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Bioaugmentation and biostimulation technologies to bioremediate soils
contaminated with herbicide mixtures

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I dedicate this thesis to the large number of people who have supported me while I worked to obtain this degree, including my family members, friends, coworkers, and my major professors, Tom Moorman and Alan DiSpirito. I especially want to thank my husband, Mark, who has provided love and support without question, and my parents, Charles and Kay Struthers, who always supported me in all I chose to do. I also want to dedicate this thesis to my good friends, Alissara Reungsang, Jessica Jordan, Beth Douglass, Jennifer Welch, and Jeremy Long, who have been with me through all the good and the trying times.

TABLE OF CONTENTS

GENERAL INTRODUCTION	1
Rationale and Objectives	1
Project Description	1
Thesis Organization	2
CHAPTER 1. LITERATURE REVIEW	3
History of Remediation	3
Uses of Bioremediation	4
Pesticide Contamination and Bioremediation	4
Physical and Chemical Factors Affecting Bioremediation	5
Bioaugmentation	9
Biostimulation	21
CHAPTER 2. FACTORS AFFECTING BIODEGRADATION OF ATRAZINE BY <i>Agrobacterium radiobacter</i> STRAIN J14a AND APPLICATION OF THE ORGANISM TO BIOREMEDIATE SOIL	27
Abstract	27
Introduction	28
Materials and Methods	31
Results	39
Discussion	55
Literature Cited	63
CHAPTER 3. ORGANIC AND INORGANIC AMENDMENTS TO ENHANCE BIODEGRADATION OF HERBICIDES IN CONTAMINATED SOILS	67
Abstract	67
Introduction	68
Materials and Methods	71
Results	79
Discussion	98
Conclusions	104
Literature Cited	105
GENERAL SUMMARY	107
APPENDIX A. THE MICROBIAL POPULATIONS (BACTERIA, ACTINOMYCETES, AND FUNGI), MICROBIAL RESPIRATION, AND DEHYDROGENASE RESULTS AT EACH SAMPLING TIME FOR THE FIRST BIOSTIMULATION EXPERIMENT	109
APPENDIX B. THE MICROBIAL POPULATIONS (BACTERIA, ACTINOMYCETES, AND FUNGI), MICROBIAL RESPIRATION, AND DEHYDROGENASE RESULTS AT EACH SAMPLING TIME FOR THE SECOND BIOSTIMULATION EXPERIMENT	130
ADDITIONAL REFERENCES	150
ACKNOWLEDGMENTS	158

GENERAL INTRODUCTION

Rationale and Objectives

Widespread use of pesticides throughout the Midwest has led to the development of a large number of agricultural chemical dealerships. It is estimated that most of these dealerships have contaminated the soil or groundwater on or around the site due to accidental spills or careless mixing, loading, and storage of pesticides (Frieberg, 1991). Based on the potential number of sites needing to be remediated throughout the Midwest, economical and rapid technologies to remediate a diverse group of relatively recalcitrant pesticide contaminants need to be developed. Bioremediation, the use of biological systems to degrade environmental contaminants, is one of the most cost-effective remediation technologies in use today. Two of the strategies used in bioremediation are bioaugmentation and biostimulation. Bioaugmentation is the process in which an individual strain or consortium of microorganisms is added to a contaminated site to enhance biodegradation of a contaminant. Biostimulation is the process in which various inorganic or organic amendments are added to the contaminated site to attempt to stimulate biodegradation of the contaminant. I have used these two technologies in an attempt to enhance herbicide biodegradation in contaminated soils.

Project Description

This project was funded by a grant from the U.S. Environmental Protection Agency administered by the Robert S. Kerr Environmental Research Laboratory in Ada, Oklahoma, in conjunction with the Departments of Entomology and Geological and Atmospheric Sciences at Iowa State University in Ames, Iowa and the Agricultural Research Service (ARS) at the National Soil Tilth Laboratory in Ames, Iowa. Our investigations at the National Soil Tilth Laboratory involve the isolation and characterization of herbicide-degrading microorganisms to use as soil bioaugmentation agents to enhance degradation of specific herbicide contaminants. We have also investigated how addition of organic and inorganic amendments affect the microbial populations, microbial activity, and herbicide biodegradation capabilities in herbicide-contaminated soils collected from agrochemical

Entomology at Iowa State University will be investigated the effectiveness of phytoremediation in enhancing herbicide biodegradation of herbicide-contaminated soils. Dr. Blythe Hoyle's laboratory in the Department of Geological and Atmospheric Sciences at Iowa State University is determining the factors controlling herbicide biodegradation in subsurface environments and developing bioremediation strategies for this environment.

Thesis Organization

This thesis has been organized into chapters and contains two manuscripts prepared for publication in the journal *Applied and Environmental Microbiology*. This introduction is followed by a literature review, the two manuscripts, general conclusions, two appendices for the second manuscript (Appendix A and Appendix B), additional references cited in the general introduction and literature review, and acknowledgments. The first manuscript is entitled "Factors affecting biodegradation of atrazine by *Agrobacterium radiobacter* strain J14a and application of the organism to bioremediate soil." The isolation and characterization of the bacterium that possesses the enzymes capable of degrading the herbicide atrazine as a source of nitrogen and the use of the bacterium as a bioaugmentation agent in an attempt to bioremediate two soils contaminated with atrazine are described. The second manuscript is entitled "Addition of organic amendments and inorganic nutrients to enhance biodegradation in herbicide-contaminated soils." I present the results of several biostimulation experiments performed in a soil contaminated with the herbicides atrazine, metolachlor, and trifluralin either individually or in combinations of the three herbicides.

CHAPTER 1. LITERATURE REVIEW

History of Remediation

The industrialization of the world has led to staggering contamination problems resulting from the release of hazardous and toxic chemicals into the soil, groundwater, surface water, and air. Since 1980, the Environmental Protection Agency (EPA) has identified approximately 31,000 abandoned waste sites and it is predicted that an additional 19,000 sites exist that will require cleanup (Cookson, 1995). It has also been estimated that 295,000 leaking underground storage tanks exist in the United States (Cookson, 1995). The cost to clean up the Department of Energy (DOE), Department of Defense (DOD), and EPA projects in the United States currently being remediated is estimated at \$655 billion (Cookson, 1995).

Up until 1980 many of the compounds present at these contaminated sites were considered to be recalcitrant (resistant to any biodegradation), leading to the development of remediation technologies based on physical and chemical principles. Some of these technologies include incineration, landfilling, solidification, and thermal desorption (Cookson, 1995). Incineration and landfilling are both very costly techniques, with estimated annual costs per cubic yard of \$530 and \$670, respectively (Cookson, 1995). The thermal processes of incineration and thermal desorption will either completely destroy the wastes or reduce their volume, but may also generate toxic ash and volatile substances (Baker and Herson, 1994). Solidification and landfilling do not destroy the contaminant, and landfilling only relocates the contamination problem (King *et al.*, 1992). Additionally, both of these remediation techniques may still result in slow leaching of the contaminants into potable water supplies, which may lead to future health, environmental, and liability issues (Davidson *et al.*, 1980).

Uses of Bioremediation

During the initial attempts to clean up contaminated sites, the use of biological systems did not receive much attention. Bioremediation (the use of living agents, such as microorganisms or plants, and microbial processes to detoxify and degrade environmental contaminants) was not considered an alternative for cleanup of contaminated sites for several reasons: (1) many of the contaminants were considered recalcitrant; (2) lack of research investigating the biochemistry of microbial interactions; and (3) little information was available on the biological processes occurring, so biological systems were thought to be completely uncontrollable and unpredictable (Cookson, 1995). Despite the lack of understanding of the microbial processes taking place, microbes have been used to treat municipal wastes as early as 600 B.C. by the Romans and other ancient civilizations (King *et al.*, 1992). However, the first case which brought the most public attention to the field of bioremediation was the Exxon Valdez oil spill in 1989 on the beaches of the Prince William Sound in Alaska. In this situation, U.S. EPA scientists observed that the oil degradation by the microbes quickly became limited by lack of available nutrients. Application of nitrogen- and phosphorus-based nutrients enhanced the degradation of the oil contamination (Anon, 1990). The technology used to clean up the Exxon Valdez spill has also been applied to oil spills along the Texas Gulf Coast during 1990, resulting in successful remediation of oil spills (General Land Office Report, 1990). These successes have led to increased popularity and use of bioremediation to cleanup a variety of different contaminants.

Pesticide Contamination and Bioremediation

The EPA released a report showing the distribution of bioremediation activities on Superfund sites (those sites which were the U.S. government's priority sites for short- and long-term cleanup programs). Petroleum, creosote, and solvents were the primary compounds which were remediated, with 33, 28, and 22% of the active sites respectively, with pesticides as the fourth most common contaminant in the study with 9% of the total bioremediation activity (U.S. EPA, 1993). With a world pesticide market of \$25.5 billion, the United States' market ranks first with 32% of the world total (Ware, 1994). In the U.S.,

pesticide usage increased steadily throughout the 1960's and 1970's, reaching all-time high levels of approximately 1200 million pounds of active ingredient in the early 1980's. Since that time, the usage level has been holding steady at approximately 1100 million pounds of active ingredient (U.S. EPA, 1991). With the immense number and quantity of pesticide compounds being produced, approximately 25,000 formulated pesticides as of 1991 (U.S. EPA, 1991), and the relatively high recalcitrance of the compounds, the safe disposal of waste and surplus pesticides has become a primary concern in the United States in the last three decades. Because nearly half of all U.S. residents and, in some areas, 95% of rural residents get their drinking water from private or public wells, efforts to prevent pesticides from entering the groundwater are extremely important (U.S. EPA, 1991). Agrochemical dealership sites have been a primary focus due to the potentially high level of contamination resulting from spills and improper storage. Several researchers have noted a strong correlation between pesticide detections in public wells and close proximity of agrochemical/fertilizer dealerships in Iowa, Kansas, Wisconsin, and Illinois (Gannon, 1992; Frieberg, 1991) and in Canada (Frank *et al.*, 1987a; Frank *et al.*, 1987b). In this study I will be using soils collected from several different agrochemical dealerships to examine the biodegradation of atrazine, metolachlor, and trifluralin, which were ranked first, third, and seventh, respectively, in U.S usage in 1990-91 (Ware, 1994). In the state of Iowa, atrazine and metolachlor were the top two herbicides used in corn production, while trifluralin was the herbicide used most prevalently in soybean production in 1990 (Hartzler and Wintersteen, 1991). Physicochemical characteristics of the herbicides alachlor, atrazine, metolachlor, and trifluralin are shown in Figure 1.

Physical and Chemical Factors Affecting Bioremediation

There are several physical and chemical factors, including pH, temperature, oxygen concentration, and water content of soil, that affect the biodegradation rates of contaminants (Moorman, 1994).

Table 1. Physicochemical and biological properties of selected herbicides

	Soil $t_{1/2}$ range	Log K_{oc} range	K_H at 25°C ($\text{atm}\cdot\text{m}^3 \text{mol}^{-1}$)	Water solubility limit (g L^{-1})
Alachlor	1-5 weeks	1.63-2.28	6.12×10^{-8}	242 (at 25°C)
Atrazine	6-10 weeks	1.95-2.71	3.04×10^{-9}	33 (at 27°C)
Metolachlor	6-100 days	2.08-2.49	9.20×10^{-9}	530 (at 20°C)
Trifluralin	132 days	2.94-4.49	4.84×10^{-5}	0.3 (at 25°C)

Physical factors

pH. Soil pH values can affect both the biological and chemical aspects of contaminant degradation. Biologically, the pH can affect the growth and activity of the various groups of soil microorganisms. In general, fungi are more tolerant of low pH (< pH 5) soil conditions than bacteria and actinomycetes (Sims *et al.*, 1993). Optimum growth conditions for most bacteria and actinomycetes occur at neutral (7.0) to slightly alkaline pH values (Cookson, 1995). The pH range normally considered optimal for biodegradation of most contaminants is between 6 and 8, but each chemical has its own pH optimum depending on such factors as the degrading microorganisms, the other environmental conditions, and the contaminant's chemistry (Sims *et al.*, 1993). If the ideal pH for a particular contaminant is known, it can easily be adjusted higher with lime or lower by adding sulfur (Pope and Matthews, 1993). The half-lives for atrazine degradation were lowest at the lowest pH values and increased with increasing pH (Li and Felbeck, 1972).

Temperature. The soil temperature affects the metabolic activity and growth of soil microorganisms. As the temperature decreases, the biodegradation rate of a contaminant will decrease and essentially stops once the temperature reaches 0°C (Sims *et al.*, 1993). The desired temperature for the majority of the organisms that will be degrading the contaminants is between 18°C and 30°C, however individual microbial species will usually be most active only within a very narrow temperature range (Pope and Matthews, 1993). McCormick and Hiltbold (1966) noted that as the temperature was increased each 10°C between 15 to 35°C, the atrazine degradation rate increased two to three-fold. Getzin (1968) noted that the transformation degradation rates for the insecticide diazinon increased as the temperatures were increased in ten degree intervals from 15°C to 35°C.

Oxygen concentration and redox potential. The vast majority of microorganisms responsible for bioremediation of contaminants are aerobic organisms, i.e. they require free oxygen to degrade the compound. Therefore, for optimum degradation of most contaminants, the oxygen concentration must be maintained at a level greater than 0.2 mg L⁻¹ (ppm) for dissolved O₂ or at a minimum air-filled pore space of 10% (Sims *et al.*, 1993). However, once the oxygen in the environment has become depleted, the facultative anaerobic and obligate anaerobic organisms will become the dominant microbes. As the oxygen is depleted and the system becomes more reduced, a corresponding increase in the

electron density results in an increased negative potential. The measurement of the oxygen status and related electron density is the redox potential (Sims *et al.*, 1993). Several of the more recalcitrant and halogenated compounds appear to be the best candidates for anaerobic degradation. Willis *et al.* (1974) showed that there is a critical anaerobic redox potential (Eh) range (+150 to +50 mV) that must be reached to achieve rapid degradation (1 ppm completely degraded in 8 days) of the dinitroaniline herbicide trifluralin. Helling (1976) reviewed several studies and concluded that there was much faster trifluralin loss from anaerobic soil than aerobic soil, but the principal reaction was reduction of the nitro groups and not ring cleavage.

Water content. The water content of soil is of critical importance in determining the extent of microbial metabolic activity. The microbes performing the contaminant degradation rely on the soil water to transport nutrients and organic C sources to the microbe and carry harmful waste products away from the cell (Paul and Clark, 1989). The water also affects the pH, aeration status, type and concentration of soluble materials, osmotic pressure, and the water flow of the soil (Paul and Clark, 1989). Recent experimental and field experience shows that a matric potential of -0.33 to -0.5 bars may be the optimum available soil water content (Sommers *et al.*, 1981). The persistence of the insecticide diazinon increased with decreasing soil moistures, with the insecticide at 2% moisture being the most persistent and the 30% moisture treatment showing the highest degradation percentage (Getzin, 1968).

Chemical factors

Bioavailability. The bioavailability of the contaminant to the microorganisms capable of degrading the contaminant governs the fate of organic contaminants in soil and the subsurface. Bioavailability is defined as the accessibility of organic contaminants for uptake and metabolism by microorganisms, plants, invertebrates, and vertebrates. Decreased bioavailability of the contaminant often results from sorption to the solid surfaces in the environment (Alexander, 1994) and/or entrapment inside the physical matrix of the soil, sediment, or aquifer materials by intragranular diffusion (Wood *et al.*, 1989; Ball and Roberts, 1991). Several factors affect the sorption of organic compounds, including the concentration and type of solutes in the surrounding solution, the type and quantity of clay minerals, the amount and nature of organic matter in the soil or sediment, pH, temperature,

and the characteristics of the specific compound involved (Alexander, 1995). Additionally, several different studies have shown that contaminants become increasingly more resistant with time to extraction and biodegradation. Phenanthrene and 4-nitrophenol were aged in sterilized loam, muck, and sand. The amount of phenanthrene and 4-nitrophenol recovered by butanol extraction decreased with increasing duration of the aging process. As the amount of aging increased, the degree of 4-nitrophenol and phenanthrene mineralization decreased, and the mineralization rates usually became slower in each of the three soil types (Hatzinger and Alexander, 1995). Alachlor degradation was determined over a concentration range of 10 to 10,000 $\mu\text{g g}^{-1}$ (ppm) in a clay loam and a sandy loam. As the concentration of alachlor increased, the relative persistence of the herbicide also increased, indicating decreased bioavailability of the alachlor, saturation of degradation processes, alachlor toxicity, or solubility problems. The mineralization rate and formation of degradation products decreased at higher concentrations, with the 10,000 ppm treated soil having half-lives of approximately 13 years (Gan *et al.*, 1995).

Bioaugmentation

Bioaugmentation is the process in which microorganisms that possess the enzyme systems capable of degrading a particular contaminant are added to a contaminated site to enhance the contaminant biodegradation. Whenever it has been decided that bioremediation is going to be used at a contaminated site, a debate occurs as to whether the site bioremediation plan should manipulate the indigenous microbial species or introduce a single species or consortium of microorganisms that are known to degrade the contaminant of interest (King *et al.*, 1992). Inoculation with organisms may be necessary for several reasons. First, the microorganisms acting on certain pollutants may be absent from a particular site or be present in such low numbers that they are unable to come in contact with the contaminant. Low indigenous populations of degraders may occur in low biomass soils, recently contaminated soils (insufficient time to adapt and grow), or in soils where the toxicity of one or more of the spill components has reduced the degrading populations (Forsyth *et al.*, 1995). The growth of the degrading populations may be suppressed by competition with other indigenous populations for inorganic nutrients or predation by protozoa (Wiggins and Alexander, 1988). Many different types of predators

and parasites, such as protozoa, bacteriophages, myxobacteria, cellular slime molds, and *Bdellovibrio*, exist in soil and water environments and may dramatically suppress the bacterial numbers. Protozoan grazing becomes a major factor when bacterial densities reach 10^6 CFU g^{-1} soil or greater, which can easily happen when a microbial population is degrading a contaminant. If the bacterial growth rate is slower than the protozoa predation rate, the added microorganisms can be severely suppressed or completely eliminated (Gurijala and Alexander, 1990; Sinclair and Alexander, 1989; and Wiggins and Alexander, 1988). Inoculation may also markedly reduce the acclimation period required to attain the ability to degrade the contaminant. Adding a microbial population with known degradative capabilities can be used to start the remediation process with little or no lag period (Alexander, 1994; King *et al.*, 1992; Bewley, 1992).

One of the primary reasons for the failure of the augmentation strain upon addition to soil and ground water for remediation of hydrocarbon contaminants is the inability of the strain to compete with the indigenous microbes for the C, N, P, K, and O_2 sources available in the habitat the organism is occupying. Often one or more of these nutrients is limiting at the remediation site and the energetic demands placed on the augmentation organism during the degradation of the contaminant will deplete the essential nutrient(s) present in the environment. Hydrocarbon degraders are widely distributed in the environment and addition of inorganic nutrients and O_2 will enhance degradation of these contaminants, therefore inoculation with an augmentation organism is usually ineffective. However, for other more recalcitrant compounds, such as pesticides and PCBs, inoculation may be advantageous because the compounds may not support growth of the inoculation organism due to low concentrations or cometabolic degradation.

If the contaminant is initially present at or metabolized to a concentration that is similar to or lower than its K_s value for the contaminant, the organism will grow slowly and eventually be outcompeted by the indigenous organisms (Alexander, 1994). The K_s is a kinetics growth constant that represents the substrate concentration at which the rate of growth is half the maximum rate for a particular growth substrate. Additional chemical and physical factors such as the temperature, pH, and salinity of the environment or the presence of toxic substances may result in death or suppression of the degradative capacity of the augmentation organism. These factors can be overcome by using similar conditions

to the remediation site when performing the enrichment for the augmentation organism (Alexander, 1994).

Introduced species often outperform the indigenous microorganisms in bioreactor and some land treatment technologies. This may not always be the case, however, especially in situations where the contaminants are particularly recalcitrant or are present in extremely high concentrations. A thorough bioassessment screening and biotreatability study should be conducted to determine if bioremediation can be utilized at a particular site. Soil bioassessment screenings usually include measurement of pH, metal content (such as Fe, Cu, and Mn), nutrient content (N, P, K), specific organic C contaminant concentration, and the total organic C content, along with microbiological measurements such as the microbial nutrient content, numbers of total heterotrophic bacteria and specific contaminant degraders, and shake-flask biodegradation testing measuring oxygen or contaminant consumption under various conditions. Biotreatability studies are conducted to determine the general rate of degradation of the target contaminant (King *et al.*, 1992; Cookson, 1995).

The record of successes in enhancing biodegradation of organic contaminants through the addition of microorganisms to soils, aquifers, and surface waters *in situ* is inconsistent. However, attempts to bioaugment soil for enhanced pesticide degradation appear to improve the overall rate of degradation in most cases. I will describe some of the more notable bioaugmentation success stories involving degradation of pesticides.

