ evolution rates (mg/L/day) for Dumfries, VA from radiotracer studies. Incubation temperature of 20°C (68°F).

Initial Concentration, mg/L	Soil Depth, ft			
	9	12	55	80
Methanol				
100	0.37			0.33
500	0.23			0.61
1000	0.28			0.67
TBA				
10		9.09×10^{-3}	1.50×10^{-2}	
50		2.08×10^{-2}	1.86×10^{-1}	
100		1.70×10^{-1}	1.12×10^{-1}	

The microcosm approach is believed to require smaller soil quantities which can provide more data than the ^{14}C-labeled substrate method and would seem to be the better option. The ^{14}C tracer method would appear to be an accurate screening technique, however, and would also provide valuable information from degradation studies of volatile substances.

V. SUMMARY AND CONCLUSIONS

This study was conducted for the purposes of; (1) investigating the use of ^{14}C-tracer technique as a means of assessing initial rates of biodegradation of chemicals of environmental concern, as a means of estimating inhibitory concentrations of such substances and as a means of determining the potential degradability of such substances and providing a detailed investigation into the biodegradation of methanol and tertiary butyl alcohol (TBA), on a site-specific basis, for the Atlantic Richfield Company.

This thesis investigated the use of a modification of the heterotrophic activity assay, the radiorespirometric technique, and the following conclusions were reached, based on the results of the research described in this thesis:

1. Methanol is readily degraded, under both aerobic and anoxic conditions, by the soil microorganisms present at the Williamsport, PA and Dumfries, VA sites investigated, up to the apparent inhibitory concentration.
2. The apparent inhibitory methanol concentration is approximately 3000 mg/L (as methanol), under both aerobic and anoxic conditions.
3. Tertiary butyl alcohol is degraded very slowly, under both aerobic and anoxic conditions, by the soil microorganisms present at both sites investigated.
4. This assay modification as used in this study, may be unreliable when using a recalcitrant substance such as TBA.

5. The ^{14}C tracer technique appears to hold promise as a screening tool for chemicals of environmental concern.
6. The data generated by the ^{14}C tracer technique compare favorably with other biodegradation research techniques, specifically the Clark cell and microcosm approaches.

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APPENDIX A

TABLE A1. Groundwater Parameters at the Soil Sampling Sites.

Parameters	Location		
	Pennsylvania	New York	Virginia
Cl^-	9.94 mg/L	8.00 mg/L	12.24 mg/L
Br^-		0.06	0.32
NO_3^-	53.40	1.54	0.11
NO_2^-		1.20	0.56
SO_4^{2-}	27.64	52.00	8.10
PO_4^{3-}	ND	ND	ND
FE	0.44	0.05	4.23
CA	22.60	73.80	2.88
Mg	4.6 5	14.40	3.88
Na	2.78	33.40	13.50
K	1.09	3.50	4.54
TOC	0.1 ppm	1.7 ppm	1.3 ppm
TOX	none	none	50.0 ppb
Alkalinity (as $CaCO_3$)	none	180 mg/L	none
Dissolved Oxygen	6.7	0.7	0.2
Temperature (^oC)	11.0	10.0	10.0
pH	4.73	7.81	4.50

ND - Not Detectable

TABLE A2. Bacterial Population in Subsurface Soil from Pennsylvania.

Depth (m)	Soil Extract, cfu/g	AO Direct Count
0	$3.0 \pm 0.3 \times 10^7$	$5.6 \pm 1.9 \times 10^7$
3-4	$3.5 \pm 2.1 \times 10^3$	$3.9 \pm 1.4 \times 10^7$
9	$1.4 \pm 0.8 \times 10^5$	$4.6 \pm 2.7 \times 10^7$

TABLE A3. Bacterial Population in Subsurface Soil from New York

Depth (m)	Soil Extract, cfu/g	AO Direct Count
0	$1.0 \times 10^7 \pm 4.0 \times 10^6$	$1.0 \times 10^8 \pm 4.1 \times 10^7$
2	$9.3 \times 10^5 \pm 1.1 \times 10^5$	$7.6 \times 10^7 \pm 3.8 \times 10^7$
3-4	$1.1 \times 10^6 \pm 8.5 \times 10^4$	$8.0 \times 10^7 \pm 6.4 \times 10^7$

TABLE A4. Bacterial Population in Subsurface Soil from Virginia.

Depth (m)	Soil Extract, cfu/g	AO Direct Count
0	$9.7 \times 10^6 \pm 5.7 \times 10^5$	$1.0 \times 10^8 \pm 4.1 \times 10^7$
3	$< 10^3$	$7.3 \times 10^7 \pm 3.7 \times 10^7$
4.5	$3.3 \times 10^6 \pm 4.0 \times 10^5$	$3.1 \times 10^7 \pm 2.2 \times 10^7$
9	$5.6 \times 10^5 \pm 7.1 \times 10^3$	$3.9 \times 10^7 \pm 3.4 \times 10^7$
15-17	$5.2 \times 10^6 \pm 9.2 \times 10^5$	$1.1 \times 10^8 \pm 6.4 \times 10^7$
24-25	$9.8 \times 10^5 \pm 5.3 \times 10^4$	$7.3 \times 10^7 \pm 5.6 \times 10^7$
31	$1.1 \times 10^5 \pm 2.8 \times 10^4$	$4.8 \times 10^7 \pm 4.5 \times 10^7$

APPENDIX B

Table B1. Experimental results of 100 mg/L methanol degradation using moist soil weight of 0.10 grams under aerobic conditions. Williamsport, PA site soil from depth of 6 to 8 feet (1.8 to 2.4 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	0.1008	3053.4		
2		0.1029	2180.5	2268.2	0
3		0.1033	1570.6		
4	2	0.1032	2375.6		
5		0.1012	2963.1	2949.6	0.01
6		0.1030	2039.9		
7	3	0.0994	3494.9		
8		0.1035	4807.3	3656.1	0.16
9		0.1013	2666.1		
10	5	0.1021	24097.8		
11		0.1035	18975.6	23457.3	4.50
12		0.0985	27298.4		
13	7	0.1012	16497.3		
14		0.1026	19658.6	18108.5	3.33
15		0.1012	18169.6		
16	9	0.1025	17065.7		
17		0.0987	15626.8	15972.5	2.86
18		0.1021	15225.1		
19	10	0.1012	51867.4		
20		0.1022	21208.5	37185.4	7.51
21		0.0992	38480.4		

