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**THE EFFECTS OF *PHANEROCHAETE CRYSPORIUM* ON THE
DEGRADATION OF TOLUENE IN FRESHLY
CONTAMINATED SITES**

A Thesis Presented

by

STEVEN M. WILKINS

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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Plant and Soil Science

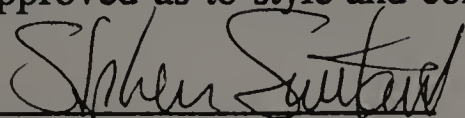
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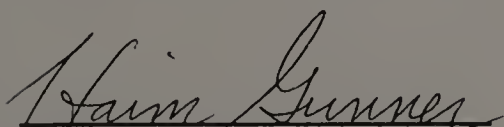
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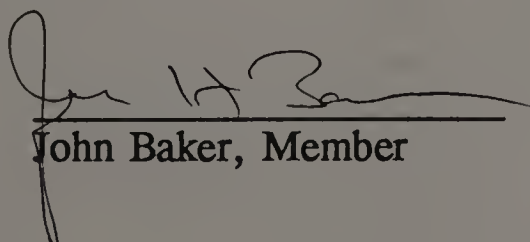
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ABSTRACT

THE EFFECTS OF *PHANEROCHAETE CHRYSOSPORIUM* ON THE DEGRADATION OF TOLUENE IN FRESHLY CONTAMINATED SITES

FEBRUARY 1995

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A bench-scale series of columns simulated the effects of adding the white-rot fungus *Phanerochaete chrysosporium* to a site newly contaminated with toluene. A series of 12 columns, 38×200 mm, were packed with approximately 130 grams of soil from the A horizon of a fine sandy loam. A humidified air stream was passed through the column at a rate of 5 ml/min under negative pressure. Throughout the different runs, one or two columns were autoclaved twice (2nd autoclaving 24 hrs after the first) and served as sterile controls. The fate of the toluene was assessed using [U-ring-¹⁴C]toluene. The volatilized toluene was collected in traps placed in the exit line. For all the experiments the rate of mineralization in the sterile controls was negligible. Organic matter was added in the form of peatmoss and/or wood-chips at ≤ 5 g /column. The columns supplied with organic additions generally showed the greatest amount of mineralization. The greatest amount of mineralization of the toluene in

columns amended with organic material was 8% which was twice as much as the maximum for columns containing no organic additions. Addition of nitrogen at 2.4 mg/kg and 24 mg/kg slightly increased the mineralization rate in comparison with columns receiving no added nitrogen. However, this increase in mineralization of the toluene due to the addition of nitrogen was slight. In all experiments, mineralization of toluene by *P. cryosporium* eliminated far less toluene from the soil than other methods of remediating a contaminated soil such as bioventing. Therefore, this fungus seems to have limited potential for the remediation of toluene in contaminated soils.

TABLE OF CONTENTS

	<u>Page</u>
ABSTRACT	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
Chapter	
1. LITERATURE REVIEW	1
2. INTRODUCTION	7
Remediation Processes	9
Composting.	9
Bioventing and Air Sparging.	10
Aerobic Bioreactors	12
Incineration	12
Land Farming	12
3. MATERIALS AND METHODS	14
Experimental Setup	14
Fungal Culture.	14
Radiolabeled Compounds.	14
Soil and Packing.	14
Sterilized Control.	15
Incubation Apparatus.	15
Initiation of Incubation.	15
Monitoring Mineralization Assay	16
Chromic Acid Digestion.	17
Data Acquisition.	18
Tenax Trap Extraction.	18
Tenax Trap Reconditioning.	18
4. RESULTS	20

Initial Toluene Mineralization Experiment Utilizing <i>P. cryosporium</i> .	20
Mass balances.	20
Mineralization products (¹⁴ CO ₂ Evolved).	21
Toluene Mineralization Utilizing <i>P. cryosporium</i> Experiment #2 . .	24
Mass balances.	24
Toluene Mineralization with Reversed Airflow.	27
Mass Balances.	27
Toluene Mineralization with Reversed Airflow Repeated.	30
Mass Balances.	30
Nitrogen Experiment	33
Mass balances.	33
5. DISCUSSION	37
REFERENCES	41

LIST OF TABLES

Table	Page
1. Percentages of added [U-ring- ¹⁴ C]-labeled toluene recovered in different fractions from the soil during the first April run.	22
2. Percentages of added [U-ring- ¹⁴ C]-labeled toluene recovered in different fractions for the soil.	25
3. Percentages of added [U-ring- ¹⁴ C]-labeled toluene recovered in different fractions for the soil.	28
4. Percentages of added [U-ring- ¹⁴ C]-labeled toluene recovered in different fractions for the soil.	31
5. Percentages of added [U-ring- ¹⁴ C]-labeled toluene recovered in different fractions of the soil.	34

LIST OF FIGURES

Figure	Page
1. Mineralization of toluene based on the accumulation of ^{14}C from the total added ^{14}C labeled toluene. Airflow continuous from the top to the bottom of the columns.	23
2. Accumulation of ^{14}C from the degradation of ^{14}C labeled toluene. The total mass of fungi decreased from 10-g to 1-g per column.	26
3. Mineralization of ^{14}C labeled toluene based on a reversing of the airflow to a flow from the top of the column to the bottom.	29
4. Repetition of the previous experiment with the airflow flowing from the top to bottom of the column. Airflow was gradually increased to give a final flow rate equal to two pore changes per day.	32
5. Effects of addition of nitrogen in the form of NH_4NO_3 at low (2.4 mg/kg) concentrations on the mineralization of toluene by <i>P. cryosporium</i>	35
6. Effects of the addition of nitrogen in the form of NH_4NO_3 at low (2.4 mg/kg) and high (24 mg/kg) concentrations on the mineralization of toluene by <i>P. cryosporium</i> inoculated as intact or fragmented mycelium...	36

CHAPTER 1

LITERATURE REVIEW

Phanerochaete chrysosporium, a wood-rotting basidiomycete that causes white-rot of wood, has been shown to degrade lignin and hazardous environmental contaminants such as phenanthrene (Hammel, et al. 1992, Dhawale et al. 1992, Moen and Hammel 1994), polycyclic aromatic hydrocarbons (Hammel et al. 1986), thianthrene (Schreiner, et al. 1988), 2,4,5- trichlorophenoxyacetic acid (Ryan and Bumpus 1989), trichlorophenol (Joshi and Gold 1993, Armenante et al. 1994), azo dyes (Paszczyński and Grigsby 1992), pentachlorophenol (Lamar et al. 1990), PCBs, TNT (Tudor et al. 1990) phenolic and chlorinated phenolic compounds (Tudor et al. 1990, Aitken et al. 1989). Studies of the use of this fungus in the remediation of hazardous compounds originated primarily in research conducted by the paper industry. During the kraft process for paper manufacture, large quantities of dark brown effluent are produced. This effluent contains large quantities of chlorinated organics as well as high BOD, COD, and suspended solids content (Mittar, and Khanna 1992). Although, this effluent is not particularly hazardous it casts a negative image on the paper industry for aesthetic reasons. *P. chrysosporium* has proven to be effective in the degradation of the chlorinated organics as well as decreasing color content and decreasing the BOD, and COD of the wastes from the paper industry entering the streams (Fukui 1992, Leatham 1993). Recently this fungus has also proven useful for the treatment of olive mill waste waters which have properties similar to waste-waters released during the process of

paper manufacture (Sayadi and Ellouz, 1993.). Having the success in the paper industry, research began to examine the feasibility of utilizing *P. chrysosporium* for the degradation of hazardous wastes. Lignin, a natural component of woody plant tissue, is made up of phenylpropanoid units that are linked almost randomly together by a combination of carbon-oxygen and carbon-carbon bonds. The great variation in the positions and atoms involved in the bonds between the monomers makes it highly resistant to attack by microorganisms (Ortha 1991). Only a few fungi and a very few bacteria are capable of degrading lignin (Bumpus et al. 1985).

P. chrysosporium secretes polymer-cleaving extracellular enzymes into its environment specifically under conditions of nutrient limitation (Ortha et al.1991). Under conditions of nitrogen, carbon, or sulfur deficiency, this fungus secretes an enzyme system dependent on H₂O₂ that is capable of generating free-radicals that cleave lignin into soluble monomers and oligomers (Bumpus et al. 1985). These soluble monomers and oligomers usually have hydroxyls (or carbonyl oxygens) added to them at the cleavage sites. The ability of lignin peroxidase to cleave lignin into its component monomers strongly implies that this enzyme possesses the ability to break a variety of covalent C-to-C and C-to-O bonds. Thus, the low substrate specificity of this highly reactive enzyme may account for the ability of *P. chrysosporium* to metabolize TNT, PCB, and other xenobiotic compounds. By virtue of possessing this extracellular, non-site-specific enzyme system, *P. chrysosporium* would seem to have great potential for degrading contaminants at hazardous waste sites.