Biodegradation of contaminant as a C or energy source

Whenever a contaminant is degraded as a source of carbon or energy, a percentage of the carbon in the contaminant is assimilated into microbial biomass. The percentage assimilated varies for the microorganism and the compound being degraded, with higher values indicating more efficient microbes (Alexander, 1994). A portion of the carbon is mineralized (converted to CO₂) during the biodegradation process. The carbon that is assimilated is later mineralized as the cells themselves are decomposed or consumed by protozoa and other predators (Alexander, 1994).

Atrazine. Few isolated microorganisms have shown the ability to degrade atrazine as a sole C source. A *Pseudomonas* strain was isolated from a mixed culture growing on atrazine as a sole C source (Yanze Kontchou and Gschwind, 1994). Less than 0.1% of the

^{14}C added as ^{14}C -UL-*ring*-atrazine was incorporated into the cells, indicating that the cells are utilizing only the side chain C for growth and energy. The strain was capable of mineralizing 54% of the ^{14}C added. The ring carbons are completely oxidized, and therefore are unable to be assimilated. In C-limited medium, only about 10% of the triazine ring N was assimilated into biomass, indicating that the organism is using the atrazine almost solely as a C, not a N source. This *Pseudomonas* strain was added into several soils treated with 10 ppm atrazine. The bacterium was capable of reducing the half-life of atrazine in one of the soils from 57 to 3.4 days, while mineralizing about 60% of the atrazine in 50 days. The ability of the isolate to degrade atrazine was reduced at lower soil water contents, in soils with pHs lower than 7.0, and under oxygen-limited conditions (Yanze Kontchou and Gschwind, 1995).

Metolachlor and alachlor. Resting cells of the fungus *Chaetomium globosum* are capable of transforming metolachlor into at least eight different metabolites without having an alternate C source provided in the medium (McGahan and Tiedje, 1978). These results indicate that the organism is able to metabolize metolachlor as a sole C source. The general transformations involved in the degradation reactions were dehalogenation or removal of the methoxy or ethoxy substituent by hydroxylation, dehydrogenation of the 6' ethyl group, dealkylation, demethoxylation, and indoline or oxyquinoline formation. Of seven fungal isolates screened for alachlor degradation ability, only *Chaetomium globosum* showed extensive and rapid alachlor degradation (Tiedje and Hagedorn, 1975). No differences in alachlor degradation rates were observed when the fungus was incubated in either phosphate buffer or potato dextrose broth supplemented with alachlor, indicating that an additional C source was not required to stimulate alachlor degradation.

Other contaminants used as C source. Many researchers have isolated microbes capable of degrading the phenoxyacetic acid herbicides, 2,4-D and 2,4,5-T, and extensive research has been performed to characterize these organisms. A 2,4-D-degrading *Xanthobacter* sp. CP was isolated after enrichment in aerated soil columns. This organism grew on the C in the 2,4-D side-chain and several other chlorinated phenoxyalkanoic acids with an even number of C atoms in the side chain (Ditzelmüller *et al.*, 1989). Comeau *et al.* (1993) studied the effect of *Pseudomonas cepacia* BRI6001 inoculum preparation and density on the rate of 2,4-D degradation in nonsterile soil. As the inoculum density was increased by a factor of ten, the time taken to obtain complete 2,4-D degradation was

reduced by 0.5 day. At the inoculum levels, 10^8 , 10^6 , and 10^4 CFU g^{-1} soil, the nearly identical (55, 52, and 48%) amounts of mineralization indicated that the differing lag times among the treatments (0, 1, and 2 days) could be attributed to the time required to grow a sufficient BRI6001 population density to mineralize 2,4-D (Comeau *et al.*, 1993). Several researchers have studied the degradation kinetics and the genetic regulation of the genes that degrade 2,4-D (Ditzelmüller *et al.*, 1989; Greer and Shelton, 1992; Short *et al.*, 1991). Greer and Shelton (1992) monitored the degradation rates of two bacterial strains with similar maximum growth rates (μ_{max}), but very different half-saturation growth constants (K_s). In a low organic matter soil, 2,4-D degradation rates were similar when applied at a rate of 10 and 100 $\mu g g^{-1}$ wet soil, but at only 1 $\mu g g^{-1}$ wet soil, the 2,4-D was degraded much more rapidly by the bacterial strain with the lower K_s value. Short *et al.* (1991) monitored degradation of 2,4-D in a soil with and without a genetically engineered organism (GEM), *Pseudomonas putida* PPO301 (pRO103), observed that degradation only occurred in the soil in which the organism was inoculated. The GEM degraded 500 $\mu g g^{-1}$ of 2,4-D to less than 100 $\mu g g^{-1}$ in 50 days and approximately 70 $\mu g g^{-1}$ of the metabolic product 2,4-dichlorophenol accumulated for a 5 weeks during the 2,4-D degradation period. The parent *Pseudomonas putida* PPO301 was incapable of 2,4-D degradation. Ditzelmüller *et al.* (1989) determined that the maximal growth rate for the 2,4-D degrading *Xanthobacter* sp. CP growing on 2,4-D was 0.13 h^{-1} , with a growth yield of 0.1 g biomass g^{-1} 2,4-D. Introduction of a 2,4,5-T degrading strain of *Pseudomonas cepacia* into test tubes of soil containing 1.0 g of 2,4,5-T kg^{-1} of soil resulted in degradation of a substantial amount of the herbicide as measured by bioassays with sensitive plants (Karns *et al.*, 1984).

A variety of other compounds have been shown to be utilized by microorganisms as a C or energy source. Daughton and Hsieh (1977) isolated a mixture of bacteria (*Pseudomonas stutzeri* and *Pseudomonas aeruginosa*) that degraded greater than 90% of 5 ppm parathion within 3 weeks in 10-g soil samples. Parathion degradation in soil was also enhanced when the soil was inoculated with an adapted two-member degrading culture (Maloney, 1988). 3,5,6-Trichloro-2-pyridinol (TCP), a metabolite resulting from the degradation of the insecticide chlorpyrifos and the herbicide trichlorpyr, was rapidly mineralized by *Pseudomonas* sp. strain M285 (Feng and Bollag, 1996). Removal of 80% to 100% of TCP from the wastewater of chlorpyrifos-manufacturing plants was achieved when it was passed through columns of M285 immobilized onto diatomaceous earth beads (Feng

and Bollag, 1996). Destruction of the herbicide chlorpropham occurred upon addition of either of two chlorpropham-degrading *Pseudomonas* species to 25-g quantities of soil (Milhomme *et al.*, 1989). Three fungal isolates, *Phanerochaete chrysosporium*, *Bjerkandera adusta*, and *Trametes versicolor*, showed enhanced degradation of the insecticide chlordane over the abiotic control after 90 days, with 42, 42, 60, and 29% of the chlordane degraded, respectively (Field *et al.*, 1995). Extensive degradation of chlordane and lindane in soil-corn cob matrices inoculated with the fungus *Phanerochaete chrysosporium* was observed, with 9 and 23% mineralization occurring in 60 days (Kennedy *et al.*, 1990). Other insecticides such as heptachlor, dieldrin, aldrin, and mirex were not degraded to any measurable degree by this fungus. Pentachlorophenol degradation by indigenous microbes occurred, but higher rates were achieved when the soil was inoculated with an *Arthrobacter* sp. (Edgehill and Finn, 1983). High concentrations of pentachlorophenol, up to 600 ppm in soil, were completely mineralized by the *Arthrobacter* sp. within 1 month (Edgehill, 1995). Addition of eight different fungal bioaugmentation agents to soils contaminated with petroleum hydrocarbons resulted in a 56 to 88% reduction in the total petroleum hydrocarbon (TPHC), compared to only a 16% reduction in the unamended soil control (McGugan *et al.*, 1995).

Biodegradation of a contaminant as an inorganic nutrient source

Microorganisms require N, P, K, S, and a variety of other elements in trace amounts to survive and multiply. It has been shown that xenobiotic compounds can be degraded to satisfy these requirements. The microorganisms commonly convert the organic complex of the compound to a simpler, inorganic form prior to incorporating it into cellular biomass (Alexander, 1994).

Atrazine. As a result of spillage at herbicide-loading and mixing sites and subsequent runoff and leaching, atrazine has been detected in groundwater at concentrations exceeding the Environmental Protection Agency's (EPA) maximum contaminant level of $3 \mu\text{g L}^{-1}$ (Parsons *et al.*, 1988; Belluck *et al.*, 1991). Due to the widespread usage and resulting groundwater contamination concerns, more research has been conducted to determine the environmental conditions which are conducive to atrazine degradation by microorganisms in the soil and groundwater and to learn more about the organisms that degrade atrazine as a C and/or N source.

Most of the atrazine-mineralizing isolates or consortiums have been isolated from N-limited enrichment cultures of soil with prior exposure to atrazine. Three *Pseudomonas* and two *Klebsiella* strains were isolated from N-limited enrichment cultures inoculated with either soil or municipal sewage and several different s-triazines as an N source. Each of these isolates was able to grow on several of eight different s-triazines (Cook and Hutter, 1981). An atrazine-mineralizing consortium was isolated from soil and added back into soil treated with 0.14 mM atrazine (Assaf and Turco, 1994a). The consortium completely degraded the parent atrazine, with 60% of the ^{14}C recovered as $^{14}\text{CO}_2$ and 30% as hydroxyatrazine. An organism capable of utilizing atrazine as the sole source of N and C and of mineralizing atrazine was isolated from an agricultural soil previously exposed to herbicide spills. In medium with atrazine as the sole source of C and N, approximately 40% of the radioactivity was recovered as $^{14}\text{CO}_2$ by 100 hours. About 50% of the radioactivity was recovered as $^{14}\text{CO}_2$ in the same time period when the cells were grown in N-limited medium containing atrazine and glucose (Radosevich *et al.*, 1995). A *Pseudomonas* sp. capable of mineralizing 80% of 100 ppm atrazine in 150 minutes was isolated in an N-limited enrichment culture from a herbicide spill site. Addition of the degrader to soil contaminated with 1500 ppm aged atrazine residue resulted in degradation of 250 ppm atrazine. When sodium citrate and the degrader were added to the contaminated soil, a decline of 70% from the initial concentration of 1500 ppm was observed (Mandelbaum *et al.*, 1995). An atrazine-mineralizing bacterial consortium, consisting of *Pseudomonas* sp., *Clavibacter michiganese*, and *Cytophaga* sp., was isolated from a soil with prior long-term exposure to atrazine. The consortium was capable of mineralizing 80% of 33 ppm atrazine in 48 hours and could also degrade the s-triazine herbicides propazine, prometryne, and atratone. Addition of the consortium into soil spiked with 1000, 500, and 100 ppm atrazine resulted in 60, 69, and 82% atrazine mineralization in 28 days (Alvey and Crowley, 1996).

Other contaminants used as inorganic source. Bacteria isolated from soils with prior exposure to the s-triazine herbicides ametryne and prometryne were capable of utilizing the methylthio group of these herbicides as a S source (Cook and Hütter, 1982). The sulfate-reducing bacterium *Desulfovibrio* sp. (B strain), isolated from an anaerobic digester, was capable of using 2,4,6-trinitrotoluene (TNT) as a sole source of nitrogen (Boopathy and Kulpa, 1992). When lactate was provided as the electron donor and sulfate as the electron acceptor, growth of the organism using TNT as the sole N source occurred

after a lag phase of approximately five days. When sulfate was absent from the medium, TNT was utilized as the sole N source and electron acceptor for growth of the organisms with a lag time of approximately three days, and with 100% removal of the TNT after eight days incubation. The isolate converted the TNT to diaminonitrotoluene and to toluene by reductive deamination, but no CO₂ was made. *Achromobacter* sp. was capable of cometabolically degrading the pesticide carbofuran for a sole N source when glucose was added as a C source (Cookson, 1995).

Members of the family *Rhizobiaceae*, including many *Rhizobium* and *Agrobacterium* strains, were able to grow on the herbicide glyphosate as the sole source of phosphorus in the presence of aromatic amino acids, although growth on glyphosate was not as rapid as on inorganic phosphate. The initial cleavage of the glyphosate was at the C-P bond achieved by a C-P lyase activity (Liu *et al.*, 1991).

Cometabolic biodegradation of the contaminant

Cometabolic transformation of a contaminant results when a microorganism transforms the contaminant, but is unable to use the substrate as a source of energy or to utilize one of the contaminant's constituent elements to produce biomass (Alexander, 1994). Most of the cometabolic reactions involve a single enzyme, resulting in hydroxylations, oxidations, denitrations, deaminations, hydrolyses, acylations, or cleavages of ether linkages (Alexander, 1994). Several ideas have been proposed to explain why cometabolism occurs. One thought is that the initial enzyme(s) converts the organic compound to a product which is not further converted to a metabolic intermediate used for biosynthesis or energy production within a cell. The initial compound may also be transformed to a product(s) that acts to inhibit late enzymatic activity which may result in mineralization or further degradation of the compound. A final explanation could be that the organism is missing a second substrate that is necessary in order to bring about a particular reaction that further degrades the compound (Alexander, 1994).

Trifluralin. Trifluralin is a member of the dinitroaniline family of herbicides. It was first developed in 1963 for use on cotton, but the registration has since expanded for use on more than 50 crops. It has been estimated that about 60% of the trifluralin is used on soybeans, 30% on cotton, and 10% on other crops (Helling, 1976). Nearly 75% of the use in the United States is in the North-central and South-central states, including Illinois, Iowa,

and Mississippi (Helling, 1976). The high Henry's law constant value (K_H) of 4.84×10^{-5} atm $m^3 \text{ mol}^{-1}$ at 23°C and high vapor pressure value of 1.1×10^{-4} mm Hg at 25°C indicate that volatilization is a significant mode of trifluralin loss from soil (Montgomery, 1993). Trifluralin has a very low water solubility of 0.3 ppm and a high range of log K_{oc} values (2.94 to 4.49 in a variety of soil types), indicating strong adsorption to soil and minimal leaching potential (Helling, 1976; Montgomery, 1993). Due to this strong adsorption to soil, bioavailability of the herbicide to organisms capable of degrading trifluralin becomes an issue. Nelson *et al.* (1983) showed that after six months incubation, 14% of the [^{14}C]-trifluralin applied was bound to the nonsterile soil, with 46, 12, 12, and 30% of the herbicide associated with the acetone:H₂O:HCl-soluble, fulvic acid, humic acid, and humin soil organic matter fractions, respectively. After sterilization of the soil, the herbicide degradation was decreased by 31% and the portion of the radioactivity associated with the fulvic acid, humic acid, and humin fractions was reduced by approximately 50% in the same time period, indicating a strong involvement of microorganisms in the fractionation of trifluralin residues in soil. Kearney *et al.* (1976) noted that the percentage of trifluralin that was unextractable or "bound" to the soil increased with time, stating that at 3, 5, and 7 months post-application 14, 24, and 25%, respectively, of the applied ^{14}C was "bound" to the soil. An increase in bound ^{14}C -trifluralin over time was also observed by Wheeler *et al.* (1979), with more trifluralin being bound in Webster soil than Cecil soil.

Despite trifluralin's susceptibility to chemical and physical degradation processes in soil and water, several organisms capable of degrading trifluralin have been isolated. These organisms degrade the trifluralin by different mechanisms in aerobic and anaerobic conditions. The aerobic organisms have been reported to N-dealkylate the side chains, reduce the nitro substituents to amine groups, and then form cyclized products (Golab *et al.*, 1979; Probst *et al.*, 1967). The N-dealkylation reactions are thought to be catalyzed by mixed function oxygenases (Laanio *et al.*, 1973). Anaerobic degradation proceeds with reduction of the side chains prior to N-dealkylation (Probst *et al.*, 1967). Only minimal amounts of trifluralin mineralization have been measured in various soils, indicating that trifluralin is usually degraded into a variety of metabolite(s) and rarely completely degraded (Messersmith *et al.*, 1971; Smith and Muir, 1984; Wheeler *et al.*, 1979). The fungus *Aspergillus fumigatus* utilized dinitramine-degrading enzymes in the presence of the cofactors NADPH and ferrous (Fe^{2+}) ion at pH 7.0 and 33°C to convert dinitramine to N^3 -

ethyl-2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine (Laanio *et al.*, 1973). Hamdi and Tewfik isolated a *Pseudomonas* sp. capable of degrading trifluralin in liquid culture (Hamdi and Tewfik, 1969b). Carter and Camper (1975) obtained 72 bacterial isolates, including 8 *Pseudomonas* spp., from soil enrichment cultures capable of degrading trifluralin. Several fungal isolates, *Aspergillus carneus*, *Fusarium oxysporum*, and *Trichoderma viride*, were shown to degrade 91, 93, and 97%, respectively of 200 ppm trifluralin in liquid culture (Zayed *et al.*, 1983). A *Streptomyces* sp. isolated from soil enrichment culture was capable of degrading 2,6-dinitro-4-(trifluoromethyl)benzenamine, a degradation product of trifluralin, into 5 different metabolic products: 3-nitro-5-(trifluoromethyl)-1,2-benzenediamine; N-[2-amino-5-(trifluoromethyl)phenyl]acetamide; N-[2-amino-3-nitro-5-trifluoromethyl)phenyl]methanesulfinamide; a diphenyldiazene; and a diphenyldiazene oxide (Lusby *et al.*, 1980).

Metolachlor and alachlor. Metolachlor and alachlor are both members of the acetanilide herbicide family. Metolachlor has been reported to be more persistent than alachlor (Zimdahl and Clark, 1982), with half-lives of metolachlor in soil ranging from 15 to 50 days (Saxena *et al.*, 1987). The soil half-life range for alachlor was 5.7 to 23 days (Montgomery, 1993). Both compounds have relatively high water solubilities, metolachlor at 530 ppm at 20°C and alachlor at 242 ppm at 25°C (Montgomery, 1993), which lead to potential leaching into the groundwater if microbial degradation and sorption do not occur rapidly. Prior to 1995, several different microorganisms had shown the ability to transform alachlor and metolachlor to metabolites, but were either unable to mineralize the ring or mineralized less than 5% of the two herbicides (Smith and Phillips, 1975; Tiedje and Hagedorn, 1975; Liu *et al.*, 1991; Pothuluri *et al.*, 1993; Liu *et al.*, 1989; McGahen and Tiedje, 1978). This type of degradation is indicative of cometabolic degradation of a compound and has been attributed to steric hindrance caused by the tertiary amine side-chain or the *ortho* position substitutions that could interfere with the enzymatic attack on the amide bond (Saxena *et al.*, 1987; Villarreal *et al.*, 1994). Ferrey *et al.* (1995) demonstrated the ability of the lignin-degrading white-rot fungi, *Ceriporiopsis subvermispora* and *Phlebia tremellosa*, to mineralize 14 and 12% of 18 µg ml⁻¹ alachlor in 122 days when grown in a 1.5% malt extract medium supplemented with *Populus tremuloides* (aspen) wood chips, while *Phanerochaete chrysosporium* mineralized approximately 6% of the ¹⁴C in the same time period. *Fomitopsis pinicola* was unable to mineralize alachlor, but did transform 92% of the alachlor to unidentified metabolites when grown in the same medium.

A screening of several microorganisms for the ability to mineralize or degrade metolachlor showed that two fungal isolates, *Fusarium* sp. and *Mucor racemosus*, one actinomycete, and two bacteria, *Bacillus circulans* and *Bacillus megaterium*, were capable of transforming metolachlor to metabolic products, but were unable to mineralize metolachlor (Saxena *et al.*, 1987). All of the isolates required an additional C source to cometabolically degrade metolachlor. The *Fusarium* sp. was capable of growing and transforming metolachlor when present up to a concentration of 300 ppm, however the actinomycete's metolachlor tolerance could not be raised above 200 ppm even when the medium sucrose concentration was doubled or a large biomass of inoculum was used. The actinomycete described by Saxena *et al.* (1987) was isolated from a soil enrichment culture with sucrose as the additional C source and was capable of degrading metolachlor to eight metabolites (Krause *et al.*, 1985). This organism used N-alkyl and alkyl side chain hydroxylation reactions and/or demethylation to produce these metabolites. All of the metabolites possessed chlorine atoms, indicating that the organism was incapable of dehalogenation of the chloroacetyl sidechain. A study by Smith and Phillips (1975) also demonstrated cometabolic degradation of alachlor by *Rhizoctonia solani*. Addition of increasing rates of sucrose to the growth medium containing the alachlor resulted in an increase in the degree of alachlor degradation. This indicates that *R. solani* does not utilize alachlor as a sole C source and that the degradation of alachlor is dependent on the presence of some other source of C. Dechlorination of metolachlor by *Phanerochaete chrysosporium*, *Rhizoctonia praticola*, *Streptomyces* sp., and *Syncephalastrum racemosum* was studied in a medium with 0.35 mM metolachlor and the alternate C sources yeast extract and sucrose (Liu *et al.*, 1991). Seven dechlorinated metabolites were produced by dehalogenation and demethylation, followed by hydroxylation of the chloroacetyl group and ring formation between the acetyl group and the benzylic ethyl side chain.