Table B2. Experimental results of 100 mg/L methanol degradation using moist soil weight of 1.00 grams under aerobic conditions. Williamsport, PA site soil from depth of 6 to 8 ft. (1.8 to 2.4 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	0.9994	2172.9	2249.7	0
2		0.9991	1998.6		
3		0.9985	2577.5		
4	3	1.0007	11862.9	16331.7	2.94
5		0.9978	14356.3		
6		1.0012	22775.8		
7	4	1.0025	17583.5	20706.2	3.90
8		0.9992	20718.1		
9		1.0025	23817.0		
10	5	1.0010	16783.5	21537.2	4.08
11		1.0022	28696.6		
12		1.0024	19131.4		
13	6	1.0018	22887.0	25178.1	4.88
14		0.9989	25611.8		
15		0.9990	27035.5		
16	7	1.0037	42199.8	37018.5	7.48
17		1.0023	19474.5		
18		1.0003	49381.1		
19	8	1.0000	41882.3	53317.6	11.05
20		1.0022	62203.9		
21		1.0006	55866.7		
22	9	0.9991	30672.7	46895.9	9.64
23		0.9985	42467.0		
24		0.9996	67548.1		

Table B3. Experimental results of 100 mg/L methanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Williamsport, PA site soil from depth of 6 to 8 ft. (1.8 to 2.4 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	4.9966	1580.0		
2		5.0131	1574.2	1605.5	0
3		5.0055	1662.6		
4	2	5.0108	26652.1		
5		5.0104	29950.0	26675.0	5.21
6		5.0055	23423.0		
7	3	5.0167	55601.3		
8		5.0144	35618.6	42796.5	8.74
9		5.0091	37169.8		
10	4	5.0158	41282.6[1]		
11		5.0068	41282.6[1]	43780.5	8.96
12		5.0083	48776.2		
13	5	5.0079	51491.4		
14		5.0123	63912.3	55936.8	11.63
15		5.0116	52403.7		
16	6	5.0114	37180.7		
17		5.0124	44981.9	44822.2	9.19
18		5.0061	52304.1		
19	7	5.0111	23781.8		
20		5.0119	33575.4	35344.9	7.11
21		5.0110	48677.6		
22	8	5.0072	61846.4		
23		5.0068	52552.4	51602.0	10.67
24		-[2]	40407.3		
25	9	5.0138	65595.2		
26		5.0173	57275.9	65675.8	13.76
27		5.0153	74156.1		

[1] Both filters for flasks 10 and 11 were accidently place in same counting vial. Actual DPM value was divided by 2 to give DPM for each flask.

[2] Soil weight not recorded.

Table B3 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
28	10	5.0095	56128.2		
29		5.0147	55720.5	49317.6	10.17
30		5.0153	36104.2		
31	13	5.0139	41647.3		
32		5.0121	17098.4	26663.6	5.21
33		5.0117	21245.3		
34	20	5.0163	34182.3		
35		5.0153	13138.6	30223.1	5.99
36		5.0144	43348.5		

Table B4. Experimental results of 100 mg/L methanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Williamsport, PA site soil from depth of 8 to 10 ft. (2.4 to 3.0 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	5.0012	2298.3		
2		5.0162	2386.2	2434.5	0
3		5.0045	2618.9		
4	2	5.0031	17414.1		
5		5.0027	5936.5	9263.9	1.39
6		4.9956	4441.2		
7	3	5.0089	9805.5		
8		5.0018	8919.2	8234.9	1.16
9		5.0076	5979.8		
10	4	5.0013	14122.2		
11		5.0033	9356.0	10040.3	1.56
12		5.0074	6642.8		
13	5	5.0034	15894.7		
14		5.0011	15440.2	17106.1	3.11
15		5.0035	19983.5		
16	6	5.0067	10276.0		
17		5.0050	25067.4	15926.2	2.85
18		5.0032	12435.0		
19	7	5.0037	10957.2		
20		5.0031	15943.6	14575.1	2.56
21		5.0087	16824.7		
22	8	5.0078	21515.0		
23		5.0094	9824.2	19665.0	3.67
24		4.9966	27655.7		
25	9	5.0012	15496.9		
26		5.0070	9632.8	15693.1	2.80
27		5.0036	21949.6		

Table B4 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
28	11	5.0035	8480.1		
29		4.9983	16127.4	10114.1	1.58
30		5.0056	5734.7		
31	12	5.0074	15325.7		
32		5.0047	21037.4	17189.7	3.13
33		5.0120	15206.1		
34	13	4.9980	9475.6		
35		5.0048	8574.1	8033.0	1.12
36		5.0119	6049.3		
37	19	4.9973	18437.4		
38		5.0067	8232.6	12675.4	2.14
39		5.0022	11356.3		
40	20	5.0147	7752.0		
41		5.0064	6131.8	7474.5	1.00
42		5.0096	8539.8		

Table B5. Experimental results of concentration effects on methanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Williamsport, PA site soil from depth of 8 to 10 ft (2.4 to 3.0 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
1	1	5.0012	3754.2		100
2		4.9943	3633.3	3511.4	
3		5.0065	3146.6		
4		4.9952	1589.7		500
5		5.0000	1726.0	1617.4	
6		4.9949	1536.6		
7		4.9900	3273.7		1000
8		5.0007	2997.5	3040.1	
9		5.0050	2849.2		
10		5.0074	7177.3		3100
11		5.0173	7654.1	7213.8	
12		5.0038	6810.1		
13		4.9946	11589.0		5000
14		5.0014	12197.4	11991.4	
15		5.0048	12189.8		
16	3	5.0090	13815.0		100
17		5.0029	15151.7	14483.4	
18		5.0014	3954.5		
19		5.0082	2560.5		500
20		4.9975	2564.8	2388.4	
21		5.0000	2039.9		
22		5.0064	3100.1		1000
23		5.0058	3700.3	2790.4	
24		4.9970	1570.7		

Table B5 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
25	3	5.0082	8123.1		3100
26		5.0015	7913.1	8218.5	
27		4.9967	8601.3		
28		5.0014	13078.7		5000
29		5.0050	13033.9	13394.6	
30		5.0011	14071.1		
31	7	5.0011	17088.0		100
32		5.0005	10819.3	16693.6	
33		5.0054	22173.6		
34		4.9976	3381.8		500
35		5.0036	3093.1	3380.8	
36		5.0028	3667.5		
37		5.0062	4332.6		1000
38		5.0064	4977.8	4734.9	
39		5.0004	4894.3		
40		4.9991	1345.9		3100
41		4.9974	9912.0	9465.6	
42		4.9972	9019.2		
43		5.0036	13436.0		5000
44		4.9987	13965.0	13385.9	
45		5.0007	12756.7		

TABLE B6. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B5. Incubation temperature of 20°C (68° F).