Ryan and Bumpus (1989) demonstrated extensive biodegradation of 2,4,5-trichlorophenoxyacetic acid by *P. chrysosporium* under nitrogen-limited conditions. After contaminated soil was mixed with water, 32.5% of this compound was mineralized to CO₂ over 30 days of incubation. Pentachlorophenol was dramatically reduced (by 98%) in soils inoculated with fungus. Only 43% of the pentachlorophenol was mineralized by the uninoculated controls. (Lamar et al. 1990). In soils contaminated with phenanthrene, the addition of *P. chrysosporium* nearly doubled mineralization (to 38%) within 21 days as compared to 20% mineralization with no inoculum (Hammel et al. 1992). Yadav and Reddy (1993) showed that *P. chrysosporium* effectively degrades benzene, toluene, ethyl benzene, and xylenes (BTEX) either separately or as a mixture. Toluene mineralization in this experiment was approximately 50% at a concentration of 5 mg/liter in a flask with pure oxygen flushed through daily.

Bacteria, often are identified as the principal agents of bioremediation (Autry and Ellis 1992) Although, many fungi also possess the ability to metabolize contaminants they are only recently attracting substantial research interest. As effectors of biodegradation, fungi have an advantage over many of their bacterial counterparts. Bacteria are small and have a certain ease with which they can contact contaminants, but they are also immobile (with some exceptions), meaning that they must be near to a contaminant before their enzymes can attack. Fungi, on the other hand can tunnel through the soil in search of nutrients. At first, the fungi begins as a small spore from which hyphae germinate and spread throughout the soil like the roots of plants. As they

spread, the hyphae may secrete wide-substrate-range extracellular enzymes, which, may cooxidize the target compound.

Today, microorganisms are only utilized in certain circumstances to degrade a limited range of contaminants. Usually, biological methods of site remediation are used to degrade hydrocarbons found in fuel spills. However, microbes have the ability to degrade an enormous variety of contaminants including both organic and inorganic compounds. For biological mineralization to occur, a compound must be available to cells either as a component of an aqueous solution or through direct contact. Several factors that influence rate and extent of the degradation of toluene include concentration, total mass of biomass, temperature, pH, nutrient and electron acceptor availability, and the adaption to the toluene by the microbes (Alvarez and Vogel 1991). Toluene has a much higher potential dissolved concentration in water than do most of the other components of gasoline. Consequently, toluene has a high availability for microbial metabolism (Klein and Jeskins 1981). The degradation of contaminants serves two purposes for the degrading microbe. First, contaminants serve as a source of food or more specifically carbon the growth of new biomass. Second, microorganisms gain energy from the partial or complete mineralization of contaminants.

Aerobic degradation of gasoline has been enhanced by diffusing air through the soil or through the groundwater which increases dissolved oxygen concentrations in the soil solution (Hincbee et al.1991). Whenever oxygen is used as the electron acceptor in the degrading of organic compounds we are talking about the process of aerobic respiration. Hydrocarbon degradation is viewed by most workers in

the field as strictly aerobic. All known metabolism pathways for hydrocarbon degradation is an oxidative attack on the molecule by a mono- or dioxygenase or a peroxide. The products of the oxygen- (or peroxide-) dependent attack on the hydrocarbon are frequently susceptible to anaerobic degradation. However, when O_2 is available to accept the reducing equivalents generated by hydrocarbon oxidation, biological degradation rates are much higher than when alternative electron acceptors must be used. Microbial enzymes utilize the molecular oxygen and use it to oxidize some part of one of the carbons in the contaminant to CO_2 with the remaining carbon used by the microbe in building new biomass. In this process the oxygen gets reduced producing water. Thus, the major products of bioremediation are CO_2 , water, and an increase in biomass.

Besides destroying a contaminant, microbial biomass also can sorb hydrophobic organic molecules. If we have ideal conditions which cause massive growth of biomass, in the path of the contaminant migration it is possible that the biomass can sorb the hydrophobic organic molecules thus retarding the plume of waste. This containment by microbial biomass is known as a biocurtain.

During the bioremediation of a site, the simple addition of nutrients and oxygen do not suffice to ensure effective degradation. The remediating system chosen must be monitored continuously for changes in the soil system. Whenever microbes are utilized to degrade contaminants, the soil chemistry environment also begins to change. Microbes increase the level of CO_2 while decreasing the concentration of O_2 in the soil atmosphere. For an example, the complete mineralization of toluene can be summarized

as follows: $C_7H_8 + 9O_2 = 7CO_2 + 4H_2O$. In addition to changes in the chemistry of the soil solution, other changes take place in the soil during bioremediation. Initially when the native populations of microbes are exposed to a toxic compound they will not degrade the waste with great rapidity. However, after a prolonged exposure to a contaminant, the native population becomes adapted to the target compound and begins to feed on it.

In some cases compounds may be present at the site in very low concentration; so low that the compound will be degraded very slowly or will remain unchanged. Other factors that can slow down the remediation at a site include the possibilities that the compound is strongly sorbed to the soil particles or that the contaminant is trapped in too small a pore for potential degradatory microbes to enter.

Most of the cases of successful bioremediation have been performed on sites contaminated with petroleum based hydrocarbons or their derivatives (Aelion and Bradley 1991). Examples of these compounds can be gasoline and fuel oils, alcohols, creosotes for wood preserving, plastics perfumes and pesticides. However, the majority of the sites are contaminated with gasoline. Benzene, toluene, ethylbenzene, and xylenes (BTEX) are the major constituents for gasoline. Toluene is more soluble than other constituents (except benzene) found in gasoline. It can serve as an electron donor for many bacteria widely distributed in nature. It is rapidly degraded relative to other; higher molecular weight components found in gasoline. As a general rule, microbes degrade BTEX readily if oxygen and nutrients are available.

CHAPTER 2

INTRODUCTION

There are 14,000 industrial sites in America producing 265 tons hazardous waste annually (Water Science and Technology Board 1993). Twelve hundred hazardous waste sites are listed on the superfund National Priorities List (NPL) (Dienemann 1992). Another 30,000 sites will be evaluated under the superfund program. The above sites are both numerous and very hazardous. But there are also tens of thousands, and possibly hundreds of thousands of smaller contaminated sites that also merit treatment.

Many of these contaminated sites contain toxic and carcinogenic compounds that persist in the environment for long periods of time with minimal microbial mineralization. Many of these compounds bioaccumulate in plants, animals, and people, thus causing health problems.

Fungal bioremediation has been neglected until only fairly recently, when laboratory and in-situ results began to reveal the vast potential of fungal bioremediation. *P. chrysosporium* has shown promise for the degradation of high molecular weight, recalcitrant compounds. However, little information exists in the literature regarding fungal potentials for mineralizing lower molecular weight compounds such as BTEX compounds. *P. chrysosporium* degrades all of the BTEX components either as individual compounds and also when presented as a mixture of BTEX compounds. Also, the degradation of these BTEX compounds did not rely upon the ligninolytic system but was

mineralized when the LIPs (lignin peroxidase) and MnP (manganese peroxidase) were absent as in the non-ligninolytic culture condition (Yadav and Reddy 1993).

BTEX compounds make up the majority of gasoline components. BTEX compounds frequently leak into the ground from USTs (underground storage tanks) at gasoline stations and airports, from pipelines, improper waste disposal and leaching landfills. BTEX components are carcinogenic and neurotoxic. BTEX compounds are also listed as priority pollutants regulated by the EPA.

There are four phases of unsaturated zone contamination: 1) air phase, where toluene is found in the soil atmosphere of the pore space in between the soil particles; 2) adsorbed phase in which the toluene sorbed onto organic material present in the soil; 3) aqueous where the toluene is present in the soil solution and 4) toluene can exist in a non-aqueous polar liquid phase.

For bioremediation to succeed, the compound intended for degradation must have certain properties. The compound to be degraded should not have too high of a vapor pressure because highly volatile compounds escape to the atmosphere before the microbes can act upon them. The compound should not be highly viscous or it may block air from reaching the invading fungus. The contaminant cannot be present at concentrations toxic for the fungus. Soil to be subjected to bioremediation should be permeable to air and water. Clay-rich soils do not lend themselves readily to bioremediation as they tend to be highly impermeable to both fluids and microbes alike. An accessible carbon source is required as well as sufficient fungal mycelium biomass

to allow for the complete mineralization of our contaminant. The soil should be appropriately moist and should have an adequate air supply .

Remediation Processes

There are two main methods of remediating a contaminated site biologically: 1) microbial inoculation, in which a site is seeded with microbes known to degrade the target compound; and 2) the bioaugmentation of naturally occurring microbial activities. In most cases bioaugmentation consists of the addition nutrients such as nitrogen and phosphorus which may be present in insufficient quantities to allow mineralization of the contaminant. Typically oxygen is provided (as air) in addition to nutrients. For bioaugmentation to succeed, the compound must be degradable by the microbes at the site. Over time, microbes capable of degrading the target contaminant will be stimulated by both the addition of nutrients and the addition of an oxygen supply. The biomass of these existing microorganisms subsequently increases to levels able to effectively mineralize the target compound.