Glutathione-S-transferases (GST) are a group of detoxification enzymes which catalyze the conjugation of reduced glutathione with a variety of electrophilic compounds (Feng, 1991). These enzymes catalyze the reactions of potentially toxic carcinogenic xenobiotic compounds, such as the acetanilide herbicides alachlor, metolachlor, and propachlor and the fungicide pentachloronitrobenzene, with the -SH group of the cysteine residue of glutathione leaving the electrophilic sites neutralized and more water soluble

(Cohen *et al.*, 1986; Feng 1991; Lamoureux and Rusness, 1989). Conjugation of glutathione with these pesticides results in formation of a peptide conjugate with the elimination of chloride. The glutathione conjugates are thought to be further metabolized by cleavage of the glutamate and glycine residues from glutathione, followed by acetylation of the free amino group of the cysteinyl residue to produce mercapturic acid (Lau *et al.*, 1980; Habig *et al.*, 1974). GST have been found in a variety of organisms including mammals, birds, reptiles, amphibians, fish, plants, insects, yeasts, algae, protozoa, fungi, and bacteria (Lau *et al.*, 1980; Shishido, 1981). Zablutowicz *et al.* (1994) demonstrated the presence of the GST pathway for alachlor dechlorination in several Gram-negative rhizosphere bacteria including *Pseudomonas*, *Enterobacter*, and *Klebsiella* species. Lau *et al.* (1980) screened a variety of organisms, including bacteria, protozoa, fungi, and algae, and found the greatest amount of GST activity in the protozoa (569 to 1958 nmol of 1-chloro-2,4-dinitrobenzene (CDNB)-GSH conjugate formed $\text{min}^{-1} \text{mg protein}^{-1}$), followed by the algae (4 to 102) and fungi (not detected to 117), and only a small amount of GST production in the bacteria screened (not detected to 3). The presence of GST was demonstrated by an enzyme assay with cell extracts from the fungus *Cunninghamella elegans* using 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates (Wackett and Gibson, 1982). The enzyme assay results indicated that two different types of GST may exist in the *C. elegans* cell extracts. Shishido (1981) demonstrated the presence of low levels of the GST enzyme in *Escherichia coli*, with a CDNB substrate assay activity of 3.78 CDNB-GSH conjugate formed $\text{min}^{-1} \text{mg protein}^{-1}$.

Zablutowicz and Dzantor (1994) showed that the fungus *Mucor* SSF1 possessed the ability to enhance degradation of alachlor and trifluralin when added into soil. Additionally, *Pseudomonas fluorescens* strain UA5-40 was able to degrade alachlor by using GST enzymes to conjugation alachlor to glutathione forming glutathione conjugate metabolites. The *Pseudomonas* strain is capable of completely dechlorinating 100 μM alachlor within 48 hours (Zablutowicz *et al.*, 1994). Addition of this strain to soil contaminated with alachlor resulted in faster degradation than occurred in the uninoculated control soil (Zablutowicz and Dzantor, 1994). These researchers also demonstrated that biostimulating substrates significantly increased the amount of growth of the soil microbial populations as well as the growth of the bioaugmentation organisms.

In Chapter 2 of this document, I will describe the atrazine degrader, *Agrobacterium radiobacter* strain J14a, which is capable of degrading 50 ppm of atrazine in approximately 72 hours in N-limited liquid culture. Addition of J14a into two different soils spiked with atrazine resulted in faster mineralization rates, reduced lag times, and enhanced atrazine degradation over the unamended control soils.

Biostimulation

Microbial growth and metabolism depends upon adequate supplies of essential macro- and micro-nutrients. Crucial nutrients for microbial growth, such as nitrogen and phosphorus, must be available to microorganisms in a usable form at the proper concentrations and ratios. The suggested C:N:P ratio for microbial growth is 100:10:1. If a soil contaminant has high carbon levels and a low nitrogen and phosphorus content, microbial biodegradation will cease once the available N and P levels are exhausted (Sims *et al.*, 1993). A small amount of potassium and sulfur are also needed for biodegradation. Therefore additions of N, P, K, and S alone or in combinations may be necessary in order to achieve degradation of a contaminant used as a C and energy source by the microorganisms in the soil.

Use of contaminant as an inorganic nutrient source

The process of adding either a C/energy source or inorganic amendment (N, P, K, or S) to soil to enhance the indigenous microbial degradation of a contaminant is termed biostimulation. If the contaminant is used as a source of inorganic nutrients, the microbial C sources could be the limiting factor in the contaminant degradation process. One example of this situation is the use of the s-triazine herbicide atrazine as an N source by certain atrazine degrading organisms. Several researchers have isolated microorganisms that are capable of utilizing the N in atrazine to utilize the ring N when additional C sources are added to the medium (Assaf and Turco, 1994b; Mandelbaum *et al.*, 1993, 1995; Radosevich *et al.*, 1995; Cook, 1987; Cook and Hutter, 1981). To determine how the C/N ratio and complexity of organic amendments affect atrazine mineralization, a soil with a history of atrazine exposure was spiked with 100 ppm atrazine and amended with a variety of organic amendments, including sodium citrate, glucose, starch, rice hulls, Sudan hay, compost,

rice-hull-water-extract, or compost-water-extract at a rate of 5% (Alvey and Crowley, 1995). The unamended control soil mineralized 73% of the atrazine after 11 weeks. Soil amended with rice hulls, starch, and compost mineralized 88, 75, and 59% of the atrazine, respectively in the 11-week time period. The two high C content amendments, rice hulls and starch, that would be predicted to be more difficult to degrade, resulted in enhanced atrazine mineralization over the unamended control soil, indicating that these two more recalcitrant amendments activated the atrazine-mineralizing portion of the soil biomass. Compost, which contained a higher N content than the other amendments added, caused inhibition of atrazine degradation from the control unamended soil, indicating that organic N sources may repress atrazine mineralization. However, the soil treated with glucose, Sudan hay, or sodium citrate mineralized <10% of the atrazine. These amendments with a high C content and little or no N caused extreme repression of atrazine mineralization for some reason which has not been explained. All treatments receiving inorganic N as $\text{Ca}(\text{NO}_3)_2$ (250 mg N 100 g⁻¹ soil) had a dramatically reduced rate of atrazine mineralization compared to the corresponding treatment without N addition. This indicates that the microorganisms are most likely utilizing the atrazine for an N source and mineralization repression was observed when a more readily utilizable inorganic N source is provided for the atrazine-mineralizing microorganisms.

Addition of C as mannitol or N as urea at varying rates to soil spiked with atrazine resulted in no difference in the amount of atrazine mineralized after 326 days (Assaf and Turco, 1994b). However, a second addition of mannitol at day 140 of the incubation resulted in a 17% increase in the amount of atrazine mineralized, suggesting that the soil microbes are using atrazine as a N source and the soil may have been C limited. The additional C will allow for a larger biomass to be formed, using the mannitol for a C source and the atrazine as a N source.

Use of contaminant as a C or energy source

The degradation of many different contaminants utilized as a C or energy source can be enhanced through biostimulation. Addition of high concentrations (500, 5,000, and 20,000 µg g⁻¹ soil) of [¹⁴C]-2,4-D to Webster soil, resulted in about 95, 80, and 60% mineralization, respectively (Davidson *et al.*, 1980). However, when the same concentrations of 2,4-D were added to Cecil soil only minimal mineralization of the [¹⁴C]-2,4-

D was observed (about 7, 0.2, and 0.2%). To increase the amount of 2,4-D mineralized in the Cecil soil, the researchers attempted to biostimulate the soil microorganisms by adding easily degradable substrates such as 1% yeast extract, 1% glucose+0.5% urea, and 2% cow manure. Only a small increase in mineralization occurred with either of these treatments, with the cow manure providing the largest 2,4-D mineralization increase from 0.2 to only 0.5% mineralization (Davidson *et al.*, 1980). These results indicate that the Cecil soil may not contain the microbial populations which possess the enzymes necessary to degrade 2,4-D or that the 2,4-D degraders were not stimulated by the amendments chosen. Another possible reason for the amendment failure is that the Cecil soil could be deficient in an inorganic nutrient needed by the 2,4-D degrading population, such as N, P, K, or S.

A study was performed to determine how biostimulation with organic and inorganic amendments effected degradation of *p*-nitrophenol, toluene, and ethylene dibromide (Swindoll, 1988). Addition of vitamins, nitrogen, phosphorus, mineral salts, and amino acids to Lula aquifer solids resulted in a much more rapid degradation rate and an increase in the mineralization of 106 ng g⁻¹ *p*-nitrophenol (PNP) from about 14% to 35%. However, addition of inorganic nutrients to soil treated with toluene and ethylene dibromide significantly decreased the percent of each compound mineralized. Addition of organic amendments, glucose or amino acids, resulted in less mineralization and longer lag times before degradation of the toluene and ethylene dibromide began than the unamended control.

A variety of different organic amendments were added to a soil contaminated with petroleum hydrocarbons (McGugan *et al.*, 1995). These amendments included straw (*Eragrostis* sp.), poultry manure, maize cobs, pine bark, barley grain, and barley beer waste. Four of the amendments (straw, poultry manure, maize cobs, and pine bark) were successful in reducing the total petroleum hydrocarbon (TPHC) concentrations by a range of 68% to 80% TPHC reduction, compared to the unamended, nonsterile control soil value of 44%. In contrast, the other two amendments, the barley grain and the barley beer waste, reduced TPHC only 43% and 54%, respectively. These low degradation extents may indicate that inorganic nutrients (N, P, and K) may be limited in the soil, therefore reducing the amount of degradation of the hydrocarbon contamination by the indigenous microbial populations.

Addition of phosphate, nitrate, sulfate, or chloride at 10 mM to lake water resulted in a decreased acclimation period over the unamended lake water for the mineralization of low

concentrations of *p*-nitrophenol (PNP), with lag times of 5, 8, 8, 12 and 13 days, respectively (Jones and Alexander, 1988). No mineralization was observed by 16 days after addition of CO_3^{2-} . Addition of a combination of N and P did not further decrease the acclimation period from the time with addition of P alone. The researchers determined that the added P resulted in a larger amount of growth of the PNP mineralizing populations present in the lake water.

Cometabolic degradation of contaminant

In cometabolism, the organisms are unable to utilize the target substrate's constituent elements nor use it as a source of energy (King *et al.*, 1992; Alexander, 1994). Alachlor was degraded cometabolically upon addition of corn and soybean stubble and an inorganic solution of NH_4NO_3 to an alachlor-contaminated soil (Felsot and Dzantor, 1990). After 56 days of incubation with 100 ppm alachlor, only 6 and 17% of the alachlor remained in the cornstalk and the soybean+N-amended soils respectively, while 60% remained in the unamended control. In the soybean amended soil, the degradation was slower than in the cornstalk amended soil, with 36% still remaining after 56 days. The dehydrogenase activity for the cornstalk and soybean+N treatments showed higher levels of activity than the unamended soil, with the soybean amended soil showing repressed levels from the unamended soil. Spiking with 1000 ppm alachlor resulted in 16% degradation of alachlor. All of the 1000 ppm treatments showed severely repressed dehydrogenase activity (Felsot and Dzantor, 1990).

Lindane, a highly chlorinated cyclic-aliphatic insecticide, was shown to be very persistent under aerobic conditions, persisting in soil up to 11 years (MacRae, 1969). This insecticide has been shown to be degraded cometabolically under anaerobic conditions by several different organisms only upon addition of either alanine, leucine, pyruvate, leucine-proline mixtures, formate, or glucose. These compounds serve as electron donors for the reduction of lindane (Cookson, 1995).

Biodegradation of pesticide mixtures

Several researchers have looked at the affect of adding various inorganic and organic amendments on biodegradation. Winterlin *et al.* (1989) collected soil cores from a highly contaminated California pesticide waste evaporation pit and uncontaminated soil from

an area 10 m from the pit and added atrazine, trifluralin, malathion, diazinon, chlorpyrifos, and parathion. They exposed the herbicide-fortified soil to various soil amendments, including organic amendments (peat, manure and corn meal), lime, acids, inorganic nutrients, and varying soil moistures in an attempt to enhance degradation of the added pesticides. The soil samples treated with manure, ammonium phosphate, and lime were very effective in reducing the half lives of the pesticides in both aerobic and anaerobic soils, compared to corn meal- and ammonium phosphate-treated soils. They concluded that for soils that are highly contaminated with a mixture of pesticides, on-site treatment with high concentrations of nutrients and lime, along with an organic source such as peat or manure, alternated between flooded and moist soil conditions should provide an acceptable procedure for reducing the pesticide concentrations.

Schoen and Winterlin (1987) investigated the effects of several soil factors on the biodegradation of mixtures of pesticides. Atrazine, captan, carbaryl, 2,4-D, diazinon, fenitrothion, and trifluralin degradation was studied by varying pH, moisture content, organic matter content, microbial activity, and pesticide concentration. They concluded that pesticide concentration was the most important factor affecting pesticide degradation, with degradation rates much slower at high (1000 ppm) levels than moderate (100 ppm) levels of the various pesticides. Atrazine degradation was optimum at low concentrations in a soil that contained a relatively large amount of organic matter.

In 1993, Dzantor *et al.* combined the techniques of landfarming and biostimulation to evaluate remediation of both low and high levels of pesticide contamination, involving mixtures of alachlor, trifluralin, metolachlor, and atrazine. The amendments applied were 2.5% sewage sludge and 5% corn meal. They also looked at persistence differences between aged residues from a contaminated site and freshly applied herbicides. Soil amendment with organic material greatly stimulated the dissipation of freshly applied alachlor, metolachlor, and atrazine, but had only a limited effect on the aged residues. Trifluralin degradation in the corn meal-amended soils was approximately 15% higher than the control soils under aerobic conditions. In all cases the freshly applied herbicide mixture degraded more rapidly than the aged residue treatments, but the degree of difference varied among the treatments. This demonstrates the necessity to begin remediating herbicide spills as soon as possible. Zablotowicz and Dzantor (1994) showed that biostimulation by addition of organic amendments (chicken litter and corn meal) and

moisture saturation had significant effects on trifluralin degradation. Only 11% of the initial trifluralin was recovered after 30 days in the saturated, chicken litter-amended treatment, compared to 68% and 83% in the unsaturated and the saturated, unamended treatments, respectively. Addition of cornmeal to soils contaminated with alachlor showed enhanced degradation over the unamended soil.

In order to determine the effects of herbicide spills on microbial activity, Dzantor and Felsot (1991) subjected soil to excessive amounts (10,000 ppm) of alachlor alone or a mixture of atrazine, metolachlor, and trifluralin. They compared the results to soils treated with normal field application rates (10 ppm). The bacteria numbers in the simulated spill samples were depressed initially, but after seven days recovered to levels similar to those in untreated controls. The fungal populations drastically decreased after only one day and became undetectable after seven and 21 days of incubation in the mixed herbicide and alachlor-only treatments, respectively. At 10 ppm, 80 to 90% of all of the herbicides degraded within one year. Under high concentrations of herbicides, only very low amounts of degradation occurred within the first year of incubation.

In Chapter 3 of this document, I will describe three experiments designed to determine the effects of biostimulation on the degradation of representatives from 3 different classes of herbicides, the *s*-triazines (atrazine), the dinitroanilines (trifluralin), and the acetanilides (metolachlor). A soil from a contaminated agricultural chemical dealership, code-named Bravo, will be spiked with 200 ppm of either atrazine, trifluralin, or metolachlor, individually or a combination of the three, and will be treated with various inorganic and organic amendments to determine which treatments provide the best alternatives for successful bioremediation.

CHAPTER 2. FACTORS AFFECTING BIODEGRADATION OF ATRAZINE BY *Agrobacterium radiobacter* Strain J14a AND APPLICATION OF THE ORGANISM TO BIOREMEDIATE SOIL

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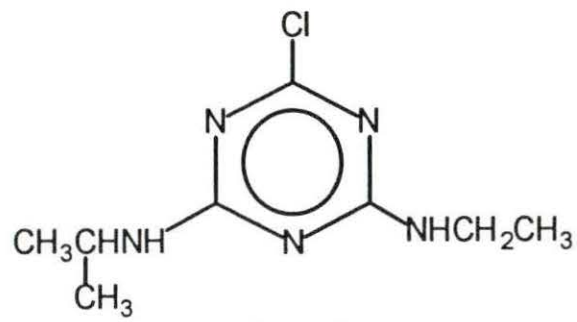
ABSTRACT

A Gram-negative, motile, rod-shaped bacterium, *Agrobacterium radiobacter* strain J14a, was isolated from an agricultural soil by enrichment using a minimal salts medium with citrate and sucrose as C sources and atrazine as its sole N source (BMA). J14a cells grown in BMA mineralized 94% of 50 $\mu\text{g ml}^{-1}$ [^{14}C -UL-*ring*]-atrazine in 72 hours with a concurrent increase in the population size from 6.2×10^6 cells ml^{-1} to 3.6×10^8 cells ml^{-1} . [^{14}C]-ethyl-side chain mineralization experiments showed that approximately 30% of the atrazine ethyl-side chain C was incorporated into J14a biomass. J14a cells grown in medium without C and N sources degraded atrazine, but the cell numbers did not increase. Metabolites produced by J14a during atrazine degradation include hydroxyatrazine (HA), deethylatrazine (DEA), and deethyl-hydroxyatrazine (DEHA). J14a cells grown in tryptic soy broth supplemented with 50 $\mu\text{g ml}^{-1}$ [^{14}C -UL-*ring*]-atrazine mineralized only 64% in 72 hr, indicating that organic N in the TSB medium slowed ring mineralization. Addition of NH_4NO_3 to BMA medium did not inhibit atrazine degradation. The bacterium produced the atrazine degrading enzymes constitutively and these enzymes remained intracellular during atrazine degradation. J14a also degraded the s-triazine herbicides ametryne, cyanazine, prometon, and simazine in N-limited medium. Addition of 10^5 J14a cells g^{-1} into a soil with low atrazine mineralization capabilities treated with 50 and 200 $\mu\text{g atrazine g}^{-1}$ soil resulted in two to five times higher mineralization than the indigenous microorganisms. Sucrose addition did not result in significantly faster mineralization rates or shorten degradation lag times. However, J14a introduction (10^5 cells g^{-1}) into another soil with an indigenous atrazine-mineralizing population, reduced atrazine degradation lag times below those in non-inoculated treatments, but increased the total mineralization only in the 50 $\mu\text{g atrazine g}^{-1}$ soil supplemented with 1% sucrose.

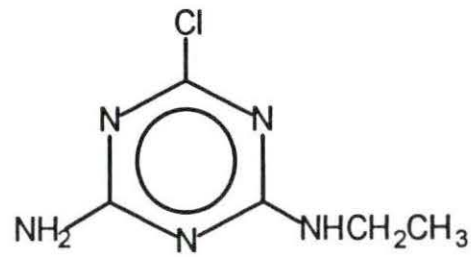
INTRODUCTION

In 1958, atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) was introduced as the first triazine herbicide (Ware, 1994). By 1991, the U.S. EPA estimated that atrazine was the most heavily used herbicide in the U.S., purchased at a rate of 75 to 90 million pounds year⁻¹ (U.S. EPA, 1991). It is used primarily for non-selective weed control of industrial or non-cropped land and selective weed control of broad-leaved and grassy weeds in corn, sorghum, sugar cane, pineapple, and certain other plants (Belluck *et al.*, 1991). Point-source contamination from pesticide mixing, loading, storage, and rinsing at agricultural chemical dealerships in the Midwest is becoming more of a concern. The U.S. EPA reported that atrazine has been found in the groundwater of approximately 25 states due to both point and nonpoint sources. In a 1987 study of Iowa public water systems, 16 of the 18 wells with detections of pesticides, including atrazine, were located within 1,000 feet of an agricultural chemical dealership (Fawcett, 1989). High levels of pesticides and nitrate occurred in soils, surface water, and groundwater at 28 dealerships in Iowa, with the maximum atrazine concentrations in soil of 1100 µg L⁻¹, in surface water 16 µg L⁻¹, and in ground water 1500 µg L⁻¹ (Gannon, 1992). The U.S. EPA maximum contaminant level in drinking water is 3 µg L⁻¹ in water. Detection of atrazine in groundwater is usually 10 to 20 times more frequent than the next most frequently detected pesticides, metolachlor, alachlor, and cyanazine (Belluck *et al.*, 1991). Due to the relative persistence and heavy use of atrazine, groundwater contamination above the EPA maximum contaminant level of 3 µg liter⁻¹ has become a national concern.