Time of Incubation days	Methanol Conc. mg/L	Average DPM Evolved	Average DPM Net	f	v mg/L/day
1	100	3511.4	571.4	1.25×10^{-3}	0.12
	500	1617.4	-	-	-
	1000	3040.1	351.1	7.70×10^{-4}	0.77
	3100	7213.8	481.8	3.52×10^{-4}	1.09
	5000	11991.4	-	-	-
3	100	14483.4	11543.4	2.5×10^{-2}	0.84
	500	2388.4	627.4	2.8×10^{-3}	0.46
	1000	2790.4	101.4	2.2×10^{-4}	0.07
	3100	8218.5	1480.5	1.1×10^{-3}	1.12
	5000	13394.6	935.6	4.1×10^{-4}	0.68
7	100	16693.6	13753.6	3.0×10^{-2}	0.43
	500	3380.8	1619.8	7.1×10^{-3}	0.51
	1000	4734.9	2045.9	4.5×10^{-3}	0.64
	3100	9465.6	2733.6	2.0×10^{-3}	0.89
	5000	13385.9	926.9	4.1×10^{-4}	0.29

Table B7. Experimental results of concentration effects on methanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Williamsport, PA site soil from depth of 8 to 10 ft (2.4 to 3.0 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
1	1	4.9874	1435.4		100
2		4.9869	1009.1	1100.8	
3		5.0057	857.8		
4		4.9985	1347.4		500
5		4.9987	822.7	1022.0	
6		5.0083	895.9		
7		4.9992	--		1000
8		4.9935	--	--[1]	
9		5.0073	--		
10		4.9942	6683.3		3100
11		5.0038	6158.2	6255.2	
12		4.9967	5924.2		
13		5.0000	11022.8		5000
14		5.0017	9142.8	10101.8	
15		4.9934	10139.9		
16	3	4.9986	4211.2		100
17		4.9971	3224.8	3020.7	
18		5.0045	1626.0		
19		5.0008	1155.4		500
20		4.9983	1383.6	1354.2	
21		5.0000	1523.6		
22		4.9993	--		1000
23		4.9962	--	--[1]	
24		5.0054	--		

[1] Indicates flasks which were believed to have received no ^{14}C-labeled toxin.

Table B7 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
25	3	4.9993	7342.5		3100
26		4.9949	7605.2	7394.5	
27		4.9993	7235.7		
28		5.0003	13841.2		5000
29		4.9943	12175.8	12823.2	
30		4.9963	12452.6		
31	7	5.0017	4316.7		100
32		4.9967	7898.2	8417.8	
33		5.0025	13038.6		
34		4.9928	4413.2		500
35		4.9955	1898.1	2451.7	
36		5.0054	1043.8		
37		4.9984	--		1000
38		5.0040	--	--[1]	
39		4.9992	--		
40		4.9928	7566.8		3100
41		5.0051	7614.3	7468.3	
42		4.9984	7223.7		
43		5.0030	12474.8		3100
44		5.0048	13350.2	12958.4	
45		5.0045	13050.2		

[1] Indicates flasks which were believed to have received no ^{14}C-labeled toxin.

TABLE B8. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B7. Incubation temperature of 20°C (68° F).

Time of Incubation days	Methanol Conc. mg/L	Average DPM Evolved	Average DPM Net	f	v mg/L/day
1	100	1100.8	-	-	0
	500	1022.0	-	-	0
	3100	6255.2	-	-	0
	5000	10101.8	-	-	0
3	100	3020.7	80.7	1.77×10^{-4}	0.01
	500	1354.2	-	-	0
	3100	7394.5	662.5	4.84×10^{-4}	0.50
	5000	12823.2	364.2	1.60×10^{-4}	0.27
7	100	8417.8	5477.8	1.20×10^{-2}	0.17
	500	2451.7	690.7	3.03×10^{-3}	0.22
	3100	7468.3	736.3	5.38×10^{-4}	0.24
	5000	12958.4	499.4	2.19×10^{-4}	0.16

Table B9. Experimental results of 100 mg/L methanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 80 to 82 ft (24.4 to 25.0 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	5.0009	2170.3		
2		5.0013	1935.0	2108.8	0
3		4.9965	2221.0		
4	2	5.0014	6257.8		
5		5.0043	3616.8	5284.8	0.52
6		5.0033	5979.8		
7	3	4.9547	17885.6		
8		4.9990	14637.7	14561.6	2.55
9		5.0019	11161.6		
10	4	4.9945	17916.3		
11		5.0003	24548.0	21232.2	4.01
12		5.0000	-[1]		
13	5	5.0086	44260.7		
14		5.0068	32267.6	38335.1	7.77
15		5.0004	38477.1		
16	6	5.0015	43095.3		
17		5.0056	25648.9	29258.8	5.78
18		5.0035	19032.2		
19	7	4.9938	18170.4		
20		5.0045	17283.4	27354.5	5.36
21		5.0027	46609.7		
22	8	5.0080	17372.7		
23		5.0006	14555.6	15626.3	2.79
24		4.9950	14950.7		
25	9	4.9948	6616.6		
26		4.9972	10588.1	6451.9	0.77
27		5.0038	2150.9		
28	10	4.9993	1689.0		
29		5.0085	-[1]	1689.0	0
30		5.0026	-[1]		

[1] Flask tipped over before filter removed, filter contaminated and not used.

Table B10. Experimental results of 100 mg/L methanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil from depth of 80 to 82 ft. (24.4 to 25.0 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	4.9800	4120.1		
2		4.9932	3349.9	3367.1	0.10
3		4.9983	2631.4		
4	2	5.0031	3451.5		
5		4.9969	3520.4	4486.3	0.34
6		4.9958	6487.0		
7	3	5.0106	4136.2		
8		5.0019	4224.2	6574.3	0.80
9		5.0017	11362.4		
10	4	5.0033	5831.3		
11		5.0065	4684.6	5056.8	0.47
12		4.9995	4654.4		
13	5	5.0076	9167.0		
14		5.0015	3833.0	5621.2	0.59
15		5.0053	3863.7		
16	6	5.0024	5385.0		
17		5.0066	5560.1	7238.8	0.95
18		4.9974	10771.2		
19	7	5.0067	8811.2		
20		4.9909	15222.9	11072.8	1.79
21		5.0004	9184.4		
22	8	4.9978	9619.2		
23		4.9962	8045.2	10951.5	1.76
24		5.0011	15190.1		
25	9	5.0035	23303.7		
26		5.0013	34485.1	27039.5	5.29
27		-[1]	23329.6		
28	10	-[1]	16415.3		
29		4.9946	6454.3	8244.8	1.17
30		4.9987	1864.7		

[1] Soil weight not recorded.