Composting

Composting is an aerobic process that has been used for years to degrade organic wastes such as leaves. Today this process is also used to enhance the degradation of organic pollutants such as oils, gasoline, and fuel oils in contaminated soils. A typical method of composting is to place a layer of contaminated soil in rows and to mix this soil thoroughly with a layer of organic material such as wood chips, sawdust, or leaves (Maynard 1994) To achieve optimum composting, the moisture content is adjusted to

50-60% of field capacity. Excess moisture of over 70% impedes the mineralization process by interfering with the aeration of the soil and by lowering the self-heating capability of the soil. Periodically, the windrows are rototilled to provide aeration and nutrients such as nitrogen and phosphorus are added.

Bioventing and Air Sparging

For groundwater and vadose zone contaminated sites, microbial biomass is usually much lower than in surface waters, and the nutrient levels are much lower as well. Typically, nitrogen and phosphorus are injected via a well to provide nutrients (Thomas and Ward 1989). However, this often does not ensure rapid, complete mineralization. If only mineral nutrients but no air were provided, the oxygen supply would be quickly exhausted and the mineralization of the target contaminant would end. The biodegradation of one liter of hydrocarbons would exhaust the oxygen supply of approximately 400,000 liters of water (Water Science and Technology Board, 1993). Thus an adequate air supply is vital for the mineralization of these hydrocarbons.

When fuel spills occur near the surface, contaminated soil is usually excavated and incinerated to prevent migration of the hydrocarbons to the groundwater where the cleanup expense increases dramatically. Other methods of cleanup include expensive physical methods such as air stripping and soil flushing (Long 1993) and pump and treat (Borden and Kao 1992). Remediation of sites using microbial activity has attracted considerable research interest and real-world application because it is less costly than the available physical methods.

Bioremediation is often thought to be accomplished almost entirely by strains of bacteria. Although bacterial may be the principal agents of biodegradation that grow at the expense of organic toxins at most contaminated sites, bioremediation may be enhanced in some situations through the introduction of a fungus. Considerable evidence has appeared in recent literature suggesting that *P. chrysosporium* can be effectively used to remediate fuel-contaminated soils.

In surface soils, oxygenation of the soil can be achieved by drainage. Air-filled pores facilitate the diffusion of oxygen, while a waterlogged soil allows only a very slow rate of oxygen diffusion incapable of meeting the respiratory demands of the decomposers. If large quantities of organic pollutants are present, sometimes the diffusion rate is still too slow, even in a well-drained sandy soil, and oxygen must be provided. The rate of air exchange can be increased by pumping or by periodic raising and lowering of the groundwater.

For a contaminated aquifer, oxygen is supplied by bubbling air into the aquifer through an air sparger. Oxygen alone is often not enough to stimulate microbes. Microbial activity in aquifers is frequently limited by nitrogen and phosphorus. Similarly, addition of only nitrogen and phosphorus frequently fails to enhance biodegradation. However, provision of both an air supply and nutrients to the aquifer usually stimulate mineralization to a significant extent (Water Science and Technology Board).

Aerobic Bioreactors

Another method of treating a contaminated soil is with the use of an aerobic bioreactor. Problems have arisen in using slurry-type bioreactors on contaminated soils due to their difficulty in design and the problems in operation resulting from the settling tendencies of the soil. However, the supernatant of a washed soil can be readily treated in this type of reactor.

Incineration

Incineration is a process by which contaminated soil is excavated and passed through giant ovens usually by means of a vibrating conveyor belt. These ovens reach temperatures as high as 2,000 degrees Celsius. The belt traveling through the oven usually vibrates thus mixing the soil and allowing an even contact between the oxygen, the heat, and the soil particles. In the burner the contaminants will react with the oxygen to produce carbon dioxide and water. When temperatures get too low hazardous contaminants (such as carbon monoxide) are released to the atmosphere (Song 1990)

Land Farming

In land farming the contaminated soil is treated in above ground treatment beds. These beds are usually lined by a plastic liner and covered with sand to allow for drainage. Perforated pipes collect the drainage for treatment. Aeration is usually provided by tilling the soil. When forced aeration is used; the off-gases frequently require treatment. This method is more commonly used at refinery sites.

Costs of these treatments vary from site to site. Generally incineration is the most expensive at \$300-1000 per ton of soil; land disposal costs around \$200-300/ton; and bioremediation costs the least (on average) at around \$50-150 per ton.

Another advantage to using in-situ bioremediation as opposed to other physical methods of treatment regardless of its cheaper costs is that in-situ bioremediation affords less of an environmental insult to the affected site and to the environment in general. In-situ remediation normally requires minimal disruption of the surface soils.

CHAPTER 3

MATERIALS AND METHODS

Experimental Setup

Fungal Culture

Wild-type strain BKM-F-1767 of *P. chrysosporium* was purchased from American Type Culture collection, Madison, Wisconsin.

Radiolabeled Compounds

[U-ring-¹⁴C]toluene (53.5 mCi/mmol) was obtained from Sigma Chemical Co., St. Louis, Missouri. The ¹⁴C-toluene was maintained at 4⁰C as a primary stock solution in anhydrous ethanol with an activity of 10⁹ DPM/ml.

Soil and Packing

Each of twelve modified 38×200-mm screw-cap test tubes contained approximately 80 to 140 g of soil. The mass of soil in the columns was kept constant except when organic matter was added. The organic matter greatly increased the volume of material to be packed in the column beyond that which could be accommodated if its mass were kept the same as that of the unamended sandy loam. Amounts and forms of carbon in some of the columns was varied to determine whether addition of organic matter affected rate or extent of mineralization. Organic amendments to the columns included peatmoss, wood chips, and sugars.

The soil used in these studies was a Typic Dystrocrept of the Narragansett series, collected behind Brook's Barn on the University of Massachusetts, Amherst campus. The soil moisture content was brought to 80% of field capacity (the optimum moisture

level for growth of the fungus) and then the columns were packed with this mixture. The soil sample was packed in 20-g increments with gentle tamping to reduce excessive void space. This packing method was the same for all columns. Thus, the physical characteristics of the soil packed in the columns was fairly uniform throughout all the experiments.

Sterilized Control

The sterilized controls were prepared by autoclaving the soil twice for 1 h at 24-h intervals. All connections were either autoclaved or sterilized with a 70% ethanol solution.

Incubation Apparatus

Customized incubators (University of Massachusetts/Amherst Glass Laboratory) after packing with soil, all openings of the screw-cap tubes were closed and sealed using threaded connectors (Chem Glass Inc, Vineland, New Jersey). Air flow was regulated by a manifold and was adjusted to the desired rate using a bubble flow meter. The flow rate was checked daily to correct possible variations of flow. Air entering the apparatus was saturated with water by passing it through deionized water to prevent drying of the soil during incubation. Air passing through the sterilized column first flowed through autoclaved glass wool to exclude contaminants. All surfaces potentially exposed to the toluene within the apparatus were of teflon, glass, or stainless steel.

Initiation of Incubation

Between 1 and 10 g of fungal mycelium were added to the soil and mixed throughout. Mixtures of ^{14}C -toluene and non-labeled toluene were appropriately diluted

with 95% ethanol to provide approximately 10^6 DPM of ^{14}C to each column. Each column received slightly less than 0.5 ml of ethanol. For all experiments, the total hydrocarbon concentration was approximately 400 ug of toluene per gram of dry column contents. Less than 1% of the total toluene was added as ^{14}C -labeled compound. To accurately determine the amount of ^{14}C added to each column, a known quantity of the mixture of ethanol and toluene was added to a scintillation vial containing ScintiVerse #2 cocktail (Fisher Scientific, Springfield, New Jersey). The tube was tightly sealed, and air circulation was begun after a 1.5-day waiting period to allow time for the radiolabeled solution to diffuse throughout the soil.

Monitoring Mineralization Assay

The toluene was added to samples of the sandy loam described above. Soil samples amended with toluene were incubated in the sealed 38×200-mm columns. Air at low pressure was introduced to the bottom of the column at a rate of 5 ml/min. Air displaced from the column was passed from the top of the columns through a glass tube containing Tenax-TA (Altech Associates, Deerfield, Illinois) which collected any toluene, or metabolic intermediates that were stripped from the columns. In later experiments the attachment points of the air inlet and exhaust lines were reversed. The Tenax traps were changed every 4 to 5 days. Hydrocarbons trapped on the Tenax were eluted with 100 ml of hexane of which duplicate 5-ml samples were withdrawn for scintillation counting in Scintillene. After the Tenax-TA traps, the displaced air was passed through an aqueous solution of 1 N NaOH, which traps the CO_2 as carbonate.