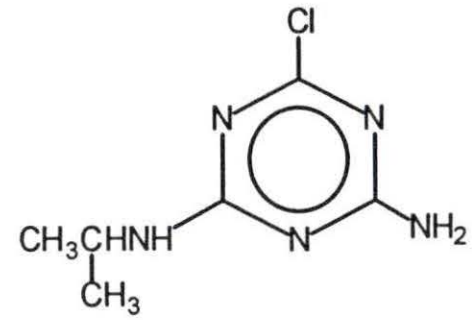
Degradation of atrazine occurs predominantly by biological processes, including *N*-dealkylation, dechlorination, and ring cleavage. The first steps in atrazine degradation appear to be *N*-dealkylation of the ethyl and isopropyl side-chains to produce deethylatrazine (DEA) and deisopropylatrazine (DIA) (Figure 1). Behki and Khan (1986) described a *Pseudomonas* sp. that dealkylated atrazine in carbon-free medium. Mougín *et al.* (1994) showed that the fungus *Phanerochaete chrysosporium* could remove the ethyl side chain to produce DEA, while the fungus *Pleurotus pulmonarius* produced four dealkylated metabolites, DEA, DIA, deethyl-deisopropylatrazine (DEDIA), and



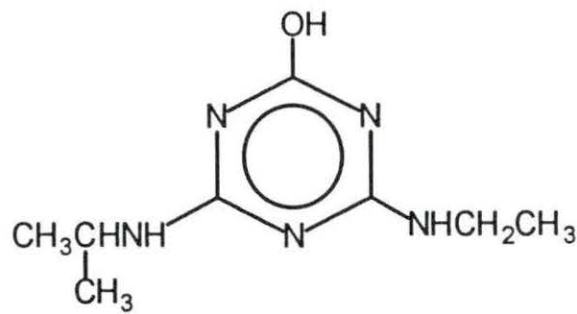
Atrazine



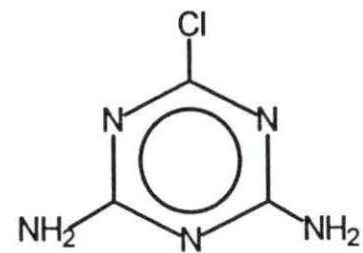
Deisopropylatrazine (DIA)



Deethylatrazine (DEA)



Hydroxyatrazine (HA)



Deethyl-deisopropylatrazine (DEDIA)

Figure 1. Structures of atrazine and metabolites.

2-chloro-4-ethylamino-6-(1-hydroxyisopropyl) amino-1,3,5-triazine (Masaphy *et al.*, 1993). *Rhodococcus* strain TE1 was capable of dealkylating the *s*-triazine herbicides propazine, simazine, and cyanazine (Behki *et al.*, 1993). Mirgain *et al.* (1993) have isolated one pure culture (*Acinetobacter calcoaceticus*), two co-cultures, and one consortium of five bacterial species from different ecosystems that were capable of dealkylating atrazine.

Several studies (Armstrong *et al.*, 1967; Erickson and Lee, 1989; and Skipper *et al.*, 1967) have shown that atrazine dechlorination in soils is a chemical process catalyzed by soil organic matter and clay mineral surfaces. The hydrolysis of atrazine to hydroxyatrazine is strongly dependent on acidic pH. However, research has shown that dechlorination is also the result of biological processes. Behki and Khan (1986) reported that two *Pseudomonas* spp. dechlorinated DEA and DIA. Mulbry (1994) purified and characterized the *s*-triazine hydrolase enzyme from *Rhodococcus corallinus* NRRL B-15444R responsible for the dechlorination of deethylsimazine and DEA. Mandelbaum *et al.* (1993b) showed that a mixed culture of bacteria could rapidly transform atrazine to hydroxyatrazine.

The final steps in atrazine degradation involve cleavage of the *s*-triazine ring to release CO₂. Yanze Kontchou and Gschwind (1994) demonstrated the ability of a *Pseudomonas* strain to mineralize atrazine and use it as a sole carbon and energy source. Several researchers have isolated pure bacterial cultures that are capable of utilizing the *s*-triazine ring as a N source (Mandelbaum *et al.*, 1995; Radosevich *et al.*, 1995). While several atrazine-degrading organisms have been isolated and characterized, very little work has been done to determine the environmental conditions that effect the biodegradation of atrazine and other *s*-triazines or the range of *s*-triazine substrates utilized by the atrazine-degrading microorganisms.

In soil, the population size of degraders capable of mineralizing atrazine varies depending on the type and characteristics of the soil being tested. Jayachandran *et al.* (1996) measured [¹⁴C-UL-*ring*]-atrazine degrader populations in eight different soils using the most probable number technique. Populations using atrazine as an N source ranged from below the detection limit (<20 cells g⁻¹ soil) to 10,530 cells g⁻¹ soil. The populations capable of mineralizing [¹⁴C-UL-*ring*]-atrazine in C-limited medium were much smaller, ranging from nondetectable to only 1630 cells g⁻¹ soil, indicating that N-limited conditions may promote atrazine mineralization in soil. Soil contaminated with 1500 µg g⁻¹ aged atrazine was inoculated with an atrazine-mineralizing *Pseudomonas* strain that utilizes

atrazine as a sole N source, resulting in degradation of approximately 17% of the atrazine (Mandelbaum *et al.*, 1995). Amending the soil with citrate increased the degradation to 70% of the aged atrazine. Addition of a *Pseudomonas* strain that utilizes atrazine as a sole C source to an unsterilized soil with 3.7% organic matter and a pH of 7.2 resulted in mineralization of over 60% of 10 μg atrazine g^{-1} soil in 49 days and an atrazine half-life of one day (Yanze Kontchou and Gschwind, 1995). However, addition of the same strain to a soil with 2% organic matter and a pH of only 5.4 and a soil with 36% organic matter resulted in much slower atrazine degradation, with half-lives of 19 and 22 days. A mixed enrichment culture of microorganisms mineralized 96% of the applied atrazine (0.56 mM) within 7 days in liquid culture (Assaf and Turco, 1994a). When this mixed culture was added to atrazine-treated soil (0.14 mM), 60% of the atrazine was recovered as $^{14}\text{CO}_2$ after 30 days incubation, resulting in a 20-fold increase in mineralization over the uninoculated soil. Addition of another atrazine-mineralizing consortium to soil spiked with 3 μg atrazine g^{-1} soil resulted in an increase in atrazine mineralization from 2.4% in the uninoculated soil to 71% in the inoculated soil (Alvey and Crowley, 1996). The corn plant rhizosphere effect appeared to enhance the long-term survival of the consortium, but the amount of atrazine mineralized was not affected by the corn plants.

In this paper we describe the isolation and characterization of a bacterial strain *Agrobacterium radiobacter* strain J14a, that is capable of utilizing atrazine as its sole nitrogen source, and investigate the effects of organic and inorganic N on atrazine degradation kinetics, the use of atrazine as the sole C and N source, and the s-triazine substrate range of strain J14a. We examine the effects of addition of J14a and sucrose into two different agricultural chemical dealership soils.

MATERIALS AND METHODS

Chemicals. Atrazine (99% purity), deethylatrazine (> 98% purity) (DEA), deisopropylatrazine (98% purity) (DIA), deethyldeisopropylatrazine (96.9% purity) (DEDIA), simazine (99% purity), and cyanazine (98% purity) were purchased from ChemService, West Chester, PA. Propazine (99% purity) was obtained from Riedel-de Haen, Seelze, West Germany. Ametryne (99.1% purity) and prometon (99.5% purity) were obtained from EPA Research Triangle Park, NC. Hydroxyatrazine (HA), deethyl-hydroxyatrazine (DEHA),

and deisopropyl-hydroxyatrazine (DIHA) were obtained from Robert Lerch (USDA-ARS Cropping Systems and Water Quality Research Unit, University of Missouri, Columbia, MO). Uniformly ring-labeled [^{14}C]atrazine ($87.77 \mu\text{Ci mg}^{-1}$, 98% radiochemical purity) and ethyl side-chain labeled [^{14}C]atrazine ($13.18 \text{ mCi mmol}^{-1}$, 98% radiochemical purity) were purchased from Sigma Chemical Co., St. Louis, MO.

Enrichment and isolation. Surface soil collected near Sheldon, Nebraska served as the inoculum for nitrogen-limited enrichment cultures grown in medium described by Mandelbaum *et al.* (1993a). This medium, designated as BMA, contains a minimal salts medium supplemented with 1 g each of sodium citrate and sucrose, 20 ml of vitamin solution (5 mg thiamine, 2 mg *d*-biotin, 2 mg folic acid, 10 mg nicotinamide, and 10 mg pyridoxine in 1 liter of Milli-Q water), 20 ml of trace elements solution, and 50 mg of atrazine in 1 liter of Milli-Q water (Millipore Corporation, Bedford, MA). After several transfers into fresh BMA liquid medium, the mixed culture J14 was obtained and was streaked for isolation onto BMA agar plates. Due to production of a large amount of polysaccharide on BMA and 0.5 strength tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) plates, it was difficult to obtain a pure culture from the culture by streaking for isolation. We used the Percoll™ (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation method (Putzer *et al.*, 1991) to separate the organisms in the culture. Isolates from each band were placed into liquid BMA medium and screened for the ability to degrade atrazine. Based on HPLC analysis of broth cultures, the isolate J14a degraded atrazine and was used in further experiments.

Isolate identification. Fatty acid profile analysis of the isolate was performed by Microcheck, Inc., Northfield, VT. Microcheck, Inc. subcultured the isolate onto TSA, incubated the plates overnight at 28°C, harvested and extracted the cells, and analyzed the cellular fatty acids of the isolate using high resolution gas chromatography. The isolate's profile was compared for similarity to the profiles of their 1700-strain database. The substrate utilization of J14a was examined using Biolog plates (Biolog, Inc., Hayward, CA). Inoculum for the Biolog plates was grown on TSA overnight and following inoculation, the plates were incubated for 24 hours in the dark at 30°C. The absorbance of the plate wells was read on an EL340 Automated Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). The substrate utilization patterns were compared with the Biolog strain database. Additional biochemical tests were performed to verify the results obtained from the fatty acid

and Biolog ID system analysis: 3-ketolactose production, the carrot tumorigenesis test, Kovac's oxidase, catalase, and the urease test. Numbers of flagella and their positions were analyzed under a transmission electron microscope (TEM) using the phosphotungstic acid negative staining technique.

Inoculum preparation. Unless stated, inoculum for all of the experiments was prepared by growing the bacteria in 50 ml of either BMA or 0.5 strength tryptic soy broth (TSB) for three days at 28°C on a rotary shaker at 120 rpm. Cultures were pelleted by centrifugation at room temperature and 7000 X g for 10 minutes. Cells were rinsed twice with 20-ml aliquots of sterilized 0.0125 M phosphate buffer (pH 7.2) and quantified by the drop plate technique. This technique was performed by preparing ten-fold serial dilutions of the cells, dispensing triplicate 25 μ L aliquots of appropriate dilutions onto 0.5-strength TSA plates and incubating the plates for 24 to 48 hours in the dark at 28°C.

Mineralization and degradation. Mineralization of [14 C-UL-*ring*]-atrazine was used to confirm the atrazine-degrading ability of the isolate. Triplicate biometer flasks (Belco, Vineland, NJ) of BMA were prepared with 50 ml of BMA with an atrazine concentration of 50 μ g ml $^{-1}$ and 100,000 dpm (1500 Bq) of [14 C-UL-*ring*]-atrazine. Three uninoculated controls were also monitored for 14 CO $_2$ production. Ten ml of 0.5 M NaOH were added to the side-arm reservoirs. The flasks were incubated in a completely randomized design at the conditions previously stated. At each sampling time the entire volume of NaOH was removed and replaced with fresh NaOH. A three-ml aliquot of the NaOH was transferred into a scintillation vial with 15 ml of Ultima Gold™ scintillation cocktail (Packard Instrument Company, Meriden, CT) and analyzed using a Packard 1600 liquid scintillation counter (Packard Instrument Company, Meriden, CT). The radioactivity counted by the LSC was corrected for quenching and background radioactivity. The final degrader population in each flask was determined by the drop plate technique. Nine additional flasks of BMA containing 50 μ g ml $^{-1}$ of unlabeled atrazine were monitored for atrazine concentration and metabolite production by HPLC and J14a cell counts by drop plate. At each sampling time, three randomly chosen flasks were sampled by removing 0.5 ml liquid, diluting with 0.5 ml of methanol (until the 72-hour sampling period), and filtering the solution through a 0.22 μ M nylon syringe filter into a 1-ml HPLC vial. The Monod and logistic growth models were used to describe atrazine mineralization. Parameters for these models were estimated from

cumulative $^{14}\text{CO}_2$ production using non-linear regression analysis (Simkins and Alexander, 1985).

To determine if side-chain C was incorporated into J14a cells, a mineralization experiment was performed using [^{14}C -*ethyl*]-atrazine. Fifty $\mu\text{g ml}^{-1}$ atrazine in BMA was used to grow the inoculum. The medium consisted of two different treatments: triplicate flasks each of BMA medium, both with and without C sources, vitamins, sucrose citrate, and 50 ml of 50 $\mu\text{g ml}^{-1}$ atrazine and 250,000 dpm (3750 Bq) [^{14}C -*ethyl*] atrazine. Three uninoculated controls were included. Sampling for $^{14}\text{CO}_2$ production proceeded as described previously. Every 24 hours from time zero, one ml of media from each inoculated flask was filtered through a 0.22 μM nylon filter and the filter was rinsed with 10 ml of phosphate buffer. The filter was placed in a scintillation vial with 15 ml of cocktail and counted on the LSC to determine the cell-associated radioactivity. Two-hundred μL of the media was removed, 15 ml of cocktail was added, and the solution radioactivity was measured. At the end of the experiment, approximately 5% of the radioactivity remained in the medium. This cell-free, filtered medium was passed through a cyclohexyl (C_8), 1000-mg solid phase extraction cartridge (United Chemical Technologies Inc.) to concentrate the analytes. The columns were eluted with two ml of methanol and the atrazine and metabolite concentrations were determined by HPLC.

In order to determine the metabolites produced by J14a during atrazine degradation, a J14a inoculum of approximately 10^{10} CFU ml^{-1} was incubated with 50 $\mu\text{g ml}^{-1}$ atrazine and [^{14}C -UL-*ring*] atrazine and the medium was analyzed by HPLC for metabolic products. The inoculum was grown in BMA and in 0.5 strength TSB (400 ml) in a rotary shaker at 120 rpm and 28°C for 72 hours. The cultures were transferred into a sterile Nalgene™ 250-ml TFE centrifuge bottle (Nalge Company, Rochester, NY), centrifuged for 20 minutes at 4000 rpm to pellet the cells, and the supernatant was removed. The cells were washed with 100 ml of phosphate buffer, combined into a 250-ml TFE bottle, and resuspended in 40 ml of BMA. Two and one-half ml of each cell suspension (TSB- and BMA-grown) containing approximately 10^{10} CFU ml^{-1} each was dispensed into 16 sterilized 25-ml glass centrifuge tubes. To eight of the 16 tubes for each cell type, an additional 2.5 ml of BMA medium was added, while 2.5 ml of BMA supplemented with 250,000 dpm (3750 Bq) of [^{14}C -UL-*ring*]-atrazine was added to the other eight tubes. The tubes were incubated on a rotary shaker at 120 rpm and 28°C. Half of the tubes for each cell type were sacrificed

at four hours and half at 24 hours. Each treatment had four replications. The samples were diluted to 100 ml with water and the cells were lysed by sonication for six minutes at a 50% duty cycle. Cellular debris was pelleted by centrifugation at 5000 rpm for 20 minutes and the supernatant was diluted to 250 ml with water. Thirteen and one-half ml of 1 M KH_2PO_4 (pH 2.5) was added to make a final solution concentration of 0.05 M KH_2PO_4 . The samples were passed through a cyclohexyl (C_8), 1000-mg solid phase extraction cartridge (United Chemical Technologies Inc., Horsham, PA) and eluted with three ml methanol to quantify the atrazine, DEA, DIA, and DEDIA. The aqueous phase was then passed through a SCX 3-ml Bond Elut cation-exchange extraction cartridge (Varian, Harbor City, CA) and eluted with 3 ml of 75% 0.5 M KH_2PO_4 (pH 7.5) / 25% acetonitrile to analyze for HA, DEHA, and hydroxydeisopropylatrazine (DIHA). Each sample was filtered through a 0.22 μm filter into an HPLC vial. Four tubes of basal minimal salts medium were spiked with a one $\mu\text{g ml}^{-1}$ standard mixture of the three hydroxylated metabolites, HA, DEHA, and DIHA, to determine the extraction procedure recovery efficiency. The non-radioactive samples were sent to Robert Lerch (USDA-ARS Cropping Systems and Water Quality Research Unit, University of Missouri, Columbia, MO) to analyze by HPLC for hydroxylated metabolite production (Lerch and Donald, 1994). The radiolabeled samples were run on a Waters HPLC with a Radiomatic radioactive detector (Packard Instrument Company) to look for atrazine and the chlorinated metabolites DEA, DIA, and DEDIA.

BMA medium was modified to assess the requirements for J14a growth and atrazine metabolism. The first treatment consisted of minimal salts medium amended with the carbon sources, trace elements, and vitamins, but without any atrazine. Treatment two was completely nitrogen limited, except for the atrazine, consisting of the basal minimal salts medium, carbon sources, atrazine, and trace elements, but no vitamin addition. The third treatment was carbon and nitrogen limited, containing only the basal minimal salts medium, trace elements, vitamins, and atrazine. Treatment four contained complete BMA medium with five grams of NH_4NO_3 . The final treatment was composed of basal minimal salts medium, atrazine, vitamins, and trace elements, without the carbon sources. Treatments were prepared in triplicate and were incubated for five days on the rotary shaker at 120 rpm and 28°C in a completely randomized design. The initial and final atrazine concentration in each flask was determined by HPLC. The inoculum size and the final populations in each flask were determined by the drop plate technique.

Two different experiments were done using 0.5-strength TSB to determine if organic N sources inhibit atrazine degradation. The first experiment used J14a cells grown in 0.5 strength TSB and 50 ml of BMA with the atrazine concentration of $50 \mu\text{g ml}^{-1}$ and 100,000 dpm (1500 Bq) of [^{14}C -UL-*ring*]-atrazine. The second experiment used J14a cells that were grown in 0.5-strength TSB medium for three generations and 50 ml of a $50 \mu\text{g ml}^{-1}$ solution of 0.5-strength TSB mineralization medium containing 100,000 dpm (1500 Bq) [^{14}C -UL-*ring*]-atrazine. Media treatments and three uninoculated controls were inoculated in triplicate biometer flasks and incubated in a completely randomized design on the rotary shaker. Sampling for both experiments occurred as described in the BMA mineralization experiment. The initial and final J14a populations were determined by the drop plate technique.

Enzyme characterization. In order to determine if the atrazine-degrading enzymes were retained within or released outside the cells to degrade atrazine, a cell-free extract of J14a was prepared by growing 50 ml of J14a in BMA medium, centrifuging the culture at 7000 rpm for 15 minutes to pellet the cells, and filtering the supernatant through a $0.22 \mu\text{m}$ disposable sterile bottle-top filtering apparatus (Corning Glass Works, Corning, NY). Five ml of the cell-free extract was added to triplicate flasks containing 50 ml of BMA medium supplemented with $50 \mu\text{g ml}^{-1}$ atrazine and $100 \mu\text{g ml}^{-1}$ chloramphenicol to inhibit the growth of any cells that passed through the filter. The flasks were incubated in a completely randomized design on a rotary shaker at 120 rpm and 28°C for 120 hours. The initial and final concentration of atrazine in each flask was determined by HPLC.

The second experiment was performed to determine if the atrazine degrading enzymes are produced constitutively or are induced. Three flasks of BMA medium were amended with $50 \mu\text{g ml}^{-1}$ atrazine and $100 \mu\text{g ml}^{-1}$ chloramphenicol and inoculated with J14a cells grown in 0.5 strength TSB for three generations. The flasks were incubated on the rotary shaker in a completely randomized design for 120 hours. The initial and final concentration of herbicide in each flask was determined by HPLC. The J14a inoculum and final population sizes were determined by the drop plate technique.

Substrate range experiment. Minimal salts medium was amended with vitamins, carbon sources, trace elements and one the following herbicide concentrations: $50 \mu\text{g ml}^{-1}$ atrazine, $5 \mu\text{g ml}^{-1}$ ametryne, $50 \mu\text{g ml}^{-1}$ cyanazine, $5 \mu\text{g ml}^{-1}$ prometon, $5 \mu\text{g ml}^{-1}$ propazine,

or $1 \mu\text{g ml}^{-1}$ simazine. Each flask was inoculated in triplicate with J14a and incubated in a completely randomized design on the rotary shaker for 120 hours. The initial and final herbicide concentration in each flask was determined by HPLC, while the inoculum and final population size in each flask was determined by drop plating.