Table B11. Experimental results of 100 mg/L methanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 9 ft. (2.7 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	4.9963	2182.9		
2		4.9936	2218.3	2303.8	0
3		4.9930	2510.1		
4	2	5.0037	7771.7		
5		5.0042	3938.5	6054.5	0.69
6		5.0021	6453.2		
7	3	5.0048	4365.0		
8		4.9948	5719.7	5064.0	0.47
9		5.0068	5107.4		
10	4	5.0098	8405.3		
11		5.0082	9236.3	8470.3	1.22
12		4.9974	7769.2		
13	5	4.9995	7062.5		
14		5.0005	12794.9	9928.7	1.54
15		5.0063	-[1]		
16	6	5.0100	27257.8		
17		4.9960	13972.3	18999.5	3.53
18		4.9955	15768.5		
19	7	4.9946	12808.1		
20		5.0088	13518.5	14521.9	2.54
21		5.0011	17239.2		
22	8	4.9917	12095.3		
23		5.0086	18033.3	16009.8	2.87
24		4.9943	17900.7		
25	9	5.0059	21579.2		
26		5.0024	31843.6	25535.4	4.96
27		5.0030	23183.4		
28	10	4.9957	-[1]		
29		5.0043	34315.4	25954.1	5.05
30		4.9943	17592.7		

[1] Solution contaminated by ethanolamine.

Table B12. Experimental results of 100 mg/L methanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil from depth of 9 ft. (2.7 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Average Percent Respired
1	1	4.9924	2463.5	2307.9	0
2		4.9957	2248.7		
3		5.0009	2211.4		
4	2	5.0109	6333.4	7346.4	0.97
5		4.9989	7635.6		
6		4.9925	8070.1		
7	3	4.9990	8069.1	5577.0	0.58
8		4.9955	3826.5		
9		5.0067	4835.4		
10	4	5.0023	8176.2	11905.1	1.97
11		5.0012	5386.9		
12		5.0005	22152.2		
13	5	4.9965	5223.4	7067.1	0.91
14		4.9983	4606.7		
15		4.9983	11371.1		
16	6	5.0012	13112.6	13914.3	2.41
17		4.9910	11133.9		
18		5.0071	17496.5		
19	7	4.9962	14265.8	10277.5	1.61
20		5.0021	6695.8		
21		4.9936	9871.0		
22	8	4.9948	14989.5	11290.1	1.83
23		4.9938	6997.4		
24		5.0107	11883.5		
25	9	4.9994	21042.9	16574.4	2.99
26		5.0100	12900.0		
27		5.0033	15780.2		
28	10	5.0064	2121.8	14181.5	2.47
29		5.0097	22884.5		
30		5.0082	17538.2		

Table B13. Experimental results of concentration effects on methanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 80 to 82 ft (24.4 to 25.0 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
1	1	4.9911	3888.1		100
2		4.9970	3419.3	3716.2	
3		4.9993	3841.2		
4		5.0039	2381.5		500
5		4.9935	1277.7	1752.8	
6		4.9975	1599.1		
7		4.9988	2485.8		1000
8		4.9988	3359.5	2852.0	
9		5.0027	2710.7		
10		4.9969	12074.0		3000
11		5.0029	8244.9	9367.3	
12		4.9950	7783.1		
13		4.9958	14270.3		5000
14		5.0018	13519.4	13641.0	
15		5.0024	13133.4		
16	3	4.9960	5899.3		100
17		4.9953	10369.6	7477.1	
18		5.0050	6162.5		
19		5.0020	2270.8		500
20		4.9965	2804.1	2599.6	
21		5.0047	2723.8		
22		5.0053	3600.6		1000
23		5.0007	3531.9	3604.9	
24		5.0000	3682.1		

Table B13 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
25	3	4.9982	9407.0		3000
26		4.9979	9301.8	9719.9	
27		4.9931	10450.8		
28		5.0009	15611.9		5000
29		5.0000	14811.5	15420.3	
30		4.9963	15837.5		
31	7	5.0017	14278.9		100
32		4.9977	6751.2	11459.8	
33		4.9989	13349.3		
34		4.9956	3376.5		500
35		4.9960	3326.6	3636.9	
36		5.0018	4207.6		
37		4.9959	4221.8		1000
38		5.0006	3569.1	4279.3	
39		4.9975	5046.9		
40		4.9956	10342.7		3000
41		4.9965	10986.9	11521.0	
42		4.9939	13233.5		
43		4.9977	15797.2		5000
44		4.9933	15749.0	15200.2	
45		4.9977	14054.4		

TABLE B14. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B13. Incubation temperature of 20°C (68° F).

Time of Incubation days	Methanol Conc. mg/L	Average DPM Evolved	Average DPM Net	f	v mg/L/day
1	100	3716.2	776.2	1.70×10^{-3}	0.17
	500	1752.8	-	-	0
	1000	2852.0	163.0	3.57×10^{-4}	0.36
	3000	9367.3	2635.3	1.93×10^{-3}	5.78
	5000	13641.0	1182.0	5.18×10^{-4}	2.59
3	100	7477.1	4537.1	9.95×10^{-3}	0.33
	500	2599.6	838.6	3.68×10^{-3}	0.61
	1000	3604.9	915.9	2.01×10^{-3}	0.67
	3000	9719.9	2987.9	2.18×10^{-3}	2.18
	5000	15420.3	2961.3	1.30×10^{-3}	2.16
7	100	11459.8	8519.8	1.87×10^{-2}	0.27
	500	3636.9	1875.9	8.23×10^{-3}	0.59
	1000	4279.3	1590.3	3.49×10^{-3}	0.50
	3000	11521.0	4789.0	3.50×10^{-3}	1.50
	5000	15200.2	2741.2	1.20×10^{-3}	0.86

Table B15. Experimental results of concentration effects on methanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil from depth of 80 to 82 ft (24.4 to 25.0 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
1	1	4.9966	2555.4		100
2		4.9970	2763.3	2647.3	
3		4.9953	2623.1		
4		4.9939	2663.3		500
5		5.0011	1312.4	1825.7	
6		4.9991	1501.3		
7		5.0023	2695.0		1000
8		4.9982	2672.1	2665.8	
9		4.9946	2630.3		
10		4.9987	9185.6		3000
11		4.9964	8300.3	8921.1	
12		4.9945	9277.3		
13		5.0046	13210.0		5000
14		4.9915	13500.4	13238.7	
15		4.9989	13005.8		
16	3	5.0002	5447.8		100
17		5.0037	3808.0	4390.3	
18		5.0027	3915.0		
19		4.9989	1364.1		500
20		5.0000	1805.3	1526.4	
21		5.0006	1409.8		
22		4.9976	3228.2		1000
23		4.9976	3165.6	3039.3	
24		5.0027	2724.0		