Samples from these traps were withdrawn daily for scintillation counting in ScintiVerse #2 for quantification of ^{14}C using a Beckman model LS 3801 liquid scintillation counter.

Chromic Acid Digestion

At the end of the incubations, the soil from the tubes was slurried with a 10:1 (by volume) solution of water and methanol. A sub-sample of this slurry was subjected to the Mebius procedure for rapid, total oxidation of organic matter in soil using $\text{K}_2\text{Cr}_2\text{O}_7$ in boiling H_2SO_4 as described by Nelson and Sommers (1982) modified to permit collection and quantification of $^{14}\text{CO}_2$. A 10-gram subsample of the slurry was combined with a 50-ml volume of a 0.7 N solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in a 500-ml sidearm Erlenmeyer flask. To this flask a 35-ml volume of concentrated H_2SO_4 was gradually added and a flow of N_2 was introduced at a flow rate of approximately 30-ml/min. The flask was brought to boiling for 45 minutes on a hot plate. At the conclusion of the digestion, the hot plate was turned off and the flask was fan cooled for 15 minutes. The exhaust gasses were swept from the flask by the N_2 after which passed through three $^{14}\text{CO}_2$ traps arranged in series. Two sidearm 12-ml test tubes each containing 7-ml of 1 N NaOH solution were placed upstream from a flask containing 75-ml of 1 N NaOH. Finally the gas lines were closed and the NaOH solutions were then assayed for ^{14}C by liquid scintillation. The digested slurry in the flask was filtered using 15-cm filter paper No.2 (W.R. Balston, LTD., England). The retained solids were dried overnight in an oven to determine the dry weight of the digested samples.

Data Acquisition

Four sets of data were analyzed for each of the columns in the experiment. First, the rate of mineralization of the added [U-ring ^{14}C] toluene was assessed by measuring ^{14}C that was captured in the NaOH solution. Two 1-ml subsamples of the NaOH traps were sampled daily for radioactivity using a Beckman liquid scintillation counter Model LS3801 (Beckman, New Jersey). Second, the amount of toluene that was vented from the incubator and onto the Tenax traps was quantified. This was also measured by scintillation counting. The last piece of data was obtained by the acid digesting of the soil applied at the end of each run.

Tenax Trap Extraction

Tenax traps were taken from their connections to the columns and were immediately eluted with 100-ml of hexane. An extra 25-ml of hexane was then washed down the trap to determine whether there were any additional counts remaining in the Tenax. The extraction direction was chosen based on the assumption that there would be a higher concentration of toluene close to the top air entry port. Therefore, hydrocarbons were washed in the direction opposite to that of air flow during incubation. Five-ml subsamples of these 100-ml and 25-ml samples of hexane were removed for scintillation counting.

Tenax Trap Reconditioning

Used Tenax traps were placed in an oven and heated at 100 to 200 $^{\circ}\text{C}$. Nitrogen was passed through the oven at not less than 25-ml/min. The traps were heated in this

manner for at least 24 hrs. A study utilizing a reconditioned trap eluted with hexane showed no residual ^{14}C remaining on the Tenax traps after the reconditioning process.

CHAPTER 4

RESULTS

Initial Toluene Mineralization Experiment Utilizing *P. cryosporium*.

Mass balances

Amounts of ^{14}C recovered during the experiment are expressed as percentages of the total [U-ring- ^{14}C] labeled toluene initially added (Table 1). In the first experiment, the unamended nonsterile control had the greatest total recovered ^{14}C at 57%. The nonsterile soil with 10 grams of added fungal mycelium had a total recovery of 36%. In general, as greater concentrations of organic matter were added in the form of peatmoss and wood chips, the total recoveries of ^{14}C declined. The unrecovered fraction of the total added ^{14}C amounted to over 80% in the two high organic addition columns (Table 1).

The least mineralization of the toluene occurred in the column with only peatmoss and wood chips. This might be attributed to sorption of the toluene to the organic matter in the column. The addition of methyl cellulose did not seem effectively to increase the mineralization of the toluene during the course of this experiment.

For all treatments the vented fraction of the radiolabeled compounds was high (Table 1) due to the volatile nature of the toluene and also probably due to the high airflow rate with regards to the size of the columns. The airflow in the columns was from the bottom to the top of the column. Because the radiolabeled toluene was added to the top of the column, it is possible that toluene remained in the column for a period too brief for the toluene to be mineralized. The nonsterile control showed the greatest

venting of the toluene at 50%. The two columns with the high organic additions had low vented recoveries at 12% and 8%. However, these low levels of recovery may be underestimates, since the overall recoveries of radiolabeled toluene from the two columns were low as well.

Mineralization products ($^{14}\text{CO}_2$ Evolved)

In the nonsterile control and the nonsterile soil amended with fungal mycelium and methyl cellulose the extents of mineralization (Table 1 and Fig. 1) were similar rates at 2.41% and 2.48% respectively. The nonsterile soil with added mycelium and had the greatest mineralization of the soils only columns in the experiment with a total recovery of ^{14}C as carbon dioxide equal to 5.7% of the total added ^{14}C . The greatest rate of mineralization was the soil column containing peatmoss and fungal mycelium, in which 7.4% of the total added was mineralized. The least degradation of the toluene occurred in the soil-free column. This column contained only woodchips and peatmoss and supported a mineralization of less than 1% of the toluene. This low rate of mineralization could be attributed to the possible sorption of the toluene to the organic material as well as to possible rapid venting of toluene from the column.

This experiment was run for four weeks. The majority of the mineralization due to the presence of the fungus occurred during the first 7 days after inoculation of the fungus (Fig. 1).

Table 1 Percentages of added [U-ring-14C]-labeled toluene recovered in different fractions from the soil during the first April run.

Fraction where ¹⁴ C was recovered in initial experiment	Nonsterile control	Nonsterile with 10-g fungal mycelium	Nonsterile 10-gram fungus & methyl cellulose	Nonsterile soil 10-gram fungus & woodchips	Wood chips 10-gram fungus & peatmoss
Mineralized	2.4	5.3	2.5	4.7	0.9
Vented	50.1	27.8	33.4	11.9	8.4
Digested	4.8	2.9	7.3	2.9	3.5
Total	57.3	36.0	43.2	19.5	12.8

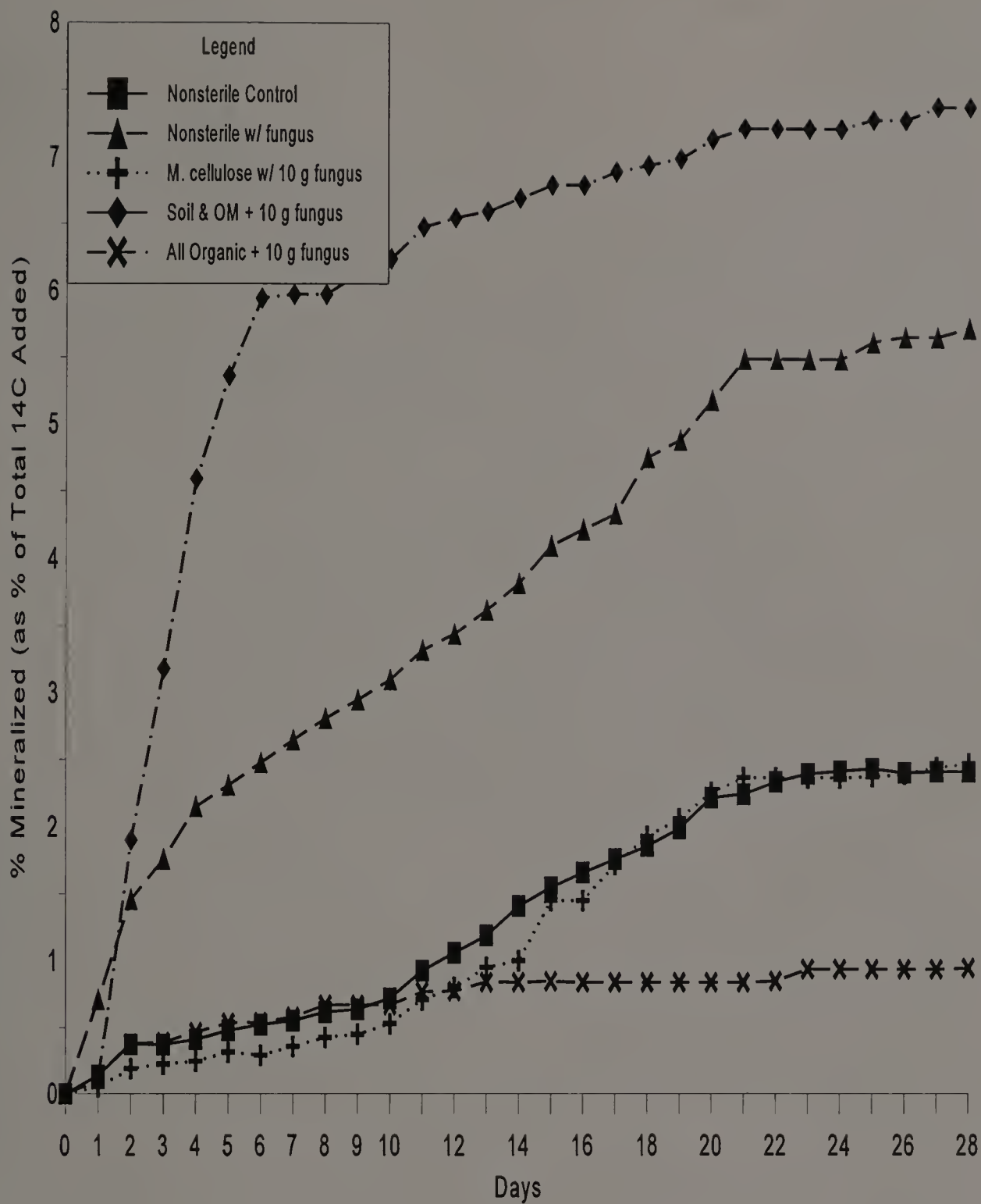


Figure 1 Mineralization of toluene based on the accumulation of ^{14}C from the total added ^{14}C labeled toluene. Airflow continuous from the top to the bottom of the columns.