HPLC analysis. HPLC was performed using a Waters HPLC system with a model 490E UV detector and a Nova-Pak C₁₈ 10-cm column contained in a Waters compression device. A 15 minute gradient method with deionized water and acetonitrile (ACN) as the mobile phases was utilized to separate atrazine, DEDIA, DIA, and DEA with detection by absorbance at 220 nm. The gradient started at 25% ACN / 75% water, reached 75% ACN / 25% water by 10 minutes, ramped back down to 25% ACN / 75% water by 13 minutes, and held at those conditions until 15 minutes at a flow rate of 1.8 ml min^{-1} . Linear calibration curves were run using external standards containing DEDIA, DIA, DEA, and atrazine. The retention times for DEDIA, DIA, DEA, and atrazine are 1.4, 2.5, 4.1, and 9.3 minutes, respectively. A modified gradient was used to determine the concentrations of the additional *s*-triazines used in the substrate range experiment. The mobile phase flow rate was increased to 2 ml min^{-1} . The gradient started at 25% ACN / 75% water, reached 75% ACN / 25% water at eight minutes, held at those conditions for four minutes, ramped down to 25% ACN / 75% water by 14 minutes, and held at those conditions for five minutes. The retention times for simazine, cyanazine, atrazine, prometon, ametryne, and propazine are 7.0, 7.1, 7.6, 9.5, 9.9, and 10.0, respectively. The [¹⁴C]-atrazine HPLC method is a 20-minute gradient with deionized water and acetonitrile (ACN) as the mobile phases to separate atrazine, DEDIA, DIA, and DEA. The gradient was held at 25% ACN / 75% water for 3 minutes, ramped up to 75% ACN / 25% water by 11 minutes, ramped back down to 25% ACN / 75% water by 16 minutes, and held at those conditions until 20 minutes at a flow rate of 1.0 ml min^{-1} . Following HPLC separation, atrazine and metabolites were quantified using a Packard RAM detector. Counting efficiency and background were determined using standards prepared from [¹⁴C]-atrazine solutions.

Soil experiment. The two soils used for this experiment were collected by scraping the top 5 cm of the surface soil at two agricultural chemical dealerships in Iowa from a location at the sites which appeared to be most impacted by the dealerships' rainfall runoff and pesticide spills. These two dealerships are code-named Alpha and Bravo and the soils collected from the sites will be given the same name. The soil from Alpha had the following

characteristics: sandy loam classification with 75% sand, 17% silt, and 8% clay; 3.2% organic C, 0.07% total N; pH 7.9; and a cation exchange capacity of 9.8. The soil from Bravo had the following characteristics: a loamy sand texture classification with 78% sand, 18% silt, and 4% clay; 2.4% organic C, 0.05% total N, and pH 6.5. Soil at both sites consisted of mixtures of soil, sand, and limestone that had been used to maintain a surface at the site. Soils were passed through a 4-mm sieve and 50 g (dry weight basis) of soil was weighed into Bellco biometer flasks. Each flask was brought to 10% moisture with Milli-Q water. The atrazine mineralizing-populations for both soils were determined by a modified [^{14}C]-atrazine most probable number (MPN) technique to enumerate pesticide degrading microorganisms (Jayachandran, submitted to Soil Biol. Biochem.) prior to the start of the experiment. Each biometer was treated with a solution containing 200,000 dpm of [^{14}C -UL-*ring*]-atrazine and additional atrazine to result in 12 flasks with $50\ \mu\text{g g}^{-1}$ and 12 flasks with $200\ \mu\text{g g}^{-1}$ for each soil. The soil was mixed thoroughly to evenly incorporate the atrazine. Ten ml of 0.5 M sodium hydroxide was added to the biometer flask sidearm to collect mineralized $^{14}\text{CO}_2$ and the samples were incubated at 25°C in the dark. After three days incubation with the herbicide, three flasks at each herbicide concentration were treated with 1% (w/w) sucrose amendment, three flasks with 10^5 J14a cells g^{-1} soil, and three flasks with both sucrose and J14a. For each treatment, an additional flask was treated with atrazine and incubated along with the [^{14}C]-atrazine-treated soils to monitor the atrazine mineralizing populations. The sodium hydroxide traps were changed periodically to monitor atrazine mineralization.

At day 63 the atrazine mineralizing populations in each soil treatment were determined by the MPN technique. Additionally, a 10 g subsample from each biometer flask was extracted twice with 30 ml methanol. The methanol was added to the soil, the samples were shaken at 190 rpm on a reciprocating shaker for one hour, allowed to sit for 24 hours, and shaken again for 30 minutes. The supernatant was removed and centrifuged at 10,000 rpm for 30 minutes to pellet out soil particles. A second-30 ml aliquot of methanol was used to repeat the extraction procedure just described. One ml of each methanol extract was mixed with 10 ml of Ultima Gold cocktail and counted on the LSC. Each extract was analyzed by HPLC using the method described earlier to determine the atrazine, DEA, DIA, and DEDIA concentrations. The soil was then air dried, powderized, and duplicate 1-g

aliquots were run on the OX500 biological oxidizer (R. J. Harvey Instrument Corporation, Hillsdale, NJ) to determine what portion of the radioactivity remained sorbed to the soil after extraction.

RESULTS

Identification. J14a is a Gram (-), motile, rod-shaped bacterium. Fatty acid analysis of J14a showed an excellent match (similarity index = 0.629) with *Agrobacterium radiobacter* and a good match (similarity index = 0.450) with *Agrobacterium rubi*. J14a also matched up very well with the Biolog profile for *Agrobacterium radiobacter*. When grown on TSA medium, J14a produced generally round, beige-colored, mucoid, opaque, smooth-edged colonies that turn rough-edged with age. J14a showed positive reaction for catalase, 3-ketolactose production, urease, and Kovac's oxidase test. Three-ketolactose production is unique to two biovars in the genus *Agrobacterium* and this positive reaction by J14a was fairly strong proof that the isolate belongs in the genus *Agrobacterium*. J14a was negative for the carrot tumorigenesis test. Under TEM the J14a cells appeared to have several peritrichous flagella.

Metabolism and growth. J14a rapidly mineralized approximately 94% of the [¹⁴C-UL-*ring*]-atrazine in BMA medium, following a short lag time of approximately 12 hours (Figure 2). Atrazine disappearance and cell growth (Figure 2) coincide with the ¹⁴CO₂ appearance. The J14a growth levels off at approximately 7.9x10⁷ CFU ml⁻¹ at 72 hours, which is the same time that the atrazine concentration drops below the HPLC detection limit of 25 µg liter⁻¹. J14a is able to maintain an initial population upon addition to medium with atrazine as the sole C and N source, but needs an additional C source in order to grow (Table 1). When incubated in the presence of 150,000 dpm of [¹⁴C-UL-*ring*]-atrazine and additional unlabelled atrazine to achieve 50 µg ml⁻¹ atrazine provided as the sole C and N source, 11% of the atrazine was mineralized by J14a in 90 hours (data not shown). J14a can degrade atrazine in the presence of easily assimilated sources of inorganic N. Addition of either KNO₃, (NH₄)₂SO₄, or NH₄NO₃ to the minimal salts medium with sucrose and citrate

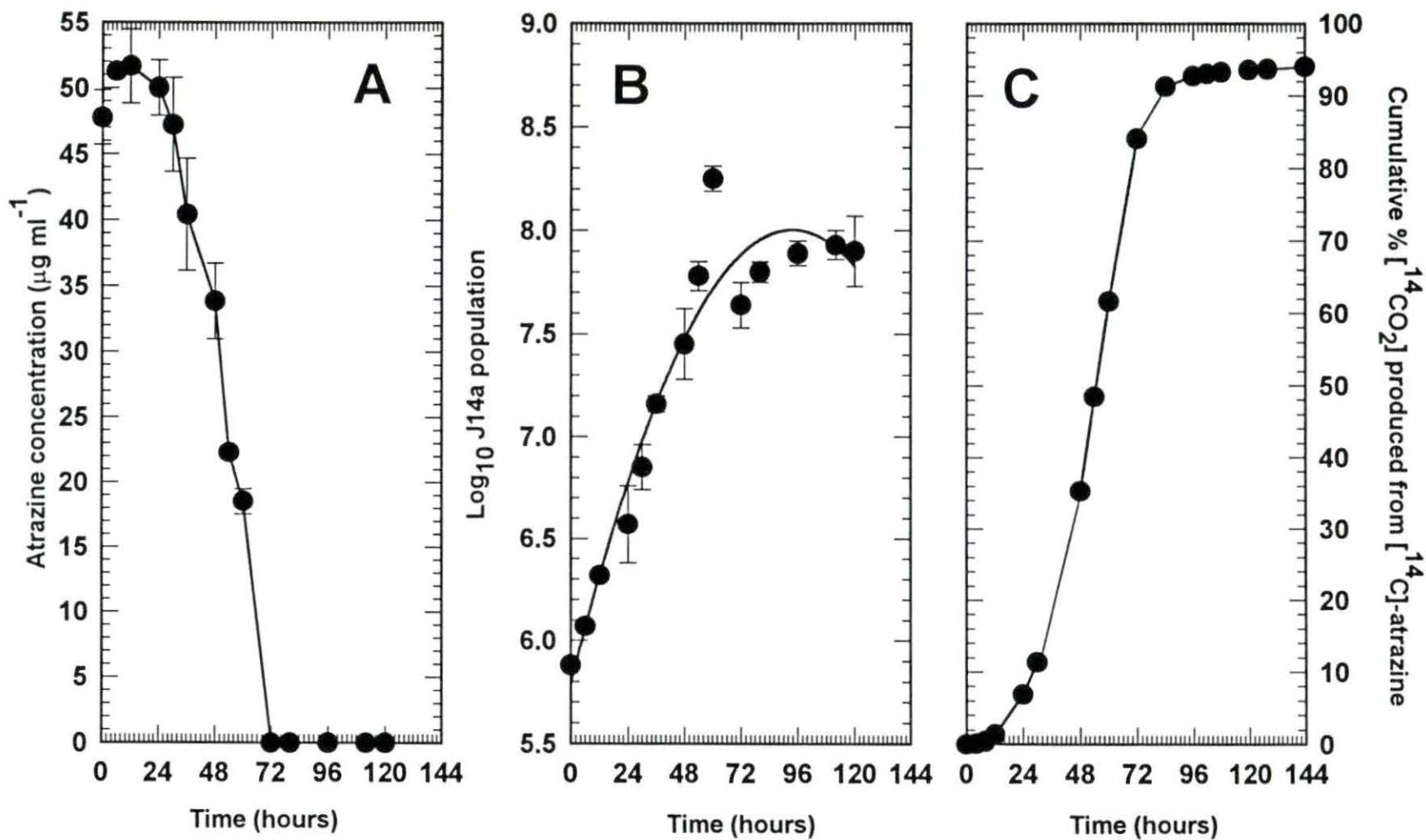


Figure 2. (A) J14a 50 $\mu\text{g ml}^{-1}$ atrazine degradation curve; (B) log_{10} population growth curve; and (C) atrazine mineralization curve upon addition of 100,000 dpm [^{14}C -UL-ring]-atrazine and additional unlabeled atrazine to achieve 50 $\mu\text{g ml}^{-1}$ in solution.

Table 1. Growth of *Agrobacterium radiobacter* strain J14a and atrazine degradation during a 120-hour incubation period

Treatment	Atrazine concentration ^a ($\mu\text{g ml}^{-1}$)	J14a population ^a (CFU ml^{-1})
Initial conditions in BMA	47.8 (2.1)	inoculum= 7.6×10^5
After 120 hours incubation		
BMA standard medium	0.0 (0.0)	8.6×10^7 (3.7×10^7)
BM without atrazine added	NA	9.1×10^5 (8.7×10^5)
BMA without N (no vitamins)	0.0 (0.0)	9.7×10^7 (8.0×10^6)
BMA without C and N	9.4 (0.9)	3.1×10^5 (3.7×10^4)
BMA with NH_4NO_3	0.0 (0.0)	1.6×10^8 (6.7×10^7)
BMA without C; with NH_4NO_3	9.6 (2.1)	4.4×10^5 (4.0×10^4)

NA= no atrazine was present in the medium at the beginning of the experiment, i.e. time zero atrazine concentration= $0 \mu\text{g ml}^{-1}$

^aThe data are expressed as the mean of three replicate samples followed by the standard deviation in parentheses

as C sources and $50 \mu\text{g ml}^{-1}$ atrazine (150,000 dpm labeled and additional unlabelled atrazine to achieve $50 \mu\text{g ml}^{-1}$) resulted in mineralization of approximately 60% of the [^{14}C -UL-*ring*]-atrazine in 90 hours for all three inorganic N sources (data not shown). Only a small amount of growth occurred in the medium without N (atrazine and vitamins), indicating that J14a cannot fix N_2 . Atrazine is mineralized in BMA medium without vitamins and containing atrazine as the sole N source (Table 1). These results indicate that the N available from the mineralization of atrazine along with the C present in the medium are capable of supporting the growth of J14a. Growth of J14a in minimal salts medium with atrazine as the N source and sucrose as the only C source resulted in the same amount of mineralization as the minimal salts medium with both sucrose and citrate as the C sources, however when citrate was provided as the only C source, less than 5% of the atrazine was mineralized (data not shown), indicating J14a is not capable of utilizing citrate as its sole C source.

Kinetics. Mineralization of [^{14}C -UL-*ring*]-atrazine was described by nonlinear regression using the Monod and logistic growth models (Simkins and Alexander, 1985). The Monod model fit the mineralization data better than the logistic model based on residual sums of squares of 1.87 and 21.1, respectively. Parameter estimates for both models (Table 2) indicate that these models are reasonably accurate estimates of the degradation process. Ring mineralization is nearly complete (94%) with little C incorporation into microbial biomass (assuming 6% incorporation) which is slightly larger than the model value of $0.03 \mu\text{g C } \mu\text{g}^{-1}$ substrate for ξ , which corresponds to 1.5% C incorporated. The difference between these values may be due to incomplete metabolism of atrazine or measurement error, because no incorporation of oxidized ring C would be expected. Previous studies indicate the triazine ring is ultimately metabolized to urea, which decomposes to produce CO_2 and NH_4^+ or NH_3 (Cook, 1987; Jutzi *et al.*, 1982; Radosevich *et al.*, 1995). The initial concentration (S_0) is near the measured value of $50 \mu\text{g ml}^{-1}$, with saturation occurring at $33 \mu\text{g ml}^{-1}$. The Monod model parameter K_s represents the substrate concentration at half of μ_{max} and was estimated to be $38 \mu\text{g ml}^{-1}$. Our studies were conducted using supersaturated solutions of atrazine ($50 \mu\text{g ml}^{-1}$). Above this concentration atrazine readily precipitates, which would limit biodegradation.

Table 2. Kinetics parameters describing [¹⁴C-UL-ring]-atrazine mineralization by *Agrobacterium radiobacter* strain J14a

Parameter	Logistic Model ^a	Monod Model ^a
S ₀	49.7 (0.5)	50.3 (0.1)
X ₀	0.3 (0.05)	1.1 (0.1)
k ₄	0.00098 (0.0003)	NA
μ _{max}	NA	0.113 (0.008)
K _s	NA	37.5 (7.8)
ζ	0.03 (0.002)	0.03 (0.0006)

^aThe parameter estimates are expressed as the mean and standard deviation of three replicate samples

NA= not applicable; the model does not estimate this parameter

S₀= initial substrate concentration (μg ml⁻¹)

X₀= amount of substrate (μg ml⁻¹) required to produce the initial population density equal to B₀ (the initial biomass); X₀=B₀/Y (yield)

k₄= rate constant; k₄=μ_{max}/K_s

ζ= the constant fraction of the radiolabeled substrate that is incorporated into the cells

μ_{max}= the maximum specific growth rate (hour⁻¹) which occurs at the higher range of substrate concentrations

K_s= a constant that represents the substrate concentration (μg ml⁻¹) at which the rate of growth is half the maximum rate

Ethyl-side chain mineralization. The ethyl side-chain mineralization experiment was used to determine if J14a incorporated the side chains into biomass. Figure 3 shows that in the medium with additional C sources, 50% of the ^{14}C is released as $^{14}\text{CO}_2$, while approximately 30% of the radioactivity is associated with the J14a biomass at 124 hours. In medium without the C sources, only about 5% of the [^{14}C -ethyl] atrazine was mineralized, with about 24% of the radioactivity being incorporated into biomass. The uninoculated control flask mineralized an average of 1.3%. The radioactivity associated with the cells for both treatments fluctuated throughout the experiment with the highest value of 37.5% for the C-amended treatment at 96 hours. In the C-amended samples, 10% of the radioactivity was removed during the sampling and only about 5% of the radioactivity remained in the medium, while in the C- and N-limited treatment, 10% of the radioactivity was removed during sampling and about 60% remained in the medium. The samples were passed through a C8 and then through a SCX cation exchange solid phase extraction column in order to determine whether the remaining radioactivity was atrazine or metabolites. The 5% radioactivity in the C-amended samples was not retained by either of the solid phase extraction columns. This ^{14}C was determined to be dissolved $^{14}\text{CO}_2$ in the medium by measuring the release of ^{14}C after acidification of the media. Nearly all of the 60% radioactivity remaining in the C- and N-limited medium was retained by the C8 column and was determined by HPLC analysis to be atrazine. The average recovery of ^{14}C as $^{14}\text{CO}_2$, [^{14}C]-atrazine and metabolites remaining in the medium, and J14a-assimilated ^{14}C was 98%. The J14a population in the C-amended medium grew from 7.27×10^6 to 2.75×10^8 CFU ml^{-1} , while the population in the C- and N-limited medium remained unchanged throughout the experiment.

Metabolite production. The metabolites produced by J14a during atrazine degradation were DEA, DEHA, and HA (Table 3). An unknown compound consistently eluted at 11 minutes during the HPLC analysis. The concentrations of DIA, DEDIA, and DIHA did not exceed the HPLC detection limit (DIA=250 $\mu\text{g L}^{-1}$; DEDIA=250 $\mu\text{g L}^{-1}$; DIHA=0.12 $\mu\text{g L}^{-1}$) during the incubation period. No apparent difference in the amount of atrazine degraded or metabolites produced was shown between tubes sacrificed at four versus 24 hours. The amount of HA produced by the cells grown in TSB is much higher than those grown in BMA, whereas the concentrations of DEHA and DEA produced were essentially the same for cells grown in TSB and BMA.

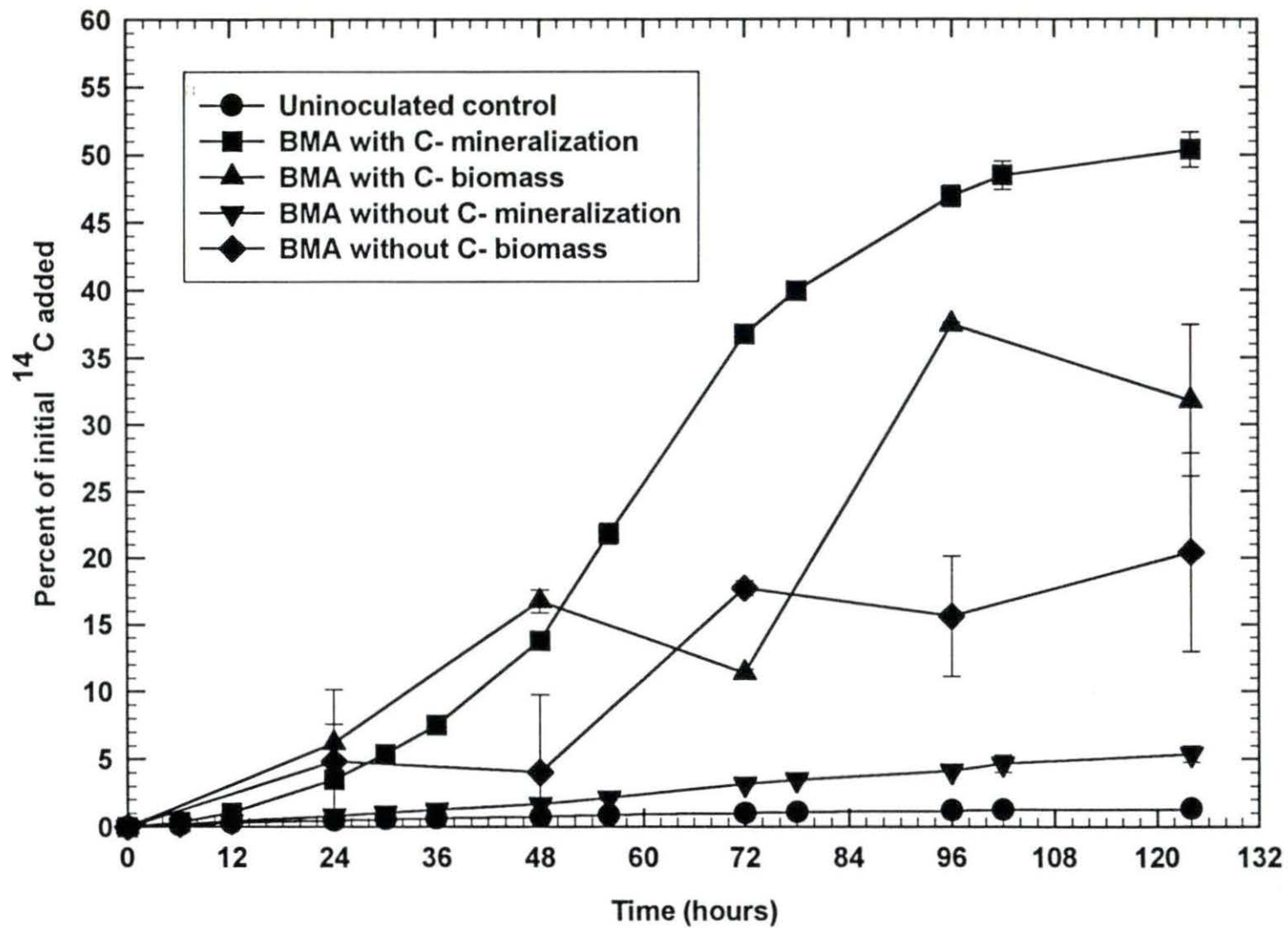


Figure 3. Mineralization and biomass incorporation of [^{14}C]-ethyl-side chain atrazine by *Agrobacterium radiobacter* strain J14a when grown under N-limiting and N- and C- limiting conditions.