Table B15 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
25	3	4.9966	8787.6		3000
26		5.0000	8707.6	8731.6	
27		4.9978	8699.6		
28		-[1]	15396.0		5000
29		5.0000	15505.9	14989.7	
30		4.9977	14067.3		
31	7	5.0018	14274.0		100
32		4.9979	7274.2	9756.5	
33		5.0000	7721.4		
34		4.9993	1628.8		500
35		5.0000	1718.3	1612.0	
36		4.9936	1488.9		
37		5.0037	3292.8		1000
38		5.0021	3239.2	3237.0	
39		4.9996	3179.1		
40		4.9951	13355.3		3000
41		5.0020	9405.9	13054.8	
42		4.9932	16403.1		
43		4.9931	10739.7		5000
44		5.0048	14492.6	13906.2	
45		5.0025	16486.2		

[1] Soil weight not recorded.

TABLE B16. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B15. Incubation temperature of 20°C (68° F).

Time of Incubation days	Methanol Conc. mg/L	Average DPM Evolved	Average DPM Net	f	v mg/L/day
1	100	2647.5	-	-	0
	500	1825.7	64.7	2.84×10^{-4}	0.14
	1000	2665.8	-	-	0
	3000	8921.1	2189.1	1.60×10^{-3}	4.80
	5000	13238.7	779.7	3.42×10^{-4}	1.71
3	100	4390.3	1450.3	3.18×10^{-3}	0.11
	500	1526.4	-	-	0
	1000	3039.3	350.3	7.68×10^{-4}	0.26
	3000	8731.6	1999.6	1.46×10^{-3}	1.46
	5000	14989.7	2530.7	1.11×10^{-3}	1.85
7	100	9756.5	6816.5	1.50×10^{-2}	0.21
	500	1612.0	-	-	0
	1000	3237.0	458.0	1.20×10^{-3}	0.17
	3000	13054.8	6322.8	4.62×10^{-3}	1.98
	5000	13906.2	1447.2	6.35×10^{-4}	0.45

Table B17. Experimental results of concentration effects on methanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 9 ft (2.7 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
1	1	5.0053	2158.8		100
2		5.0008	2169.3	2202.1	
3		5.0002	2278.3		
4		5.0000	1208.9		500
5		4.9981	1056.9	1212.5	
6		5.0009	1371.6		
7		4.9958	2542.6		1000
8		4.9975	2151.8	2324.9	
9		5.0024	2280.4		
10		5.0100	6866.4		3000
11		5.0064	14210.4	9141.9	
12		4.9925	6348.9		
13		5.0067	11089.8		5000
14		4.9935	11689.7	10801.9	
15		4.9967	9626.1		
16	3	5.0068	5012.5		100
17		5.0075	5617.8	5242.2	
18		5.0071	5096.3		
19		4.9960	1724.2		500
20		5.0064	1672.3	1725.5	
21		4.9985	1780.1		
22		5.0068	3105.4		1000
23		5.0016	1930.4	2765.3	
24		5.0070	3260.0		

Table B17 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
25	3	5.0000	8325.3		3000
26		4.9941	8686.5	8592.7	
27		4.9946	8766.3		
28		5.0078	13318.7		5000
29		5.0039	13560.3	13125.1	
30		4.9961	12496.3		
31	7	4.9900	5656.0		100
32		5.0071	5252.8	6352.1	
33		4.9951	8147.6		
34		5.0054	1887.8		500
35		5.0059	2558.7	2217.4	
36		4.9997	2205.8		
37		5.0028	3969.3		1000
38		5.0077	2664.1	3178.2	
39		5.0054	2901.1		
40		5.0015	8388.1		3000
41		5.0056	7435.5	8012.0	
42		5.0011	8212.3		
43		5.0080	25826.8		5000
44		5.0095	12060.2	16427.1	
45		5.0097	11394.2		

TABLE B18. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B17. Incubation temperature of 20°C (68° F).

Time of Incubation days	Methanol Conc. mg/L	Average DPM Evolved	Average DPM Net	f	v mg/L/day
1	100	2202.1	-	-	0
	500	1212.5	-	-	0
	1000	2324.9	-	-	0
	3000	9141.9	2409.9	1.76×10^{-3}	5.28
	5000	10801.9	-	-	0
3	100	5242.2	2302.2	5.05×10^{-3}	0.17
	500	1725.5	-	-	0
	1000	2765.3	76.5	1.68×10^{-4}	0.06
	3000	8592.7	1860.7	1.36×10^{-3}	1.36
	5000	13125.1	666.1	2.92×10^{-4}	0.49
7	100	6352.1	3412.1	7.48×10^{-3}	0.11
	500	2217.4	456.4	2.00×10^{-3}	0.14
	1000	3178.2	489.2	1.07×10^{-3}	0.15
	3000	8012.0	1280.0	9.36×10^{-4}	0.40
	5000	16427.1	3968.1	1.74×10^{-3}	1.24

Table B19. Experimental results of concentration effects on methanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil depth of 9 ft (2.7 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
1	1	5.0060	4764.2		100
2		5.0062	2290.4	3089.0	
3		5.0054	2212.5		
4		4.9975	1180.4		500
5		4.9991	1112.6	1227.3	
6		5.0060	1388.9		
7		5.0077	7301.7		1000
8		4.9925	6240.9	6697.6[1]	
9		5.0083	6550.1		
10		5.0071	6687.8		3000
11		5.0100	6429.4	6526.6	
12		5.0104	6462.6		
13		5.0074	10653.0		5000
14		5.0022	10495.1	10597.9	
15		4.9932	10645.7		
16	3	4.9970	3711.1		100
17		5.0085	5736.7	4620.4	
18		5.0039	4413.5		
19		4.9986	1288.0		500
20		5.0072	1458.5	1387.4	
21		5.0092	1415.6		
22		5.0056	2701.8		1000
23		5.0000	2470.6	2545.2	
24		5.0003	2463.1		

[1] Addition of ^{14}C-labeled toxin incorrect.