Toluene Mineralization Utilizing *P. cryosporium* Experiment #2

Mass balances

The totals of recovered ^{14}C in the different fractions appear in Table 2. The total recovered ^{14}C expressed as a percentage of the total added ranged from a low of 0.00% mineralization in the sterile control to a high of 7.5% in the nonsterile soil with the amended organic material (Fig. 2). The extent of mineralization was 4.4% in the nonsterile control and was 5.5% in the nonsterile soil with added fungal mycelium. The sterile soil (5.6% mineralization) with added fungus supported greater mineralization than the nonsterile soils. The addition of organic matter to a soil enhanced the mineralization of the toluene. However, this increase in mineralization is unlikely to be significant. The addition of high concentrations of sugars to the columns greatly inhibited the mineralization of the toluene and this was probably due to preferential utilization by the fungus of the sugar as a carbon and energy source.

The majority of the recovered radiolabeled toluene was vented onto the Tenax traps (Table 2). Replacement of the Tenax traps revealed that nearly all venting occurred during the first week of the experiment. The vented toluene as a percentage of the total added ranged from a low of 32% to a high of 112%. This high percentage of vented toluene could be attributed to the addition of the toluene to the top of the column. The air flow during this experiment flowed from the bottom of the column to the top of the column. This direction of airflow could very easily increase outgassing of the toluene to the Tenax traps.

Table 2 Percentages of added [U-ring-14C]-labeled toluene recovered in different fractions for the soil.

Fraction where ¹⁴ C was recovered	Sterile control	Nonsterile control	Nonsterile 1-gram fungus	Sterile 1-gram fungus
Mineralized	0.0	4.4	5.5	5.6
Vented	53.2	77.7	59.4	32.0
Digested	2.4	4.3	3.5	4.1
Total	55.6	86.4	68.4	41.7

Fraction where ¹⁴ C was recovered	Nonsterile 1-gram fungus peatmoss	Nonsterile 1-gram fungus sugars	Nonsterile 1-gram fungus peatmoss sugars	Nonsterile mutant fungus 1-gram	Sterile mutant fungus 1-gram
Mineralized	7.5	0.0	0.2	2.6	2.8
Vented	61.1	57.8	84.1	112.4	27.8
Digested	12.4	5.3	1.5	ND ^a	ND ^a
Total	81.0	63.1	85.8	115.0	30.6

ND^a = not determined

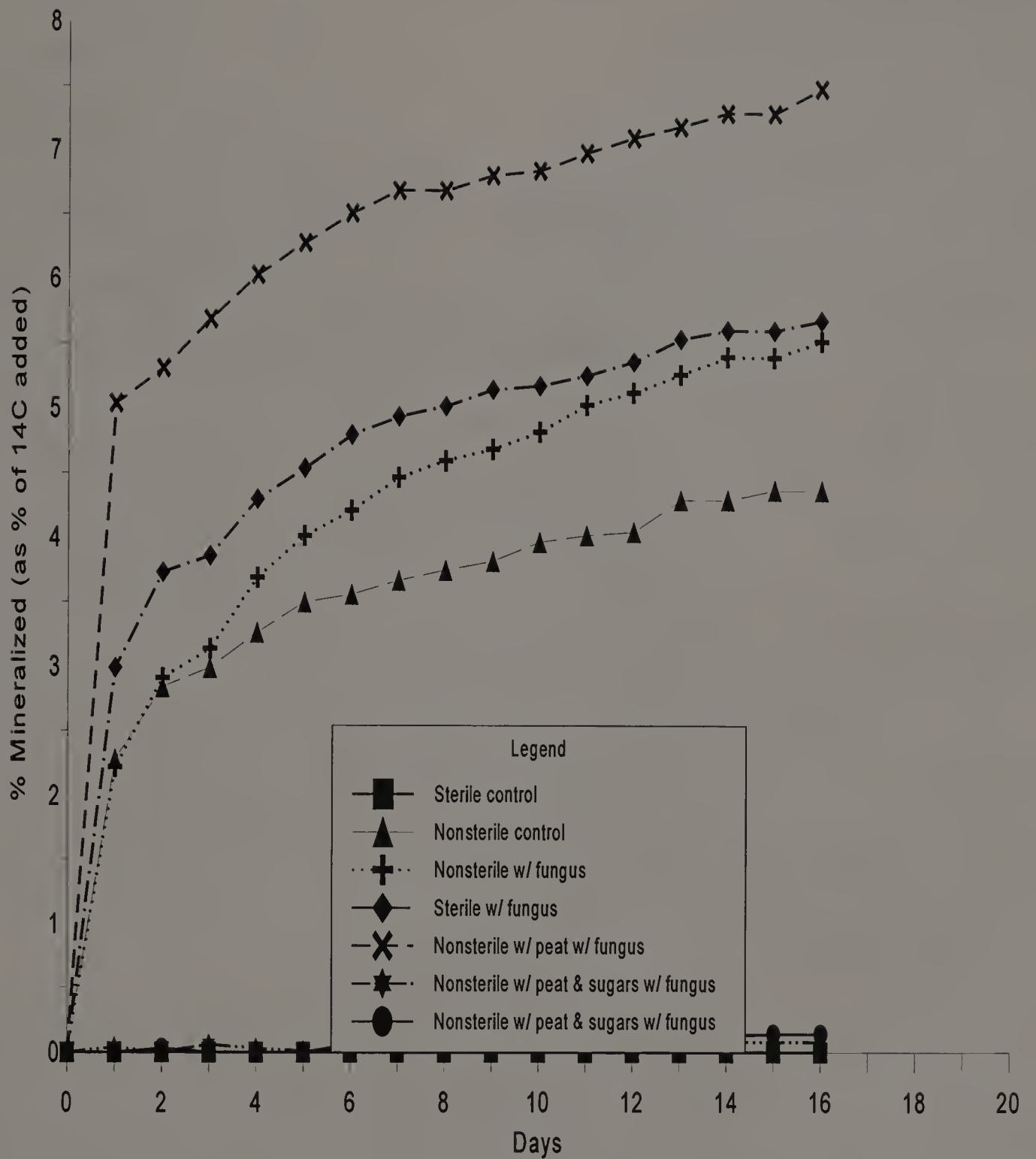


Figure 2 Accumulation of ^{14}C from the degradation of ^{14}C labeled toluene. The total mass of fungi decreased from 10-g to 1-g per column.

Toluene Mineralization with Reversed Airflow

Mass Balances

This experiment was performed with the airflow being reversed from a flow direction of bottom to top to a flow direction top to bottom. During this run the comparisons were made between sterile and non-sterile controls. The effects of the addition of *P. chrysosporium* to the columns inoculated with sterile and non-sterile soil were also examined. Mineralization in the sterile controls was negligible (Table 3 Fig. 3). The extents of mineralization in the two nonsterile controls differed by a factor of five (Table 3).. However, the mineralization rates were low and in agreement with those in other sterile columns during different runs.

The addition of fungi to the nonsterile soils lead to a greater extent of mineralization than occurred in those columns containing sterile soil samples. However, both extents were low and the apparent difference between them is of doubtful significant. The addition of organic material in the form of peatmoss also effected a negligibly small increase in the rate and extent of mineralization of the toluene.

Despite the reversed direction of airflow, in this experiment as in the last two experiments the majority of the recovered toluene was trapped on the Tenax. Although the airflow was reversed, the flow rate was still 5 ml/min run for 24 hours per day. The majority of the toluene was vented in the first few days as was seen in the prior runs. After this run extensive toluene mineralization by *P. chrysosporium* began to seem unlikely.

Table 3 Percentages of added [U-ring-14C]-labeled toluene recovered in different fractions for the soil.