Table 3. Atrazine and metabolite mass balance for the *Agrobacterium radiobacter* strain J14a atrazine degradation pathway experiment

Treatment	Atrazine* (%)	HA* (%)	DEHA* (%)	DEA* (%)	Unknown peak at 11 min* (%)
TSB / 4 hours	30.0 (0.1)	16.2 (0.8)	2.6 (0.1)	3.0 (0.3)	0.8 (1.2)
TSB / 24 hours	32.3 (8.1)	12.5 (0.2)	2.0 (0.2)	3.1 (0.6)	0.9 (1.2)
BMA / 4 hours	25.8 (2.1)	1.8 (0.1)	2.1 (0.1)	4.1 (0.2)	1.8 (0.1)
BMA / 24 hours	30.0 (21.4)	1.7 (0.01)	2.0 (0.1)	3.7 (0.01)	1.7 (0.01)

*Data are expressed as the mean percentage of the total radioactivity recovered as atrazine or the metabolite in triplicate flasks with the standard deviation in parenthesis.

Growth in TSB medium. Cells grown in TSB and then inoculated into BMA medium (Figure 4A) mineralized an average of approximately 88% of the [^{14}C]-atrazine added to the medium. The TSB-grown J14a inoculum reduced the initial lag period from about 24 hours down to only eight hours and increased the atrazine degradation rate. These events can be attributed to a larger cell yield, and therefore a larger biomass, for the cells grown in TSB versus the BMA-grown cells (5.1×10^7 CFU ml $^{-1}$ versus 6.2×10^6 CFU ml $^{-1}$). The TSB-grown J14a population grew from 5.1×10^7 to 2.6×10^9 CFU ml $^{-1}$. J14a grown for three generations in TSB mineralized only about 63% of the [^{14}C]-atrazine upon inoculation into TSB mineralization medium (Figure 4B). Thirty-seven percent of the radioactivity remained in the medium. The medium was passed through a C8 solid phase extraction column to concentrate atrazine and the chlorinated atrazine metabolites. The solid phase extraction column retained only 7% of the medium radioactivity, while 30% passed through the column with the aqueous phase. This indicates that polar metabolites such as hydroxyatrazine and hydroxy N-dealkylated metabolites were produced. The total recovery of ^{14}C was 98% of that added at the start of the experiment. The J14a population grew from 1.7×10^8 to 1.9×10^9 CFU ml $^{-1}$.

Enzyme characterization. The enzymes used by J14a to degrade atrazine appear to be constitutive and remain intracellular throughout the entire atrazine mineralization process. TSB-grown J14a cells inoculated into BMA medium are susceptible to chloramphenicol, as indicated by the lack of growth upon exposure. The cell numbers decreased from 1.7×10^8 CFU ml $^{-1}$ to 6.8×10^6 CFU ml $^{-1}$, but were capable of completely degrading $50 \mu\text{g ml}^{-1}$ atrazine present in BMA medium containing $100 \mu\text{g ml}^{-1}$ chloramphenicol. When the TSB-grown cells were inoculated into BMA medium without chloramphenicol, complete degradation of the $50 \mu\text{g ml}^{-1}$ atrazine occurred, with growth from 1.7×10^8 CFU ml $^{-1}$ to 6.8×10^8 CFU ml $^{-1}$. The cell-free extract was unable to degrade any of the atrazine, indicating that the atrazine-degrading enzymes are not released outside of the cell during atrazine degradation.

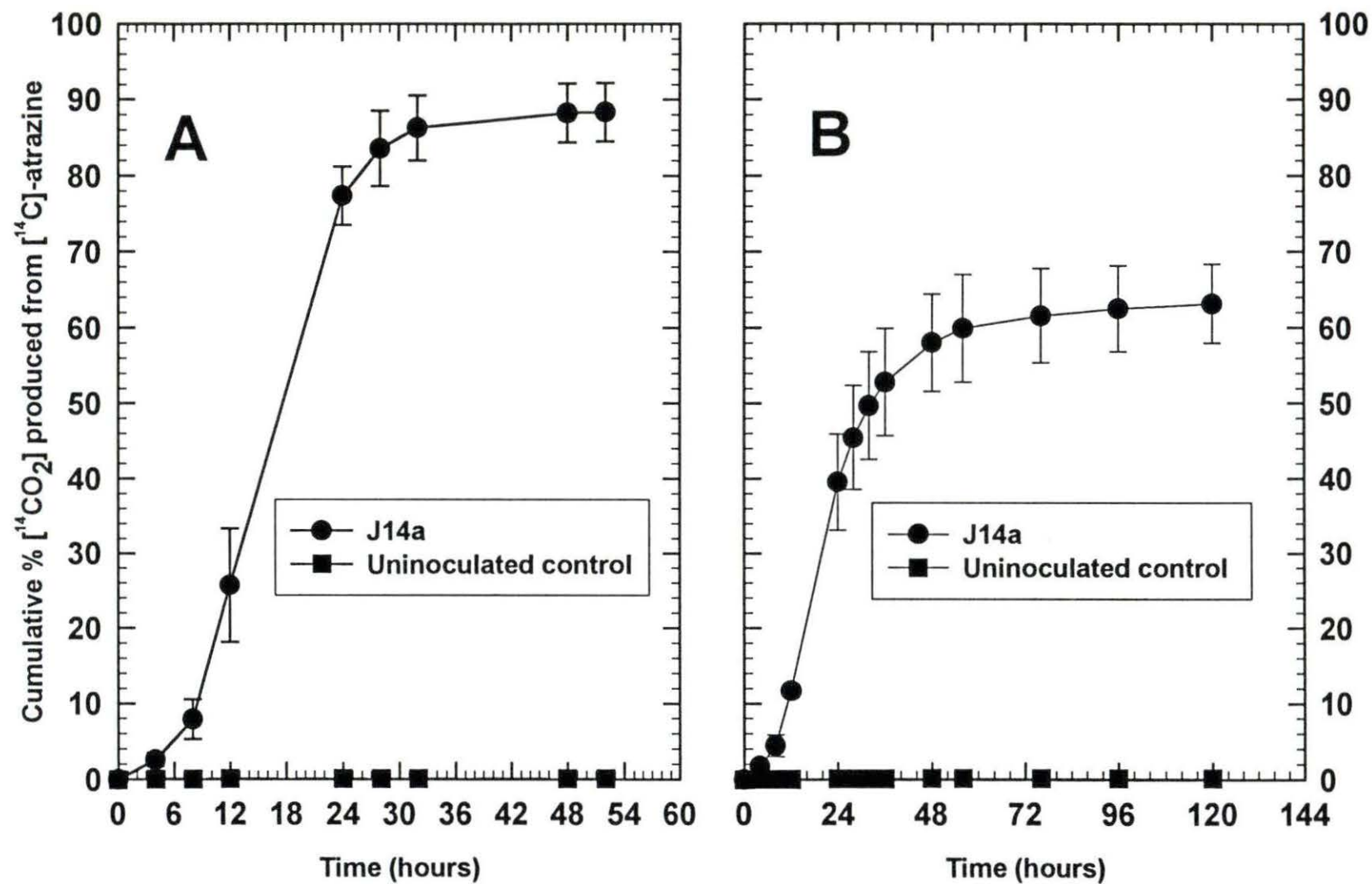
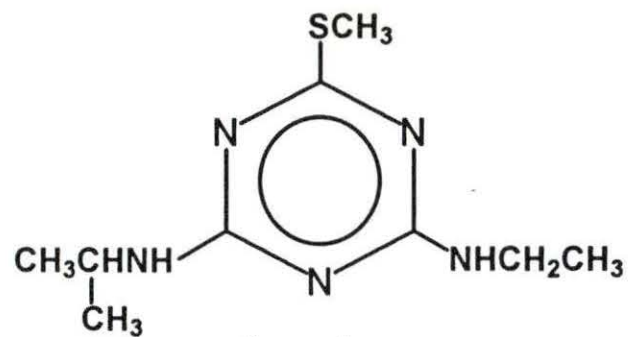


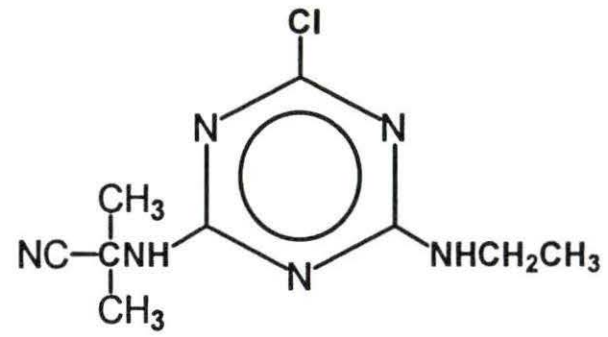
Figure 4. (A) J14a mineralization in BMA medium; (B) J14a mineralization in 0.5 TSB. Both cultures were grown in 0.5 TSB for 3 generations prior to transfer into the respective mineralization medium.

Enzyme substrate range. J14a utilized the herbicides ametryne, cyanazine, prometon, and simazine in the N-limited medium (Table 4, Figure 5). Metabolites from any of these compounds were not detected with our HPLC method. This may suggest that mineralization of some of these compounds occurred. However, J14a may have produced metabolites that our HPLC method is unable to detect. Due to solubility problems with propazine, we were unable to confidently state that J14a was capable of degrading propazine. However, additional peaks appeared in the medium by 120 hours, which could have indicated metabolite production from propazine. Additionally, an average decrease in the propazine peak size from the initial to final samples occurred indicating that a small amount (approximately 30%) of propazine was degraded.

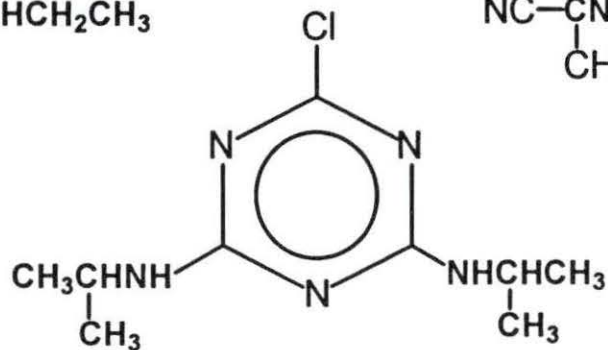
Mineralization in Alpha soil. Atrazine mineralization was enhanced by inoculation of J14a into the Alpha site soil (Figure 6). The two $50 \mu\text{g g}^{-1}$ treated soils amended with J14a mineralized five times more atrazine than the soils without the degrader (Figure 6A). No statistically significant differences were observed between the two J14a amended soils or the two soils without J14a at any of the time points; however a statistically significant difference ($p \leq 0.05$) between J14a amended and unamended soils was noted at all time points. The Alpha soil atrazine-mineralizing populations remained constant in the soils amended with J14a, with an initial population of 4.2×10^2 degraders g^{-1} soil and final populations 4.6×10^2 and 4.9×10^2 (Table 5), indicating that the population actually decreased because 10^5 J14a cells were added at day three of the experiment. The populations in the two soils without J14a declined to 49 and 23 degraders g^{-1} soil. The $200 \mu\text{g g}^{-1}$ soils (Figure 6B) showed mineralization trends similar to the $50 \mu\text{g g}^{-1}$ treatments. Both of the soils amended with J14a show enhanced mineralization (2 to 3X greater) and a faster initial degradation rate than the soils without degrader addition (Figure 6B). All of the $200 \mu\text{g g}^{-1}$ treatments contained a similar number of atrazine-mineralizing organisms ranging from 4.5 to 20 degraders g^{-1} soil. Despite the apparent differences in the total amount of ^{14}C -atrazine mineralized in the 50 and $200 \mu\text{g g}^{-1}$ treatments amended with J14a, the actual mass of atrazine mineralized is very similar in all four treatments, 26.7, 27.9, 37.6, and $28.2 \mu\text{g g}^{-1}$ atrazine (calculated from Table 6). Twice as much of the atrazine (on a mass atrazine per mass soil basis) was bound to the soil in the $200 \mu\text{g g}^{-1}$ soils as in the $50 \mu\text{g g}^{-1}$ soils. The



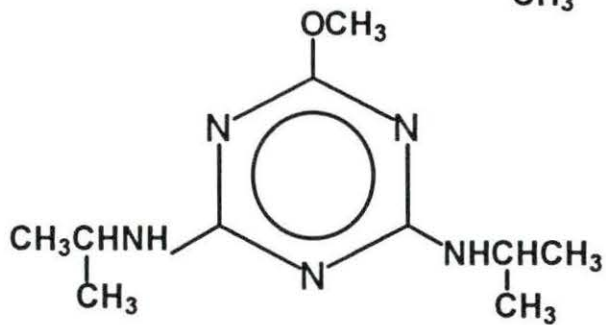
Ametryne



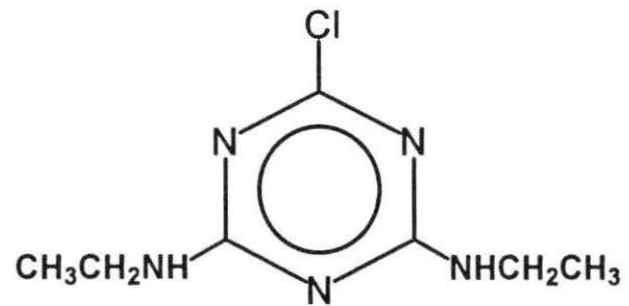
Cyanazine



Propazine



Prometon



Simazine

Figure 5. Structure of related triazines metabolized by *A. radiobacter* strain J14a.

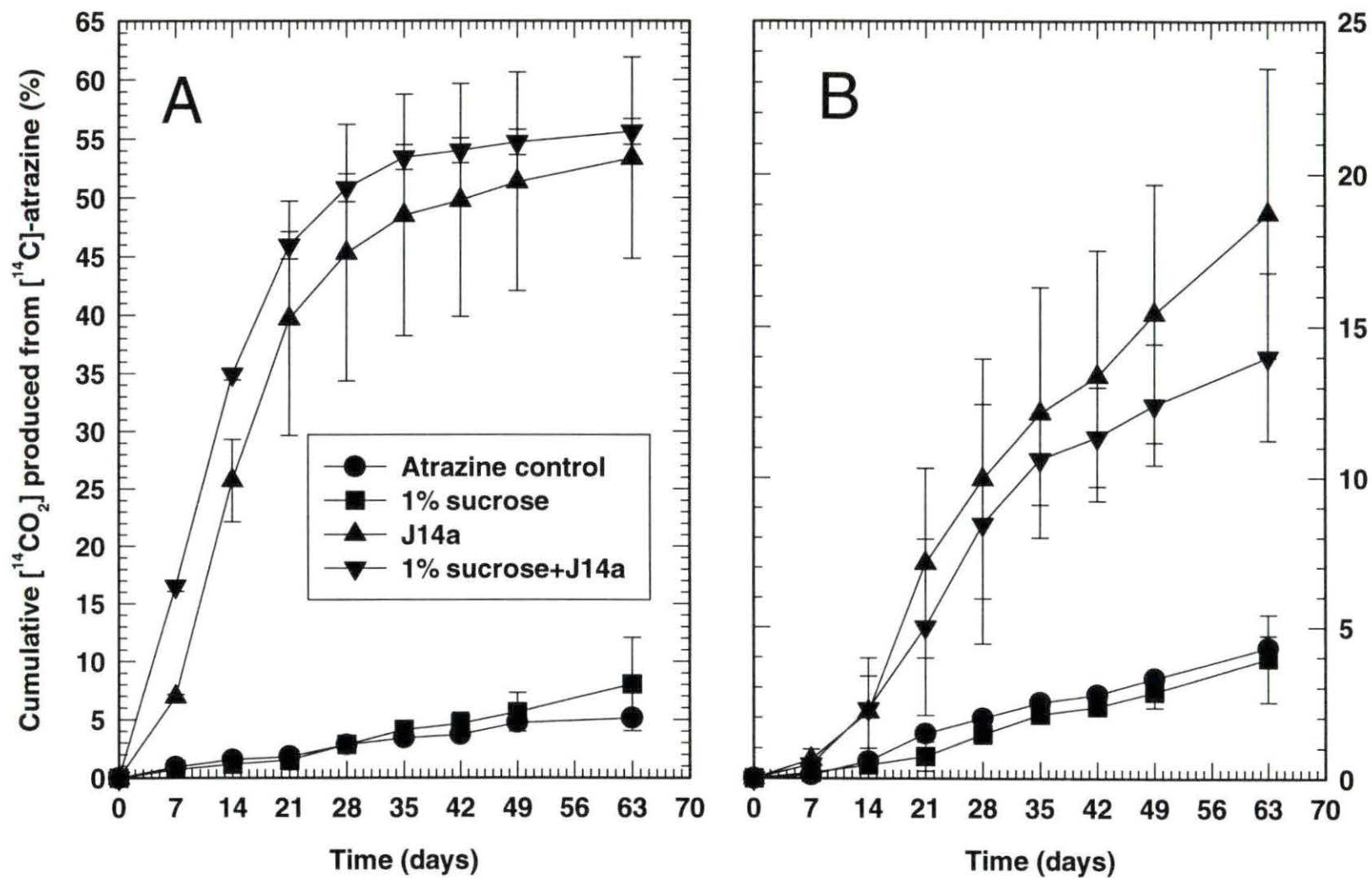


Figure 6. (A) Alpha soil mineralization with 50 µg g⁻¹ atrazine addition; (B) Alpha soil mineralization with 200 µg g⁻¹ atrazine addition. The legend for both figures is shown in part A.

Table 4. Degradation of *s*-triazines by *Agrobacterium radiobacter* strain J14a

Common name	Chemical name	Percent degradation*
Atrazine	6-Chloro-N ² -ethyl-N ⁴ -isopropyl-1,3,5-triazine-2,4-diamine	100
Simazine	6-Chloro-N',N'-diethyl-1,3,5-triazine-2,4-diamine	100
Propazine	6-Chloro-N',N'-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine	28
Cyanazine	2-((4-Chloro-6-(ethyl-amino)-1,3,5-triazin-2-yl)amino)-2-methylpropanenitrile	80
Prometon	6-Methoxy-N,N'-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine	62
Ametryne	N-Ethyl-N'-(1-methylethyl)-6-methylthio-1,3,5-triazine-2,4-diamine	71

*The percent degraded value is the average of three replicate flasks.