Table B19 (continued)

Flask No.	Incubation Time (days)	Soil Weight (grams)	DPM	Average DPM	Methanol Concentration (mg/L)
25	3	5.0010	7188.1		3000
26		4.9961	7204.6	7111.5	
27		5.0084	6941.9		
28		5.0017	10908.5		5000
29		4.9953	11649.5	11266.8	
30		5.0100	11242.5		
31	7	5.0053	17536.7		100
32		4.9993	19737.9	14865.3	
33		5.0050	7321.3		
34		5.0015	2170.1		500
35		5.0096	1822.6	2484.0	
36		4.9983	3459.4		
37		4.9909	3802.1		1000
38		4.9932	3282.4	3579.8	
39		4.9976	3655.0		
40		5.0100	7335.3		3000
41		5.0011	7622.0	7723.6	
42		5.0100	8213.5		
43		5.0079	12762.7		5000
44		4.9997	13389.3	12703.8	
45		5.0053	11959.5		

TABLE B20. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B19. Incubation temperature of 20°C (68° F).

Time of Incubation days	Methanol Conc. mg/L	Average DPM Evolved	Average DPM Net	f	v mg/L/day
1	100	3089.0	149.0	3.27×10^{-4}	0.03
	500	1227.3	-	-	0
	1000	-[1]	-	-	-
	3000	6526.6	-	-	0
	5000	10597.9	-	-	0
3	100	4620.4	1680.4	3.69×10^{-3}	0.12
	500	1387.4	-	-	0
	1000	2545.2	-	-	0
	3000	7111.5	379.5	2.77×10^{-4}	0.28
	5000	11266.8	-	-	0
7	100	14865.3	11925.3	2.62×10^{-2}	0.37
	500	2484.0	763.0	3.17×10^{-3}	0.23
	1000	3579.8	890.8	1.95×10^{-3}	0.28
	3000	7723.6	991.6	7.25×10^{-4}	0.31
	5000	12703.8	244.8	1.07×10^{-4}	0.08

[1] Addition of ^{14}C-labeled toxin incorrect.

TABLE B21. Experimental results of 10 mg/L t-butanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 55 ft (16.8 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	Average % Respired
1	1	5.0095	6120.6		
2		4.9986	5364.6	5556.2	0.02
3		5.0043	5183.4		
4	2	4.9977	4885.3		
5		4.9961	5411.9	4929.7	-0.06
6		4.9931	4492.0		
7	3	4.9988	5842.2		
8		5.0017	5207.4	5372.6	-0.01
9		4.9939	5068.2		
10	4	5.0013	5098.3		
11		4.9900	5538.5	5554.5	0.02
12		5.0075	6026.6		
13	5	5.0040	5490.0		
14		5.0020	4874.1	5291.4	-0.02
15		4.9947	5510.0		
16	6	5.0088	5252.2		
17		5.0026	5305.3	5305.0	0.01
18		4.9923	5357.6		
19	7	4.9936	5497.2		
20		5.0017	5749.2	5578.2	0.02
21		5.0006	5488.3		
22	8	5.0027	5212.4		
23		4.9930	5610.7	5289.3	-0.02
24		4.9979	5044.7		
25	9	4.9965	4882.9		
26		5.0010	5176.1	5086.3	-0.04
27		5.0071	5200.0		
28	10	4.9938	6225.9		
29		4.9945	7050.0	6685.9	0.15
30		5.0075	6781.7		

TABLE B22. Experimental results of 10 mg/L t-butanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil from depth of 55 ft (16.8 m). Incubation temperature of 20°C (68°F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	Average % Respired
1	1	4.9986	5286.1		
2		4.9945	5587.3	5450.6	0
3		4.9954	5508.5		
4	2	4.9973	5228.4		
5		5.0083	5377.9	5346.3	-0.01
6		5.0032	5432.6		
7	3	4.9950	4454.6		
8		5.0100	5085.1	4912.6	0.06
9		4.9958	5198.0		
10	4	4.9951	5957.0		
11		4.9969	6238.3	5816.1	0.05
12		5.0008	5253.1		
13	5	4.9990	5516.1		
14		5.0034	5368.0	5521.7	0.01
15		5.0027	5681.1		
16	6	4.9973	5665.6		
17		5.0012	5911.6	5742.2	0.04
18		4.9995	5649.4		
19	7	4.9981	6178.9		
20		5.0100	5736.0	5857.8	0.05
21		5.0015	5658.4		
22	8	4.9925	5566.7		
23		4.9978	5823.0	5716.4	0.04
24		5.0012	5759.6		
25	9	4.9992	5692.4		
26		5.0088	5394.7	5606.7	0.02
27		5.0087	5733.1		
28	10	5.0055	6405.0		
29		5.0012	5932.7	6168.9	0.09
30		4.9956	-[1]		

[1] Solution contaminated by ethanolamine.

TABLE B23. Experimental results of 10 mg/L t-butanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 12 ft (3.7 m). Incubation temperature of 20°C (68°F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	Average % Respired
1	1	4.9982	6637.9		
2		4.9975	6269.6	6472.0	-0.02
3		5.0043	6508.5		
4	2	5.0029	5480.0		
5		5.0090	5690.5	5689.3	-0.12
6		4.9967	5033.5		
7	3	4.9960	6343.8		
8		5.0081	6182.3	6027.5	-0.08
9		5.0030	5556.5		
10	4	5.0016	-[1]		
11		4.9982	6116.2	6353.6	-0.04
12		5.0085	6590.9		
13	5	5.0077	6498.9		
14		5.0034	5991.0	6360.3	-0.04
15		5.0026	6499.4		
16	6	5.0007	5321.9		
17		4.9967	6162.4	5799.1	-0.11
18		5.0040	5913.0		
19	7	5.0069	5664.2		
20		4.9922	8454.8	7261.1	0.07
21		5.0041	7664.3		
22	8	5.0073	6254.6		
23		5.0075	6708.0	6368.5	-0.04
24		5.0072	6142.9		
25	9	5.0015	7134.5		
26		5.0047	6952.8	6868.3	0.03
27		4.9987	6517.7		
28	10	5.0064	5937.7		
29		4.9953	6426.1	5987.5	-0.08
30		4.9973	5598.8		

[1] Flask tipped over before filter removed.

TABLE B24. Experimental results of 10 mg/L t-butanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil from depth of 12 ft (3.7 m). Incubation temperature of 20°C (68°F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	Average % Respired
1	1	5.0003	6890.8		
2		5.0053	6839.1	6639.1	0
3		5.0069	6187.3		
4	2	5.0036	7649.1		
5		5.0065	-[1]	7399.7	0.09
6		4.9961	7150.3		
7	3	5.0031	5970.4		
8		5.0047	5986.8	5996.5	-0.08
9		5.0080	6032.4		
10	4	5.0000	6739.7		
11		4.9981	6038.2	6536.7	-0.02
12		5.0029	6832.2		
13	5	5.0042	6528.1		
14		5.0029	6697.6	6557.7	-0.01
15		5.0045	6447.3		
16	6	4.9987	6104.3		
17		4.9974	6104.9	6134.2	-0.06
18		4.9992	6193.4		
19	7	4.9997	8194.1		
20		5.0092	8163.0	8170.7	0.18
21		4.9947	8154.9		
22	8	4.9934	-[1]		
23		5.0071	-[1]	7194.7	0.06
24		5.0000	7194.7		
25	9	4.9963	6636.1		
26		5.0058	6155.5	6662.1	0
27		5.0048	-[2]		
28	10	5.0052	5428.1		
29		5.0076	6070.1	5689.0	-0.12
30		4.9968	5568.7		

[1] Filter contaminated with splashed liquid during shaking.