Fraction Where ¹⁴ C was Recovered	Sterile control	Sterile control	Nonsterile control	Nonsterile control	Nonsterile with fungus (1-gram)
Mineralized	0.02	0.03	0.3	1.4	2.2
Vented	91.1	68.1	78.8	60.4	62.8
Digested	5.4	12.9	10.6	12.8	7.2
Total	96.5	81	89.7	74.6	72.2

Fraction where ¹⁴ C was recovered	Nonsterile with fungus (1-gram)	Sterile with fungus (1-gram)	Sterile with fungus (1-gram)	Nonsterile with fungus (1-gram) peatmoss	Nonsterile with fungus (1-gram) peatmoss
Mineralized	1.7	0.7	1	0.8	0.9
Vented	71.4	75.7	74.8	70.9	79.8
Digested	6.2	7.9	9.42	8.7	8.1
Total	79.3	84.3	85.2	80.4	88.8

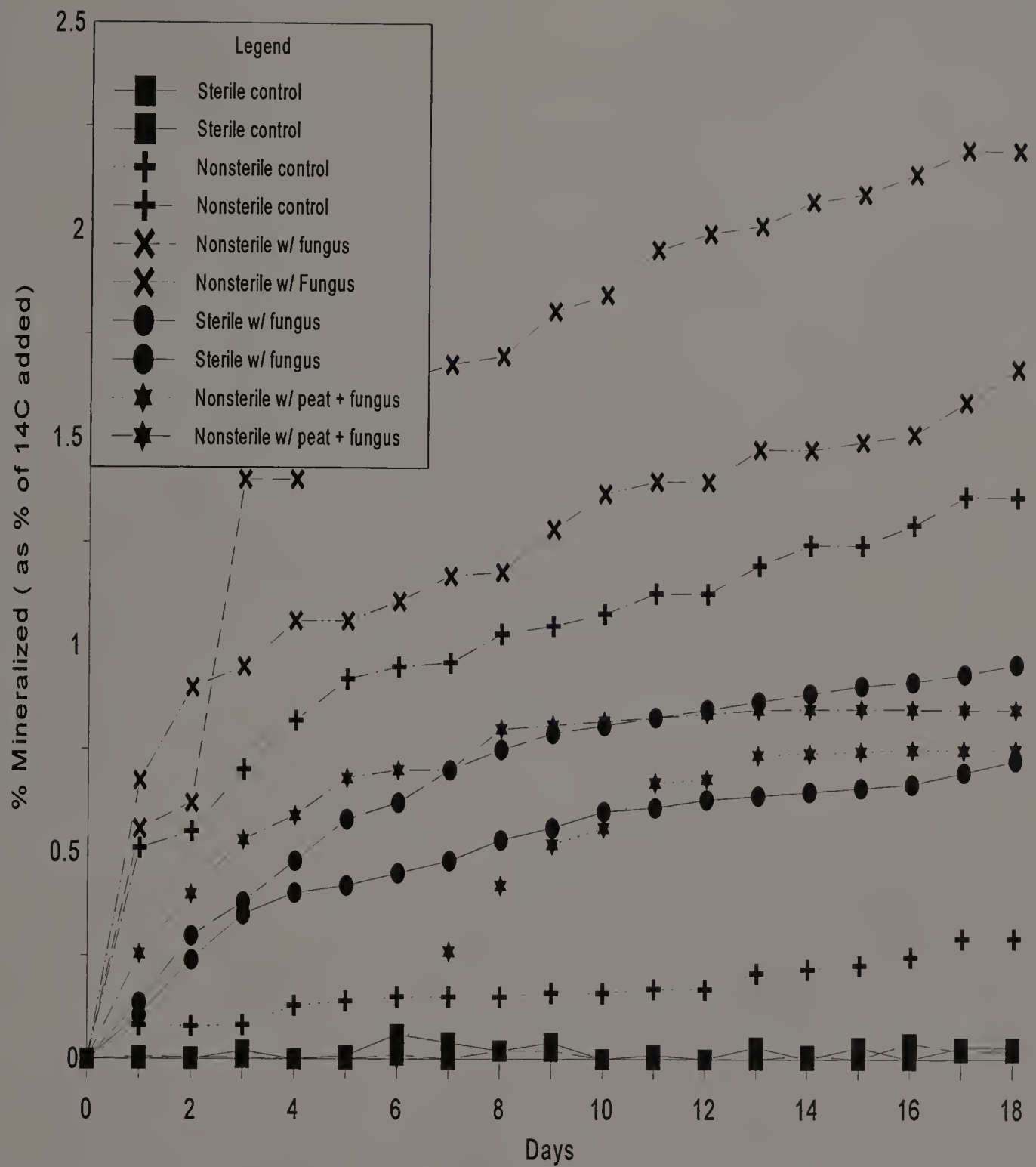


Figure 3 Mineralization of ¹⁴C labeled toluene based on a reversing of the airflow to a flow from the top of the column to the bottom. Samples amended with peat were also inoculated with *P. cryosporium*.

Toluene Mineralization with Reversed Airflow Repeated

Mass Balances

The previous experiment was repeated hoping that a great reduction in flow rate with airflow from top to bottom of the column, would stimulate increased mineralization of the toluene. During this run we again wanted to compare the addition of *P. chrysosporium* to sterile and nonsterile controls, and to determine as to whether or not the addition of this fungus does stimulate mineralization of toluene. The extent rate of toluene mineralization in the sterile control was negligible, and the nonsterile controls also mineralized only about 0.5% of the total added (Fig. 4 or Table 4). The addition of *P. chrysosporium* did have a greater effect on the mineralization rates of toluene in the nonsterile soils as opposed to the sterile soils.

Again the greatest factor in toluene removal from the columns was from venting (Table 4). The direction of airflow was from top to bottom even though the pumps were turned on for only one hour per day. One hour a day with the pumps turned on to give 5ml/min gives us approximately two pore changes per day. Even with this greatly reduced rate of airflow, the toluene still vented from the column.

Table 4 Percentages of added [U-ring-¹⁴C]-labeled toluene recovered in different fractions for the soil.

Fraction where ¹⁴ C was collected November 1993	Sterile control	Sterile control	Nonsterile control	Nonsterile control
Mineralized	0.3	0.3	0.6	1.8
Vented	75.7	76.2	15.6	61.1
Digested	13.7	9.6	10.8	9.5
Total	89.7	86.1	27.0	72.4

Fraction where ¹⁴ C was collected November 1993	Sterile 1-gram fungus	Sterile 1-gram fungus	Nonsterile 1-gram fungus	Nonsterile 1-gram fungus	Sterile peat 1-gram fungus
Mineralized	1.4	0.9	2.7	3.3	0.5
Vented	46.2	73.1	46.4	54.2	38.2
Digested	9.9	3.4	8.4	5.8	11.2
Total	57.5	77.4	57.5	63.3	49.9