Table 5. Atrazine-degrading MPN populations 63 days following introduction of 10^5 *Agrobacterium radiobacter* strain J14a cells gdw^{-1} soil and 1% (w/w) sucrose into Alpha and Bravo soils at day three

Treatment	Alpha (cells g^{-1} soil)	Bravo (cells g^{-1} soil)
Background population	420	1400
<u>Day 63 populations</u>		
50 $\mu\text{g g}^{-1}$ atrazine	20	11,000
50 $\mu\text{g g}^{-1}$ atrazine+sucrose	23	4600
50 $\mu\text{g g}^{-1}$ atrazine+J14a	490	7900
50 $\mu\text{g g}^{-1}$ atrazine+sucrose+J14a	460	17,000
200 $\mu\text{g g}^{-1}$ atrazine	20	2300
200 $\mu\text{g g}^{-1}$ atrazine+sucrose	45	54,000
200 $\mu\text{g g}^{-1}$ atrazine+J14a	<20	28,000
200 $\mu\text{g g}^{-1}$ atrazine+sucrose+J14a	20	49,000
Average soil population	167	19,500

Table 6. ^{14}C mass balance at day 63 in the soils from the Alpha and Bravo sites^a

Treatment	Mineralized (%)	Extractable (%)	Bound (%)
<u>Alpha soil</u>			
50 $\mu\text{g g}^{-1}$ atrazine	5.1 (0.1) a ^b	65.7 (8.3) a ^b	16.7 (1.0) c ^b
1% sucrose	8.1 (4.1) a	64.7 (3.4) a	19.4 (1.1) d
J14a	53.4 (8.5) b	20.0 (2.4) b	14.4 (1.5) b
1% sucrose+J14a	55.7 (1.1) b	12.7 (0.3) b	10.8 (0.4) a
200 $\mu\text{g g}^{-1}$ atrazine	4.4 (0.4) a	71.5 (4.9) a	9.0 (1.4) a
1% sucrose	4.0 (1.5) a	69.1 (2.9) a	7.8 (0.7) a
J14a	18.8 (4.7) c	55.9 (12.7) a	9.9 (2.2) a
1% sucrose+J14a	14.1 (2.8) c	60.0 (2.0) a	8.0 (0.2) a
<u>Bravo soil</u>			
50 $\mu\text{g g}^{-1}$ atrazine	51.9 (1.6) a	11.6 (2.3) a	21.0 (3.6) b
1% sucrose	56.6 (2.6) a	13.3 (4.3) a	19.0 (3.1) b
J14a	57.0 (3.8) a	11.2 (1.7) a	19.4 (2.8) b
1% sucrose+J14a	68.6 (0.4) b	5.9 (1.7) a	12.1 (5.4) a
200 $\mu\text{g g}^{-1}$ atrazine	35.3 (5.7) c	30.9 (10.7) b	26.4 (0.8) c
1% sucrose	30.0 (11.9) c	31.0 (12.3) b	20.1 (3.1) b
J14a	29.6 (14.9) c	30.8 (11.2) b	22.1 (5.9) b
1% sucrose+J14a	25.5 (2.7) c	34.4 (3.9) b	20.8 (0.6) b

^aEach value is the mean of three replications with the standard deviation in parenthesis

^bDifferent letters following the standard deviation values indicate that the treatment means within each soil type are significantly different from each other at the $p \leq 0.05$ level. No letter following the standard deviation indicates that all of the conditions necessary to complete the One Way ANOVA and Student-Newman-Keuls t-tests in the Jandel Scientific SigmaStat program were not met by the treatment mean and standard deviation values.

recoveries of ^{14}C for the Alpha 50 and 200 $\mu\text{g g}^{-1}$ soils ranged from 79 to 92%, with an average recovery of 85%.

Bravo soil mineralization. The Bravo site soil contained an active indigenous atrazine-mineralizing population of 1.4×10^3 degraders g^{-1} soil, (Table 5), which rapidly mineralized [^{14}C -UL-*ring*]-atrazine applied at various concentrations (Chapter 3, Figure 2). In the soils treated with 50 $\mu\text{g g}^{-1}$ atrazine (Figure 7A), inoculation of J14a increased the initial atrazine mineralization rate over those soils without J14a; however, only the J14a+sucrose soil showed a significantly larger amount of atrazine mineralization throughout the entire incubation period. The soils treated with 200 $\mu\text{g g}^{-1}$ atrazine (Figure 7B) did not show statistically significant differences in the final amount mineralized, but the two soils amended with J14a had a more rapid initial degradation rate than those without J14a. The Bravo site soil contained larger atrazine degrading populations than the Alpha site soil, with an average population size of 19,500 cells g^{-1} soil versus only 167 cells g^{-1} soil in the Alpha soil (Table 5). The Bravo soil tended to support larger populations in the 200 $\mu\text{g g}^{-1}$ than the 50 $\mu\text{g g}^{-1}$ soils. The soil from Bravo showed a higher portion of bound residue than the Alpha site soil, with overall levels of bound residues in Bravo and Alpha soils of 20% and 12%, respectively (Table 6). The radioactivity recoveries for the Bravo treatments ranged from 81 to 93%, with an average recovery of 86%.

DISCUSSION

Atrazine degrading microorganisms. Prior to this work, the bacterial isolates capable of degrading *s*-triazines have been classified into three main taxonomic groups: (a) the genus *Pseudomonas* (Behki and Khan, 1986; Mandelbaum *et al.*, 1995; Yanze Kontchou and Gschwind, 1994); (b) the genus *Klebsiella* isolated from sewage sludge (Cook, 1987; Cook and Hutter, 1981; Hapeman *et al.*, 1995); and (c) the genus *Rhodococcus* (Behki *et al.*, 1993; Cook, 1987; Mulbry, 1994; Shao and Behki, 1995; Van Zwieten and Kennedy, 1995). In addition to these atrazine-degrading isolates, Alvey and Crowley (1996) have identified a consortium of bacteria, including a *Pseudomonas* sp., a *Clavibacter* sp., and a *Cytophaga* sp., which is capable of mineralizing atrazine when added to liquid culture and soil in combination. We have isolated an atrazine-degrading organism from an additional genus, *Agrobacterium*. *Agrobacterium* sp. and *Pseudomonas*

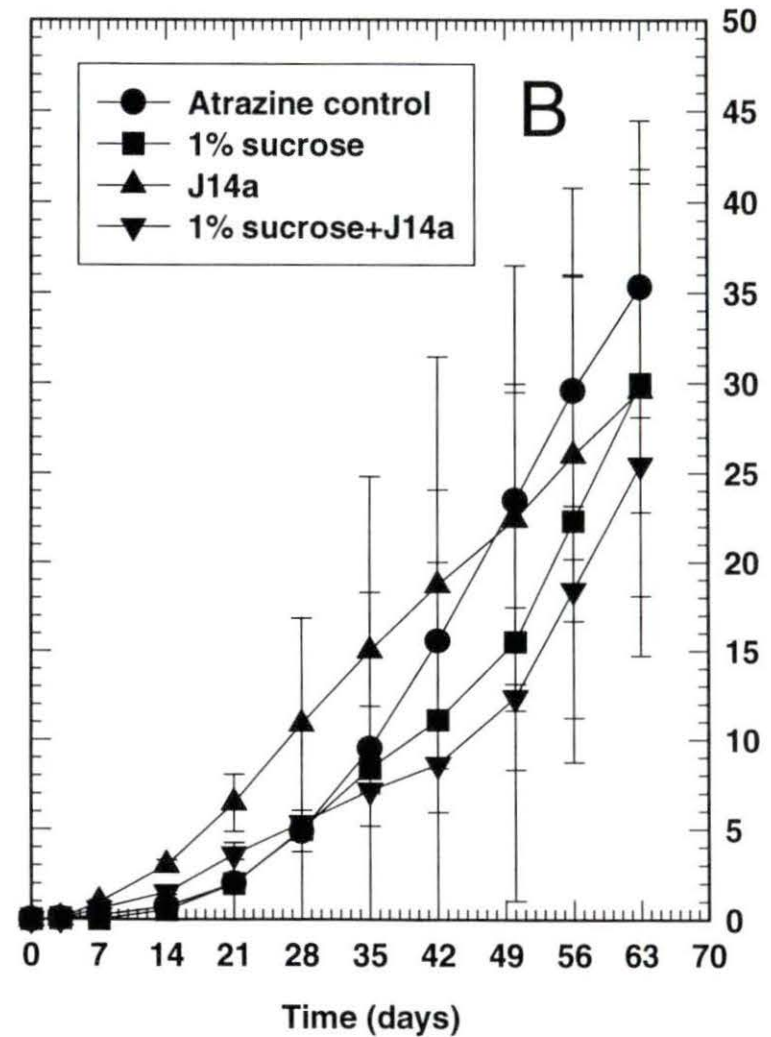
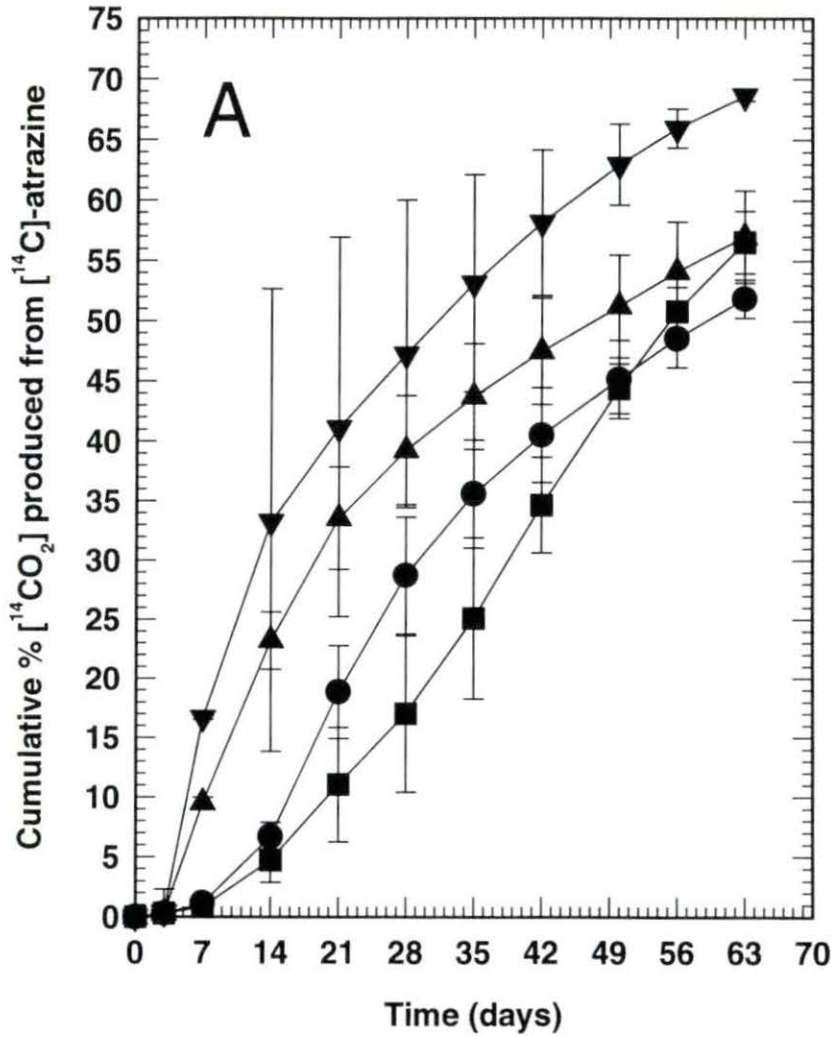


Figure 7. (A) Bravo soil mineralization with 50 µg g⁻¹ atrazine addition; (B) Bravo soil mineralization with 200 µg g⁻¹ atrazine addition. The legend for both figures is shown on part B.

alcaligenes were reported to biodegrade atrazine in a synergistic reaction (Mirgain *et al.*, 1993). Additionally, Radosevich *et al.* (1995) isolated a bacterium capable of partial atrazine mineralization that was inconclusively identified by the API-NFT test as *Agrobacterium radiobacter*.

Recently, three bacterial species have been reported that are capable of atrazine mineralization. Two of these microorganisms, *Pseudomonas* sp. (Mandelbaum, 1995) and an unidentified Gram (-) organism (Radosevich *et al.*, 1995) mineralize atrazine for the N in the triazine ring. The third isolate, *Pseudomonas* sp., uses atrazine as a sole C and energy source (Yanze Kontchou and Gschwind, 1994).

J14a metabolite production. J14a produces three metabolites, HA, DEHA, and DEA, along with CO₂ during the degradation of atrazine. The production of these three metabolites indicates that J14a produces enzymes that perform the three main steps in the atrazine degradation process, N-dealkylation, dehalogenation, and ring cleavage, concomitantly. Because both HA and DEA are present, it appears that the N-dealkylation and dehalogenation enzymes are produced and function concomitantly. The lack of DIA and hydroxydeisopropylatrazine (DIHA) accumulation indicates that either (a) J14a preferentially removes the ethyl over the isopropyl side chain, which is consistent with other reports (Assaf and Turco, 1994b; Behki *et al.*, 1993; Mougín *et al.*, 1994) or (b) removal of the isopropyl side chain is the rate limiting step of the J14a atrazine metabolic pathway and these metabolites are utilized immediately by additional enzymes once they are produced. The larger concentration of HA in the TSB-grown cell treatment indicates either that (a) the organic N (or some other compound) in the TSB is repressing only the ring-cleavage enzymes, but not inhibiting the dechlorination enzymes; or (b) the atrazine-degrading enzymes of BMA-grown cells could be more synchronous, resulting in a condition where no metabolites accumulate.

J14a growth and utilization of atrazine. The growth of J14a under N-limited conditions demonstrates that J14a metabolizes atrazine for use as a sole N source. J14a released approximately 95% of the ¹⁴C from the [¹⁴C-UL-ring]-atrazine as ¹⁴CO₂, which is consistent with the fact that the ring C is in a completely oxidized state and indicates that complete mineralization of the atrazine has occurred. The ζ kinetic parameter estimate of 0.03 for both the Monod and logistic models indicates that essentially none of the ¹⁴C from

the atrazine ring structure was incorporated into J14a biomass. This is consistent with the atrazine metabolism pathway described in previous reports where the atrazine *s*-triazine ring is cleaved and NH_4^+ and CO_2 are produced (Behki and Khan, 1986; Cook, 1987; Cook and Hutter, 1981).

Mineralization of [^{14}C -ethyl]-atrazine showed that approximately 25 to 30% of the N-ethyl side-chain was assimilated into cell biomass by 124 hours for both the C- and N-limited treatments, which is comparable to substrate incorporation data seen in previous experiments in which microorganisms degrade recalcitrant molecules (Alexander, 1994). Evidently the amount of C incorporated into biomass from the atrazine is not enough to support growth, because the J14a cells grown in C- and N-limited [^{14}C]-ethyl side-chain medium incorporated the same percentage of atrazine as the C-amended flasks, but were unable to grow. The cells grown in C amended medium assimilated the C from the sucrose into biomass and were able to grow. J14a is capable of degrading and utilizing atrazine under C and N limiting conditions. Under these conditions, it is unable to grow, but is able to maintain the initial population size. This indicates that the ethyl- and isopropyl-sidechains of atrazine at this concentration do not provide enough C to support growth of this J14a population.

J14a degrades atrazine in the presence of the inorganic nitrogen sources NH_4NO_3 , KNO_3 and $(\text{NH}_4)_2\text{SO}_4$. Mandelbaum *et al.* (1995) showed that NH_4NO_3 suppressed atrazine degradation by a *Pseudomonas* sp. One might have predicted that J14a also would have preferentially used a more easily utilizable N source, such as the NH_4^+ or the NO_3^- , rather than utilizing the N present in the atrazine sidechains and ring structure. The population size in the medium with NH_4NO_3 addition is larger than that in the standard medium. This result suggests that once the atrazine N was depleted, the J14a cells switched over to utilize the N in the NH_4NO_3 and the excess C remaining from the sucrose and sodium citrate to produce additional biomass. These results are an indication that the J14a atrazine metabolizing enzymes are produced constitutively and, therefore, are not regulated by inorganic N concentrations in the environment.

Rapid mineralization of atrazine by J14a cells grown for several generations in TSB without exposure to atrazine, along with the results from the TSB mineralization experiments, provide further evidence that the J14a atrazine-degrading enzymes are produced constitutively. Other studies have concluded that atrazine-degrading enzymes

can be constitutively produced (Cook, 1987). Constitutive enzyme production suggests that J14a does not need exposure to atrazine or a degradation product in order to begin degrading atrazine when added to a spill site; therefore, the potential for immediate atrazine degradation is present. The TSB mineralization curves also indicate that mineralization continues upon exposure to organic N sources, such as the organic components of tryptic soy broth. However, as indicated by the incomplete mineralization of atrazine in Figure 3B (only about 63%), when J14a is grown in TSB mineralization medium, it does appear that partial inhibition of the atrazine degradation is achieved upon exposure to organic N or some other compound in the TSB.

The atrazine-degrading enzymes of J14a demonstrated a wide substrate range degrading capability upon exposure to a variety of *s*-triazine substrates in N-limited medium. J14a was capable of degrading every *s*-triazine substrate tested, unlike the *Pseudomonas* sp. isolated by Yanze Kontchou and Gschwind (1994), which could not degrade cyanazine or ametryne. This indicates that the enzymes used by J14a to degrade atrazine are not specific for atrazine. The non-specificity, constitutive production, and rapid degradative capabilities of the J14a degradative enzymes indicate that the organism would be ideal for addition to a spill site with a mixture of *s*-triazine herbicides.

Addition of J14a to soils with different characteristics resulted in two different patterns of activity. These differences in patterns of atrazine biodegradation in soil may be due to a variety of factors including inoculum survival, microbial competition, atrazine sorption, protozoa predation, and soil physical and chemical properties. In the Alpha soil, J14a augmentation was successful in partially remediating the soil at both herbicide concentrations, resulting in both a faster mineralization rate and a much higher total of mineralized atrazine than that by the indigenous microbial populations. Due to the apparent short survival period of J14a in soil, more complete bioremediation of the Alpha soil may be achieved if J14a inoculum were added to the soil at several time points throughout the incubation period. Bioaugmentation did not seem necessary in the Bravo soil, achieving only a reduction in the initial degradation lag time and the faster initial degradation rates. The indigenous Bravo atrazine-mineralizing populations were capable of achieving essentially the same degree of bioremediation by the end of the incubation period.

Addition of sucrose did not seem to enhance the mineralization of atrazine in the Alpha soil or the 200 $\mu\text{g g}^{-1}$ treatment in the Bravo soil, but did allow for significantly higher

mineralization in the Bravo 50 $\mu\text{g g}^{-1}$ atrazine treatment amended with J14a. In the Alpha soil this may have been due to the rapid growth of a fungal population which covered the surface of the soil with a mat, possibly outcompeting J14a for the sucrose and limiting the diffusion of the O_2 supply into the soil. In a soil containing 1500 ppm aged atrazine residue, Mandelbaum *et al.* (1995) observed a much larger effect on atrazine degradation with the addition of sodium citrate than the atrazine mineralizing by the *Pseudomonas* strain alone, with 35% and 17% of the 1500 ppm atrazine degraded, respectively. However, the greatest treatment effect was observed when a combination of sodium citrate and the *Pseudomonas* strain was added to the soil, with a decline of 70% of the 1500 ppm observed.

The J14a cells added to the Alpha and Bravo soils did not survive the entire 63 day incubation period. Atrazine-mineralizing populations (as determined by the MPN technique) in the J14a-augmented soils were similar to the indigenous population size in the unaugmented soils. The J14a cells may have been outcompeted by the indigenous populations once the easily degradable C supply was depleted. Both the Bravo and Alpha soils are considerably limited in terms of C (Bravo soil, 2.4% organic C; Alpha soil, 3.2% organic C), N (Bravo soil, 0.05% N; Alpha soil, 0.07% N), and possibly phosphorus. The Bravo soil has a fairly low microbial respiration rate of $3.7 \pm 2.0 \mu\text{g CO}_2$ produced gdw^{-1} soil min^{-1} , indicating that only a small amount of the C present in the soil is bioavailable for microbial utilization. Nutrient limitations will limit the amount of atrazine that can be degraded by J14a and may favor the indigenous populations that are already adapted to the oligotrophic conditions in the soil. Yanze Kontchou and Gschwind (1995) inoculated soils with their *Pseudomonas* strain, preincubated the cells in the soil for 21 and 42 days, added 10 μg atrazine g^{-1} soil, and incubated the soils for an additional two and seven days. The *Pseudomonas* inoculum survived the six-week incubation period without atrazine exposure and degraded 98% of the atrazine in the 21-day incubated soil and 91% of the atrazine after 42 days of incubation. Survival of an atrazine-mineralizing consortium (determined by the MPN technique) in soil spiked with three μg atrazine g^{-1} was 30 times greater when added to soil with corn plants versus survival in unplanted soil; however, no significant difference was observed in the amount of atrazine mineralized in the planted versus unplanted soil, with 84 and 71% mineralized, respectively (Alvey and Crowley, 1996).

In all three of the studies involving the addition of atrazine-mineralizing bacterial strains into soil, the researchers air-dried the soil and rehydrated it to a set moisture content

(Alvey and Crowley, 1996; Mandelbaum *et al.*, 1995; Yanze Kontchou and Gschwind, 1995). Air-drying soil almost always results in the death of desiccation-sensitive microbial populations and reduces the diversity of the indigenous soil populations. A portion of these organisms that perished could have been atrazine-degrading organisms and therefore would be unable to compete with the bioaugmentation organisms for the available atrazine. Reduction in the soil microbial population size and diversity will enhance the survivability of the added organisms, because the added isolates will also have less competition for the available C and energy sources and the inorganic nutrients. Mandelbaum *et al.* (1995) mixed their aged, highly contaminated soil (4% atrazine w/w) with atrazine-free soil from an adjacent field to reduce the concentration of atrazine to 1500 ppm. This could provide less competition for the added organism because the added soil had not been exposed to the atrazine spill and probably contained lower populations of atrazine-degrading microorganisms.