[2] Flask tipped over before filter removed.

TABLE B25. Experimental results of concentration effects on t-butanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 55 ft (16.8 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
1	1	4.9945	7440.9		
2		4.9963	6613.4	6650.2	10
3		5.0058	5896.4		
4		4.9987	30818.5		
5		5.0068	67904.4	41825.0	50
6		5.0078	26752.2		
7		4.9947	55891.2		
8		4.9900	62810.1	57818.9	100
9		4.9995	54755.4		
10		4.9992	191337.0		
11		5.0007	177268.8	177639.8	300
12		4.9979	164313.5		
13		4.9964	260672.6		
14		4.9945	301209.1	283978.2	500
15		5.0100	290052.9		
16	3	4.9965	7355.0		
17		4.9935	7392.8	6984.5	10
18		5.0000	6205.7		
19		4.9948	24353.6		
20		5.0058	12252.3	21595.2	50
21		5.0018	28179.6		
22		5.0088	55039.5		
23		5.0027	91921.8	67233.4	100
24		5.0081	54738.9		
25		5.0031	303391.9		
26		5.0055	157116.0	205503.7	300
27		5.0026	156003.3		
28		5.0089	277659.7		
29		4.9974	252538.5	263041.5	500
30		4.9926	258926.2		

TABLE B25. (continued)

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
31	7	5.0033	6909.5		
32		5.0000	6633.9	6553.9	10
33		5.0065	6118.2		
34		4.9963	8026.9		
35		5.0029	35091.0	25102.9	50
36		4.9991	32190.7		
37		4.9954	61585.8		
38		5.0016	54252.8	57279.3	100
39		5.0064	55999.3		
40		5.0090	157138.6		
41		4.9945	167878.6	160330.6	300
42		5.0083	155974.5		
43		5.0045	243259.0		
44		4.9979	277860.4	263584.6	500
45		5.0071	269634.3		

TABLE B26. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B25. Incubation temperature of 20°C (68° F).

Time of Incubation, days	t-butanol conc., mg/L	DPM evolved	DPM net	f	v mg/L/day
1	10	6650.2	1234.4	1.50×10^{-3}	0.02
	50	41825.0	15337.8	3.73×10^{-3}	0.19
	100	57818.9	9160.0	1.12×10^{-3}	0.11
	300	177639.8	17516.7	7.11×10^{-4}	0.21
	500	283978.2	35709.5	8.69×10^{-4}	0.43
3	10	6984.5	1568.7	1.91×10^{-3}	0.01
	50	21595.2	-	-	0
	100	67233.4	18574.5	2.26×10^{-3}	0.08
	300	205503.7	45380.6	1.84×10^{-3}	0.06
	500	263041.5	14772.8	3.60×10^{-4}	0.06
7	10	6553.9	1138.1	1.39×10^{-3}	0
	50	25102.9	-	-	0
	100	57279.3	8620.4	1.05×10^{-3}	0.01
	300	160330.6	207.5	8.42×10^{-6}	0
	500	263584.6	15315.9	3.73×10^{-4}	0.03

TABLE B27. Experimental results of concentration effects on t-butanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil from depth of 55 ft (16.8 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
1	1	5.0023	5656.6		
2		5.0025	5635.6	5684.5	10
3		5.0080	5761.2		
4		5.0024	26481.6		
5		4.9953	29587.9	27901.1	50
6		4.9981	27633.8		
7		5.0015	48444.0		
8		4.9926	48704.4	50012.9	100
9		5.0073	52890.3		
10		5.0089	140669.3		
11		5.0030	132274.3	145491.4	300
12		4.9944	163530.5		
13		5.0067	237189.8		
14		5.0045	242312.3	248880.9	500
15		5.0032	267140.6		
16	3	5.0013	6096.7		
17		5.0041	5852.8	5816.9	10
18		5.0009	5501.1		
19		5.0019	31261.2		
20		4.9978	29465.7	30220.2	50
21		5.0063	29933.6		
22		4.9939	55068.5		
23		5.0000	51971.8	53228.2	100
24		4.9969	52644.2		
25		4.9995	172734.4		
26		5.0024	170757.7	170020.7	300
27		4.9935	166569.9		
28		4.9907	269156.6		
29		5.0039	256675.9	264366.9	500
30		4.9923	267268.2		

TABLE B27. (continued)

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
31	7	5.0100	5802.6		
32		5.0061	6317.7	5970.0	10
33		5.0085	5789.8		
34		4.9964	31569.8		
35		4.9949	32433.6	31739.3	50
36		4.9951	31214.4		
37		4.9908	53360.6		
38		4.9990	55071.8	54725.4	100
39		5.0061	55743.8		
40		5.0030	172976.6		
41		4.9958	157130.7	163846.6	300
42		4.9992	161432.6		
43		4.9963	274844.1		
44		4.9990	276902.0	273623.9	500
45		4.9967	269125.4		

TABLE B28. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B27. Incubation temperature of 20°C (68° F).