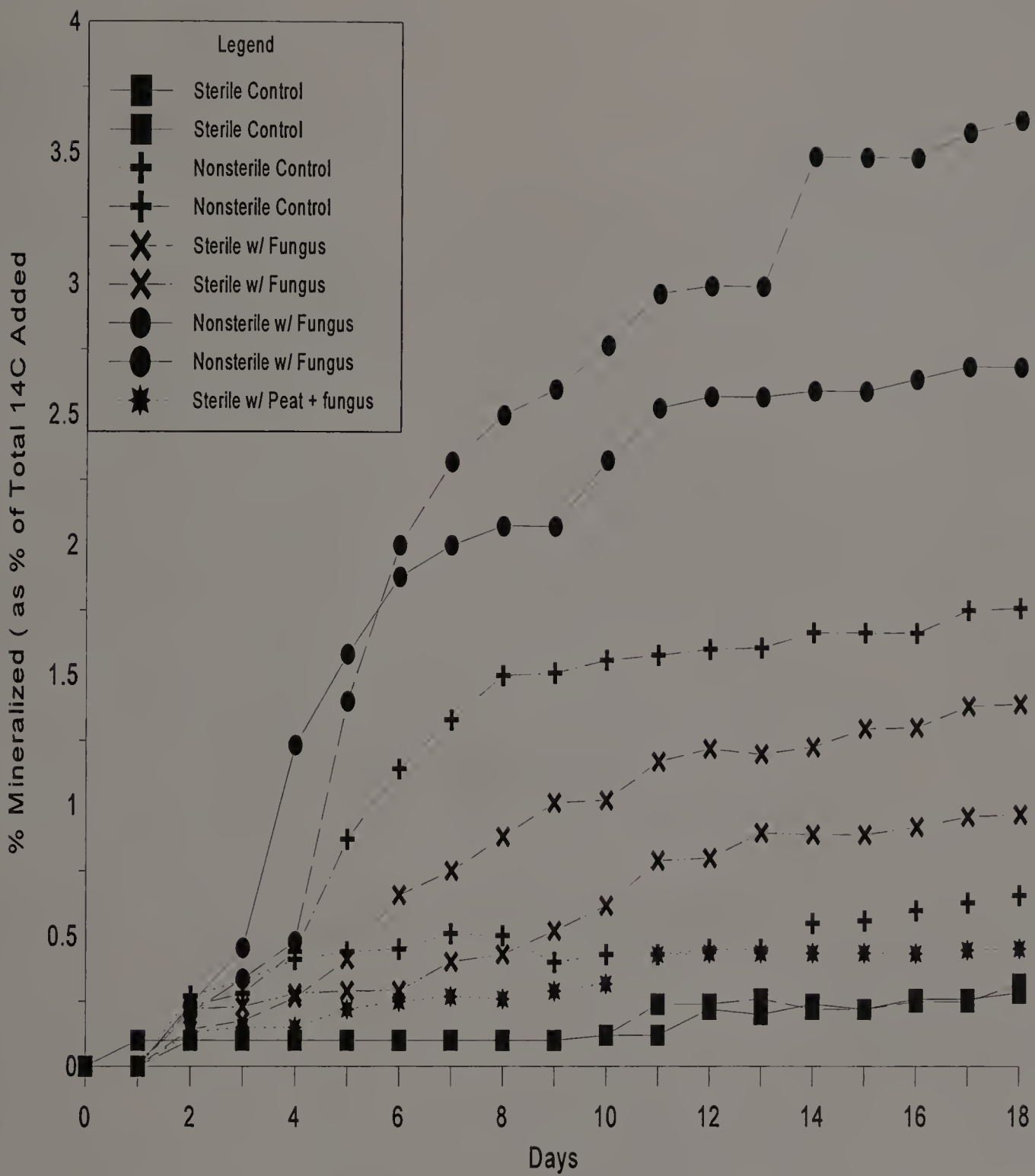


Figure 4 Repetition of the previous experiment with the airflow flowing from the top to bottom of the column. Airflow was gradually increased to give a final flow rate equal to two pore changes per day.

Nitrogen Experiment

Mass balances

During the last and final experiment, an evaluation was made of the effect of an addition of nitrogen (in the form of NH_4NO_3) on the mineralization of toluene. The most extensive mineralization did occur in the column containing the most added nitrogen (Fig. 6). However, in a replicate column no more toluene was mineralized than in those columns amended with nitrogen at the lower level (2.4 mg/kg).

Again, as in previous runs, the majority of the recovered ^{14}C was vented. The soil columns during this run had less air passing through them than in all of the other experiments. The airflow was from the top of the column to the bottom. The airflow rate was 5 ml/min. There was a rest period of three days after the radiolabeled toluene was added to the columns. During this rest period, the columns received no air flow. On the fourth and fifth day, air was provided at 5 ml/min for 15 minutes. On the 6th day airflow was maintained for 30 minutes, and on the seventh and all subsequent days, the columns airflow was induced for a period sufficient to give the columns two pore changes (approximately 1 h) per day. After a week the columns were white with conidia and showed evidence of mycelial networks within the columns. The vast majority of the radiolabeled toluene was vented within five days of the initiation of airflow. Unfortunately, no practical means existed for decreasing the rate of loss by outgassing of toluene from the columns. Had the supply been restricted by lengthening the rest period or by reducing the rate or duration of airflow, the fungus was thought to likely revert to its protected spore stage instead of spreading throughout the column.

Table 5 Percentages of added [U-ring-¹⁴C]-labeled toluene recovered in different fractions of the soil.

Fraction where ¹⁴ C was recovered	Sterile control 2.4mg/kg N	Nonsterile control 2.4 mg/kg N	Nonsterile control 2.4 mg/kg N	Nonsterile with fungus (1-gram) 2.4 mg/kg N	Nonsterile with fungus (1-gram) 2.4mg/kg N
Mineralized	.05	1.1	1.2	1.2	1.3
Vented	58.5	51.6	41.4	47.6	24.9
Digested	13.3	9.9	15.2	12.5	5.7
Total	71.6	62.6	57.8	61.3	31.9

Fraction where ¹⁴ C was recovered	Nonsterile with fungus (1-gram) no N	Nonsterile with fungus (1-gram) 24 mg/kg N	Nonsterile with fungus (1-gram) 24 mg/kg N	Nonsterile with fungal fragments ^a (1-gram) 24 mg/kg N	Nonsterile with fungal fragments (1-gram) 2.4 mg/kg N
Mineralized	1.8	4.4	2.3	2.0	1.4
Vented	31.5	24.8	50.2	56.4	44.6
Digested	11.9	4.2	11.9	10.8	3.3
Total	45.2	33.4	64.4	69.2	49.3

^a *P. cryosporium* was fragmented by blending for 120 seconds.

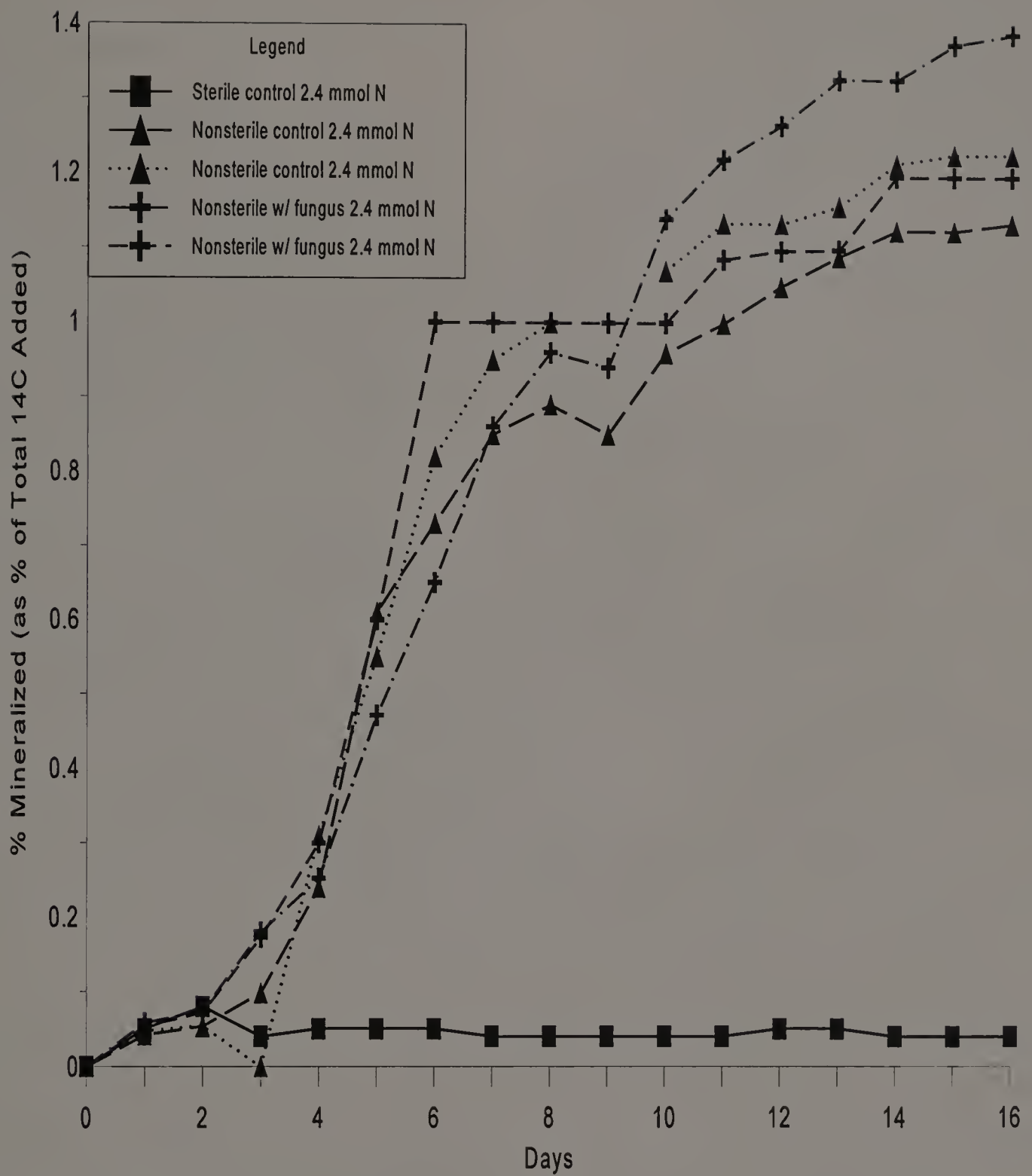


Figure 5 Effects of addition of nitrogen in the form of NH_4NO_3 at low (2.4 mg/kg) concentrations on the mineralization of toluene by *P. cryosporium*.

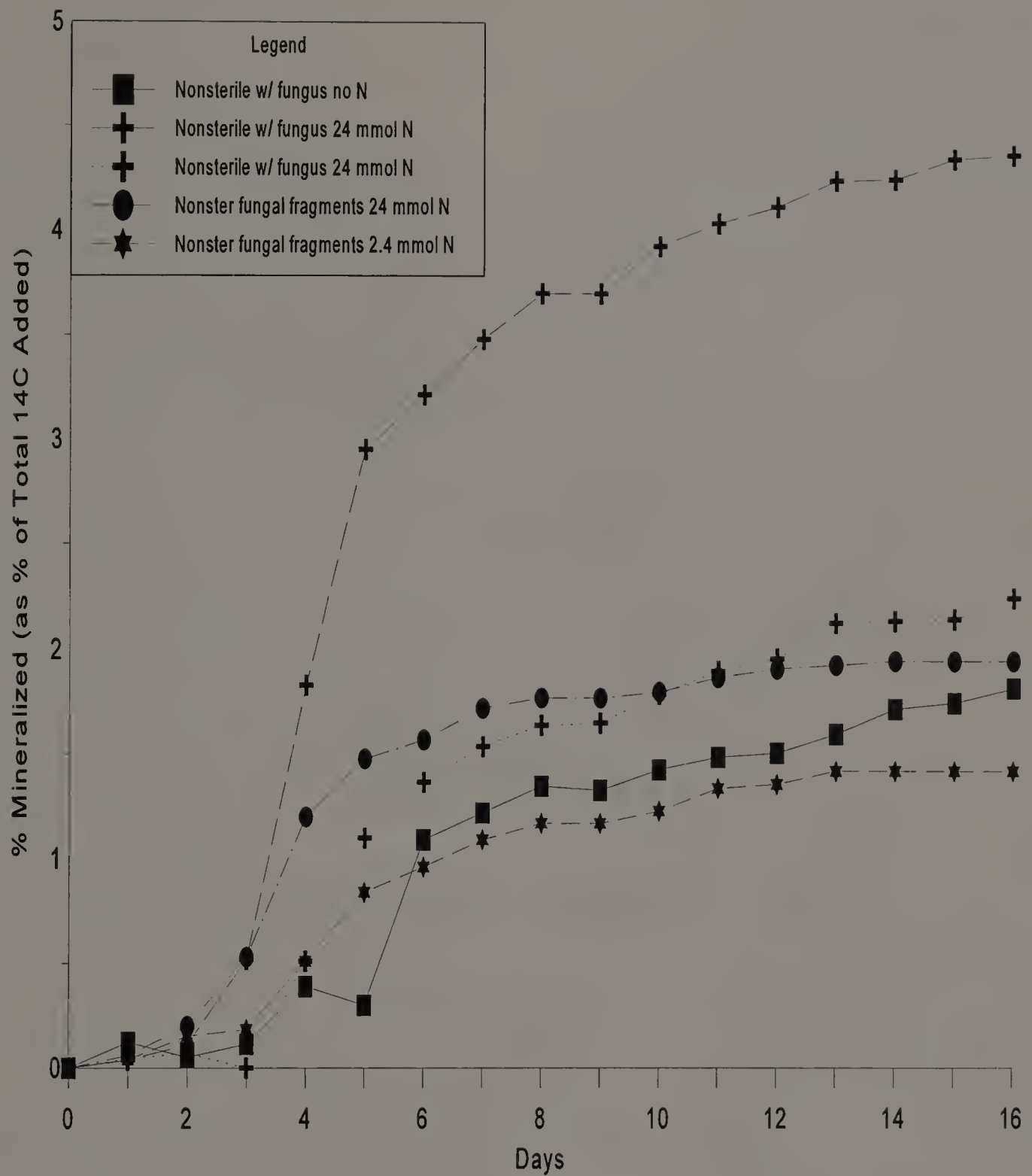


Figure 6

Effects of the addition of nitrogen in the form of NH_4NO_3 at low (2.4 mg/kg) and high (24 mg/kg) concentrations on the mineralization of toluene by *P. cryosporium* inoculated as intact or fragmented mycelium.

CHAPTER 5

DISCUSSION

In this study we examined the mineralization of toluene at high levels (400 μ g/g) by the white-rot fungus *Phanerochaete cryosporium* in a sandy loam. Over the years much research has been devoted to *P. cryosporium* and its ability to degrade recalcitrant molecules such as phenanthrene, lignin (Ortha et al. 1993), and azo dyes (Ollikka et al. 1993) but nothing was found in the literature regarding the degradation of gasoline components by this fungus.

The first experiment was designed to check biodegradation of the toluene under a variety of conditions. Some columns received organic matter while others received substantial organic additions in the form of peat moss or wood-chips. In all of the treatments the mineralization was very low. However, from a glance at the columns it was apparent that the fungus was indeed growing. The sides of the columns were covered with white conidia, and mycelial networks could be seen growing into the soil within the column. It was also interesting to note that as the organic addition levels increased the amount of evolved ^{14}C -toluene greatly decreased. Possibly this was due to sorption of the toluene onto the organic material. The vented toluene was much lower in the columns with organic additions than those without the organic additions. Further digestion of the organic material probably would have released this sorbed toluene. However, the digesting of high levels organics in our digestion system was dangerous and resulted in the expulsion

of the containment cap from the digestion flask with ensuing release of radioactive materials into the laboratory.

The second experiment was very similar to the first experiment in that the effects of organic matter additions were examined. These columns had less organic material than those of the first experiment to allow soil digestion to proceed in a safe manner. Also, sterile soils with added fungi were compared to nonsterile soils with added fungi. Again the mineralization of the toluene was low. As in the first experiment the majority of the ^{14}C -labeled toluene was vented onto the traps. At the conclusion of this experiment it seemed likely that the experimental design was flawed and allowed the majority of the toluene to escape from the column before the fungi had a chance to degrade it.

During the third experiment, differences between sterile and non-sterile controls were examined, as were the effects of *P. cryosporium* inoculation into columns containing sterile and nonsterile soil. This experiment was almost a duplicate of the second experiment except for a reversal in the direction of gas flow.

During the first two experiments the airflow was from the bottom to the top of the column. Because our radio-labeled toluene was added to the top of the column, it was thought that reversing the airflow would prevent the toluene from escaping too quickly from the column. An increase in mineralization due to the longer resident time of the toluene was an anticipated consequence of this airflow reversal.

However, the majority of the toluene again was vented onto the traps well within the first week of the experiment, and the mineralization of the toluene was still low.

During the fourth experiment air again flowed from the top of the column down to the bottom with airflow direction making little difference. It was thought possible that so much air was flowing through the column that toluene was blown out. In this study airflow was reduced from 5-ml/min to a constant 5-ml/min for one hour per day, or approximately two pore changes per day. The sides of the columns again became covered with white conidia, and networks of mycelia seemed to ramify throughout the soil. The amount of vented toluene decreased from the levels measured in previous experiments, but the extent of mineralization of the toluene did not increase.

During the final experiment the addition of nitrogen in the form of ammonium nitrate was tested to see if it increased the extent of toluene mineralization. In one column the addition of ammonium nitrate at 24 mg/kg appeared to double the extent of mineralization compared to that in the columns receiving no added nitrogen. However, a replicate column with the same concentration of ammonium nitrate exhibited showed no such increase in the rate of mineralization.

Yadav and Reddy (1993) showed that toluene was degraded by the white-rot fungus *P. cryosporium*. At toluene concentrations of .5 mg/liter in broth culture under laboratory conditions with pure oxygen purged through daily, the mineralization of toluene approached 80%. However, as the level of toluene increased to 20 mg/liter under those same laboratory conditions, the mineralization of toluene decreased to approximately 30% of the total added. In the experiments reported here the level of toluene added to the soil was 400 mg/kg of soil. Had

toluene been introduced at low levels, mineralization of toluene in this study might have increased greatly. However, the addition of toluene at levels likely to be found in a site contaminated with toluene was deemed to be a desirable component of the experiment design for the studies reported here.

P. cryosporium did not effectively mineralize toluene at 400 mg/kg under any of the conditions tried in these experiments. It seems probable that the toluene was added at too high a concentration. However, another reason for the minimal degradation of the toluene in our experiments can be inferred from a consideration of this fungus in its natural environment.

P. cryosporium lives in the insides of rotting trees in the presence of large quantities of organic matter especially lignin, cellulose, and hemicellulose. But, during these experiments the fungi was asked to operate in a potentially toxic environment with meager quantities of organic material compared to its preferred conditions in a rotting log. A better choice for toluene-mineralizing fungi might be to look at litter-degrading fungi, which might be expected to have a better ability to thrive in the lower organic content of our experiments. Litter-degrading fungi thrive in areas of soil with lower levels of organic materials.

Phanerochaete cryosporium does not appear to possess the capability to mineralize toluene at high concentrations (400 mg/kg). Consequently, this fungus seems inappropriate for the remediation of a soil contaminated with toluene at concentrations high enough to be of any practical concern..

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