Time of Incubation, days	t-butanol conc., mg/L	DPM evolved	DPM net	f	v mg/L/day
1	10	5684.5	268.7	3.27×10^{-4}	0
	50	27901.1	1413.9	3.44×10^{-4}	0.02
	100	50012.9	1354.0	1.65×10^{-4}	0.02
	300	145491.4	-	-	0
	500	248880.9	612.2	1.49×10^{-5}	0.01
3	10	5816.9	401.1	4.88×10^{-4}	0
	50	30220.2	3733.0	9.09×10^{-4}	0.02
	100	53228.2	4569.3	5.56×10^{-4}	0.02
	300	170020.7	9897.6	4.02×10^{-4}	0.04
	500	264366.9	16098.2	3.92×10^{-4}	0.07
7	10	5970.0	554.2	6.75×10^{-4}	0
	50	31739.3	5252.1	1.28×10^{-3}	0.01
	100	54725.4	6066.5	7.39×10^{-4}	0.01
	300	163846.6	3723.5	1.51×10^{-4}	0.01
	500	273623.9	25355.2	6.17×10^{-4}	0.04

TABLE B29. Experimental results of concentration effects on t-butanol degradation using moist soil weight of 5.00 grams under aerobic conditions. Dumfries, VA site soil from depth of 12 ft (3.7 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
1	1	4.9996	8223.2		
2		5.0007	-[1]	7408.4	10
3		4.9992	6593.6		
4		4.9971	34078.8		
5		4.9977	33806.7	34524.8	50
6		5.0082	35688.9		
7		4.9988	56323.5		
8		5.0035	58881.1	58476.1	100
9		5.0012	60223.8		
10		5.0082	177272.8		
11		5.0025	182648.6	176178.2	300
12		5.0012	168613.1		
13		4.9990	299907.9		
14		4.9986	270934.2	286488.0	500
15		5.0014	288621.9		
16	3	4.9975	7210.4		
17		4.9920	5988.7	6236.0	10
18		5.0056	5508.8		
19		5.0032	32331.0		
20		5.0055	60454.2	49209.8	50
21		5.0070	54844.2		
22		4.9963	38959.9		
23		5.0081	31441.2	41522.7	100
24		4.9962	54167.0		
25		4.9988	163129.9		
26		4.9941	191365.1	175687.2	300
27		5.0054	172566.6		
28		4.9968	343020.3		
29		5.0031	267952.9	320865.6	500
30		4.9980	351623.6		

[1] Flask tipped over before filter removed.

TABLE B29. (continued)

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
31	7	5.0006	5933.5		
32		5.0075	6587.6	6717.2	10
33		4.9915	7630.6		
34		5.0057	33792.3		
35		5.0074	36166.1	34277.0	50
36		4.9960	32872.6		
37		5.0080	67406.1		
38		5.0089	59162.0	62146.9	100
39		5.0009	59872.5		
40		4.9976	178101.1		
41		5.0026	180553.3	179843.1	300
42		4.9960	180875.0		
43		5.0092	305483.7		
44		4.9981	298717.5	297189.8	500
45		5.0091	287368.2		

TABLE B30. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B29. Incubation temperature of 20°C (68° F).

Time of Incubation, days	t-butanol conc., mg/L	DPM evolved	DPM net	f	v mg/L/day
1	10	7408.4	746.7	9.09×10^{-4}	0.01
	50	34524.8	3257.9	7.93×10^{-4}	0.04
	100	58476.1	3528.6	4.30×10^{-4}	0.04
	300	176178.2	6801.3	2.76×10^{-4}	0.08
	500	286488.0	-	-	0
3	10	6236.0	-	-	0
	50	49209.8	17942.9	4.37×10^{-4}	0.07
	100	41522.7	-	-	0
	300	175687.2	6310.3	2.56×10^{-4}	0.03
	500	320865.6	30196.6	7.35×10^{-4}	0.12
7	10	6717.2	55.5	6.76×10^{-5}	0
	50	34277.0	3010.1	7.33×10^{-4}	0.01
	100	62146.9	7199.5	8.76×10^{-4}	0.01
	300	179843.1	10466.2	4.25×10^{-4}	0.02
	500	297189.8	6520.8	1.59×10^{-4}	0.01

TABLE B31. Experimental results of concentration effects on t-butanol degradation using moist soil weight of 5.00 grams under anoxic conditions. Dumfries, VA site soil from depth of 12 ft (3.7 m). Incubation temperature of 20°C (68° F).

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
1	1	5.0060	6454.5		
2		4.9967	5642.9	6328.9	10
3		5.0011	6889.4		
4		5.0052	31263.3		
5		5.0072	33706.1	33149.6	50
6		4.9948	34479.3		
7		5.0100	55533.2		
8		5.0085	55097.1	55489.2	100
9		4.9950	55837.4		
10		5.0060	172245.4		
11		4.9973	162147.3	169527.3	300
12		5.0057	174189.2		
13		5.0015	297069.5		
14		4.9976	261668.9	280580.6	500
15		4.9978	283003.3		
16	3	5.0004	6929.7		
17		4.9991	6608.2	6700.9	10
18		4.9935	6564.9		
19		5.0021	37495.0		
20		4.9945	34033.2	38294.5	50
21		5.0000	43355.4		
22		5.0068	69896.7		
23		4.9983	67169.8	68875.0	100
24		4.9942	69558.4		
25		5.0071	178430.6		
26		4.9904	290301.7	218537.5	300
27		4.9972	186880.1		
28		5.0068	308377.2		
29		5.0035	314338.9	308051.2	500
30		5.0084	301437.4		

TABLE B31. (continued)

Flask No.	Incubation Time, days	Soil weight grams	DPM	Average DPM	t-butanol Conc., mg/L
31	7	4.9954	6319.2		
32		5.0052	7009.6	6850.7	10
33		5.0027	7223.2		
34		5.0057	33253.0		
35		5.0100	36995.1	34639.0	50
36		4.9969	33668.8		
37		5.0052	60667.3		
38		4.9947	58408.3	58846.4	100
39		4.9975	57463.5		
40		4.9992	173557.6		
41		5.0050	182978.3	178326.4	300
42		5.0100	178443.3		
43		5.0052	307188.1		
44		5.0000	291209.5	319049.1	500
45		4.9991	358749.6		

TABLE B32. Calculations for the fraction of organic evolved as $^{14}CO_2$, f, and $^{14}CO_2$ evolution velocity, v, for the data presented in Table B31. Incubation temperature of 20°C (68° F).

Time of Incubation, days	t-butanol conc., mg/L	DPM evolved	DPM net	f	v mg/L/day
1	10	6328.9	-	-	0
	50	33149.6	1882.7	4.58×10^{-4}	0.02
	100	55489.2	541.8	6.60×10^{-5}	0.01
	300	169527.3	150.4	6.10×10^{-6}	0
	500	280580.6	-	-	0
3	10	6700.9	39.2	4.77×10^{-5}	0
	50	38294.5	7027.6	1.71×10^{-3}	0.03
	100	68875.0	13927.6	1.70×10^{-3}	0.06
	300	218537.5	49160.6	1.99×10^{-3}	0.20
	500	308051.2	17382.2	4.23×10^{-4}	0.07
7	10	6850.7	189.0	2.30×10^{-4}	0
	50	34639.0	3372.1	8.21×10^{-4}	0.01
	100	58846.4	3899.0	4.75×10^{-4}	0.01
	300	178326.4	8949.5	3.63×10^{-4}	0.02
	500	319049.1	28380.1	6.91×10^{-4}	0.05

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