In addition to air-drying the soil, the other bioaugmentation studies also added a higher number of degraders as the soil inoculum. In our study only 1×10^5 J14a cells were added g^{-1} air-dried soil. Mandelbaum *et al.* (1995) added 2×10^6 cells g^{-1} air-dried soil and Alvey and Crowley (1996) added 4.5×10^6 cells g^{-1} air-dried soil. Yanze Kontchou and Gschwind (1995) inoculated the soil with 2.8×10^{10} cells g^{-1} air-dried soil and also a 5-fold and 25-fold dilution of the same inoculum. As the dilution factor increased, the initial atrazine degradation rate decreased. Ramadan *et al.* (1990) conducted a study in lake water to determine the effect of inoculum size on the degradation of *p*-nitrophenol. They determined that a species able to degrade a chemical in a rich medium culture may fail to degrade the same chemical if added as a small inoculum to oligotrophic environments due to elimination by protozoan grazing or nutrient deficiencies the organisms to which the organism may not be acclimated.

It has been shown by time-course experiments for atrazine sorption by whole aggregates analyzed using a modified first-order regression equation that almost all of the sorption of atrazine to soil organic matter occurs within the first 24 hours after addition of the herbicide to the soil (Novak *et al.*, 1994). We allowed 3 days of incubation of the soil with the spiked atrazine prior to addition of J14a to the soil, most likely resulting in more atrazine bound residue and less atrazine dissolved in the soil water. It did not appear that either Alvey and Crowley (1996) or Yanze Kontchou and Gschwind (1995) aged the soil with

the atrazine prior to addition of the degraders, enhancing the bioavailability of atrazine for their atrazine degraders. As time increases, the concentration of atrazine in the solution and water-desorbable phases will decrease, while the proportion of atrazine which is hydrophobically bound to soil organic matter or has diffused into grains of the soil matrix (bound residue) increases and becomes much less bioavailable (Alexander, 1994; Alexander, 1995; Ball and Roberts, 1991; Wood *et al.*, 1989, 1990). Therefore, addition of J14a to a site contaminated with aged atrazine residues may result in only limited atrazine mineralization. The atrazine-mineralizing *Pseudomonas* sp. isolated by Mandelbaum *et al.* (1995) was capable of degrading 250 ppm of 10-year aged atrazine residues within a three-week incubation period when added at a rate of 2×10^6 cells g^{-1} air-dried soil. However this aged, atrazine-contaminated soil containing 4% (w/w) atrazine was mixed with atrazine-free soil from an adjacent field which could lead to increased bioavailability of the atrazine due to dissolution into the soil water of the added soil. Yanze Kontchou and Gschwind (1995) noted that preincubation of atrazine in a soil with high organic matter content (36%) lowered the bioavailability of the atrazine and limited the atrazine degradation rate by the *Pseudomonas* sp.

Despite the higher percentage of C in the Alpha versus the Bravo soil, 3.2 and 2.4% respectively, there were higher bound residue levels in the Bravo soil, especially in the $200 \mu g g^{-1}$ treatments. One possible explanation for the higher amount of bound residue in the Bravo soil, is that the organisms indigenous to Bravo soil may produce more hydroxyatrazine (HA) than the Alpha site organisms during atrazine degradation. HA ($\log K_{om}=2.0-2.8$) sorbs more strongly to soil organic matter than atrazine ($\log K_{om}=1.6-2.0$), making HA unavailable for further degradation and resulting in an accumulation of radiolabeled material in the bound residue fraction (Brouwer, 1990).

Another possible explanation for the low numbers of the atrazine-degrading microorganisms is predation by protozoa. Several other researchers have attributed dramatic decreases in actively growing augmentation and indigenous soil, sewage, and lake water microbial populations to active bacteriophagous protozoa populations which consume the bacteria (Gurijala and Alexander, 1990; Mallory *et al.*, 1983; Ramadan *et al.*, 1990; Sinclair and Alexander, 1989; Wiggins and Alexander, 1988).

The rapid degradation of atrazine, constitutive expression of the degradative enzymes, and the wide substrate range of strain J14a indicate that J14a should be effective

as a bioaugmentation agent. Despite these characteristics, our results indicate that competition and survival of the inoculum are important to the effectiveness of introduced degraders. Nevertheless, the addition of J14a to soils freshly contaminated by atrazine or other *s*-triazines herbicides should result in extensive degradation if a fresh supply of inoculum is provided periodically throughout the remediation time period.

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CHAPTER 3. ORGANIC AND INORGANIC AMENDMENTS TO ENHANCE BIODEGRADATION OF HERBICIDES IN CONTAMINATED SOILS

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ABSTRACT

Herbicide contamination of the surface soil and groundwater by agricultural chemical dealerships due to accidental spills and careless mixing, storage, and disposal of herbicides is a major concern in the United States. Soil collected from three agricultural chemical dealerships, two in Iowa (code-named Alpha and Bravo) and the third in Illinois (code-named Echo), was screened for the ability to mineralize atrazine. Approximately 70% of applied [^{14}C -UL-*ring*]-atrazine was mineralized in Bravo and Echo soils when applied at $5\ \mu\text{g}\ \text{atrazine}\ \text{g}^{-1}\ \text{soil}$, but only 45% to 55% was mineralized at $100\ \mu\text{g}\ \text{atrazine}\ \text{g}^{-1}\ \text{soil}$. However, when $200\ \mu\text{g}\ \text{atrazine}\ \text{g}^{-1}\ \text{soil}$ was applied to the Bravo soil in combination with $200\ \mu\text{g}\ \text{trifluralin}$ and $200\ \mu\text{g}\ \text{metolachlor}\ \text{g}^{-1}\ \text{soil}$, atrazine mineralization ceased, and the atrazine mineralizing populations declined from 1950 to only 110 cells $\text{g}^{-1}\ \text{soil}$. A herbicide-contaminated soil collected from Echo site was not capable of mineralizing atrazine when applied at either the 5- or $100\text{-}\mu\text{g}\ \text{g}^{-1}$ concentration. In an attempt to enhance the microbial biomass size and activity, the Bravo soil was spiked with the $200\ \mu\text{g}\ \text{g}^{-1}\ \text{soil}$ combination of atrazine, metolachlor, and trifluralin and was amended with two different levels of organic (0.5% or 5% w/w) and inorganic (1 or 2 ml $50\ \text{g}^{-1}\ \text{soil}$) amendments. These amendments included municipal compost, sawdust, cattle manure, peat, a corn fermentation byproduct, cornstalk, and an inorganic solution containing CaSO_4 , K_2HPO_4 , and NH_4NO_3 to achieve an approximate N:P:K:S ratio of 10:1:1:1. Several amendments enhanced microbial activity and increased microbial populations, however only the manure (0.5% and 5%), 5% peat, and 5% cornstalk treatments significantly increased atrazine degradation over the unamended, herbicide-spiked treatment. Metolachlor degradation was enhanced over the unamended, herbicide-spiked treatment by virtually all of the amendments, with the higher amendment rate showing more degradation in all cases. Essentially the same amount of trifluralin was degraded in each of the amended samples as in the

unamended treatment. Because none of the amendments were completely effective in remediating soil contaminated with the herbicide mixture, the Bravo soil was treated with each of the three herbicides alone and was amended with 5% sawdust and 5% manure to examine whether biostimulation enhanced degradation of the individual herbicides in Bravo soil. Sawdust-amendment of the Bravo soil enhanced the degradation of trifluralin, while amendment with manure resulted in enhanced metolachlor degradation. Sawdust amendment appeared to repress degradation of atrazine-treated soil, while manure addition did not affect degradation compared to the unamended, treated soil. Application of trifluralin with [¹⁴C]-atrazine at 200 µg g⁻¹ yielded similar amounts of atrazine mineralized as the soil treated with atrazine alone, while the metolachlor applied with atrazine in the 200 µg g⁻¹ combination resulted in complete inhibition of atrazine mineralization, indicating a toxic metolachlor effect.

INTRODUCTION

It is estimated that 90% of the agricultural chemical dealerships in the state of Iowa have some degree of detectable contamination, with up to half of the sites having groundwater contamination exceeding the state's cleanup guidelines due to accidental spills or careless mixing and loading of the chemicals (Gannon, 1992). Since nearly half of all U.S. residents and, in some areas, 95% of rural residents get their drinking water from private or public wells that tap into groundwater supplies, efforts to prevent pesticides from entering groundwater are extremely important (U.S. EPA, 1991). In several midwestern states in the United States, including Iowa, Kansas, Wisconsin, and Illinois (Frieberg, 1991; Gannon, 1992) and Canada (Frank *et al.*, 1987a; Frank *et al.*, 1987b) correlations were observed between the occurrence of herbicide-contaminated private and public wells and the presence of nearby agricultural chemical dealerships. In a 1987 study, 16 of the 18 Iowa public water system wells with detections of pesticides other than atrazine were within 1,000 feet of an agricultural chemical dealership (Fawcett, 1989). A total of \$1.8 million had been spent as of 1992 to determine the level of contamination in the soil and groundwater at 22 Iowa agricultural chemical dealerships (Gannon, 1992). Only 5 of the 22 sites have conducted or are planning to conduct remediation. The Iowa Fertilizer and Chemical Association estimated that it could cost between \$50 and \$100 million to assess, monitor,

and remediate the contaminated dealerships in Iowa alone (Gannon, 1992). With these costs increasing in the future, it is important to conduct research on potentially economical and efficient strategies to remediate these sites.

Several studies have investigated the effects of biostimulation, the addition of nutrients to enhance degradation of a contaminant, on remediating a single herbicide contaminant in soil (Alvey and Crowley, 1995; Assaf and Turco, 1994; Jones and Alexander, 1988; Mitra and Raghu, 1986; Schmidt and Alexander, 1985; Swindoll *et al.*, 1988; and Zablutowicz and Dzantor, 1994). After a single mannitol addition to soil contaminated with atrazine, only 39% of the atrazine was mineralized after 326 days incubation (Assaf and Turco, 1994). Addition of a second aliquot of mannitol at day 140 resulted in an additional 17% mineralization of atrazine. Amendment of DDT-contaminated soil with rice straw decreased the persistence of DDT, from 66% to only 22% remaining after 70 days, and enhanced the formation of the metabolite DDD (Mitra and Raghu, 1986). Addition of chicken litter to unsaturated and saturated soil treated with trifluralin resulted in 13% and 72% more degradation than the unamended soil, respectively, in 30 days, while addition of soybean meal resulted in 37% and 57% more trifluralin degradation in the same time period (Zablutowicz and Dzantor, 1994). Addition of cornmeal to the same unsaturated soil contaminated with alachlor, resulted in a 17% increase in the amount of degradation over the unamended soil.

However, in most cases, herbicides are present at agricultural chemical dealerships in mixtures and in some areas at high concentrations. Only a few studies have focused on the interactive effects on the microorganisms and herbicide persistence when multiple herbicides occur at high concentrations (Dzantor *et al.*, 1993; Dzantor and Felsot, 1991; Schoen and Winterlin, 1987; Winterlin *et al.*, 1989). These results indicate that herbicides can also be toxic to the microbial populations at high concentrations. Dzantor *et al.* (1993) landfarmed soil contaminated with $\geq 100 \mu\text{g g}^{-1}$ trifluralin and alachlor and $\geq 20 \mu\text{g g}^{-1}$ atrazine and metolachlor. Dehydrogenase activity was highest in the plots amended with organic material, but about the same amount of alachlor was degraded in the amended and unamended plots, with 85-95% and 75-85% degradation in the plots, respectively. A similar trend was observed in the trifluralin-contaminated plots with 70-80% degradation in the corn-meal amended plots and 60-75% degradation in the unamended plots. Dzantor and Felsot (1991) treated soil with

10 and 10,000 $\mu\text{g g}^{-1}$ of alachlor alone and as a mixture of alachlor, trifluralin, and atrazine, with the lower concentration representing typical field application rates and the high concentration simulating a spill site. Bacterial populations were initially depressed in the high-concentration soil, but recovered after 7 days' incubation, while the fungal populations dropped to below detection limit after 7 and 21 days in the mixed herbicide and alachlor only treatments. Dehydrogenase activity was reduced dramatically after only 1 day and had not recovered by day 125. Herbicide degradation was minimal in the high concentration treatments; however, little difference was observed in the alachlor degradation applied alone or as a mixture after one year.

The effect of several soil factors, including soil type, pH, moisture content, organic matter content, microbial activity, and pesticide concentration, on the degradation of a mixture of pesticides (atrazine, captan, carbaryl, 2,4-D, diazinon, fenitrothion, and trifluralin) was studied (Schoen and Winterlin, 1987). The pesticide concentration was the most important variable with lower rate constants and longer half-lives at 1000 $\mu\text{g g}^{-1}$ than at 100 $\mu\text{g g}^{-1}$ rate of addition. Winterlin *et al.* (1989) also studied degradation of aged residues and freshly applied herbicides, including atrazine, trifluralin, malathion, diazinon, chlorpyrifos, and parathion. They observed that the soil pH was a major factor in pesticide degradation, with half-lives of most pesticides being shorter in the highest pH soils under anaerobic conditions, while the half-lives were longer at the lowest pHs in the aerobic treatments. They recommended a remediation treatment consisting of alternating moist and flooded conditions with addition of a heavy lime treatment in combination with an organic source such as manure to effectively remediate pesticide contaminated waste sites.

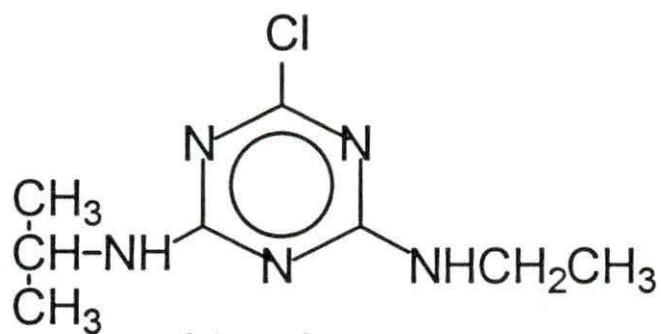
Due to the presence of high concentrations of herbicide mixtures at contaminated dealership sites, it can be predicted that a toxic effect on the degrading microorganisms exists (Moorman, 1989). Because an effective indigenous population most likely existed at most of these sites prior to contamination with the herbicides, biostimulation may be necessary to overcome the toxic herbicidal effects (Kruger, 1996). The amendments are thought to work by stimulating the metabolic activity of the degrading populations or by eliminating a nutrient limitation which may exist in the environment. Biostimulation is predicted to be particularly effective in enhancing the degradation of those herbicides which are degraded cometabolically, such as metolachlor and trifluralin.

In this study we collected two soils from agrochemical dealerships in Iowa and two soils from a dealership in Illinois and determined if the indigenous microbial populations were capable of mineralization of atrazine. We then looked at how amendment addition into one of the Iowa dealership soils affected the biodegradation of atrazine, metolachlor, and trifluralin, which were ranked first, third, and seventh in usage during 1990-91 (Ware, 1994) (Figure 1). We evaluated the effects of the herbicide combinations and amendments on microbial activity and population density.

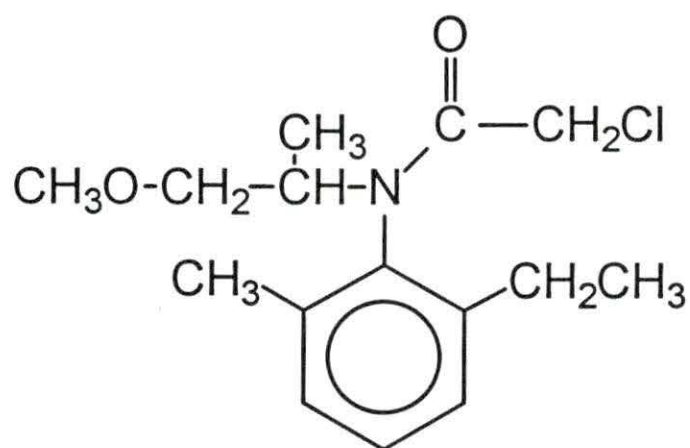
MATERIALS AND METHODS

Chemicals. Atrazine (92.2% purity) and metolachlor (97.3% purity) were received as a gift from Ciba Crop Protection (Greensboro, NC). Trifluralin (99.0% purity) was purchased from ChemService, West Chester, PA. Uniformly ring-labeled [^{14}C]-atrazine ($87.8 \mu\text{Ci mg}^{-1}$, 98% radiochemical purity) was purchased from Sigma Chemical Co., St. Louis, MO. 2,3,5-Triphenyl formazon and 2,3,5-triphenyltetrazolium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Fisher Scientific HPLC-grade ethyl acetate was used for the herbicide residue extractions and Baxter and Jackson Chrompure HPLC solvent grade methanol was used for the dehydrogenase assay.

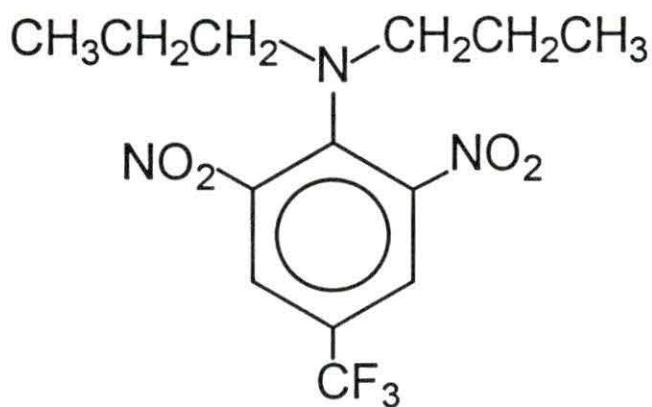
Soil collection and preparation. We obtained soil samples from two Iowa agricultural chemical dealerships (code-named Alpha and Bravo) and an Illinois dealership (code-named Echo). The top 10 to 14 cm of soil at the Alpha site were collected using hand trowels, placed into Whirlpak bags, and put on ice for transport back to the laboratory. The soil was mixed thoroughly, passed through a 2.5-mm sieve, and stored at 4°C. The Alpha soil had the following characteristics: a sandy loam classification, with 75% sand, 17% silt, and 8% clay; 3.2% organic C; 0.07% total N; and pH 7.9. At the Bravo site, soil was collected from a drainage pathway area on the site. The upper 5 cm of soil was scraped up with shovels and placed into 40-gallon stainless steel garbage cans for transport back to the laboratory. The soil was then passed through a 4-mm sieve and stored at room temperature. The soil had the following characteristics: a loamy sand texture classification with 78% sand, 18% silt, and 4%



Atrazine



Metolachlor



Trifluralin

Figure 1. Structures of atrazine, metolachlor, and trifluralin.

clay; 2.4% organic C, 0.05% total N; and pH of 6.5. The water content equivalent to half-bar water potential was determined in a pressure plate apparatus, but this value, 4.7% moisture, was lower than the field moisture value of 6.3% so the moisture content was not adjusted for the first biostimulation experiment. However, due to difficulties with maintaining this moisture content throughout the first experiment, the Bravo soil water content was adjusted to 10% moisture for the remaining experiments.

Two different soil samples were collected from the Echo site in Illinois. The first soil sample was collected from an area (site 1) at the edge of the dealership site that was not impacted by dealership activities. This soil (E1) acted as a control soil for the site to obtain background microbial measurements. The upper 5 cm of the surface soil were placed in a plastic-lined container for transport back to the laboratory. This soil had the following characteristics: loam texture classification with 40% sand, 42% silt, and 18% clay and a pH of 6.6. The second soil collected from the Echo site was from a highly contaminated area on the site located next to the mixing and loading pad (site 2). This soil was collected by scraping away the overlying gravel and collecting the upper 10 cm of surface soil with a shovel. This soil will be referred to as Echo site 2 (E2). Both Echo soils were passed through a 4-mm sieve and stored at room temperature. The soil was classified as sandy loam with 70% sand, 18% silt, and 12% clay and a pH of 6.4. It contained $>1000 \mu\text{g g}^{-1}$ of trifluralin, $>1000 \mu\text{g g}^{-1}$ pendimethalin, $180 \mu\text{g g}^{-1}$ atrazine, $130 \mu\text{g g}^{-1}$ alachlor, and trace amounts of additional herbicides as determined by ethyl acetate extraction and gas chromatography (Kruger, 1996). CaCl_2 desorption analysis was done on the E2 soil to determine what portion of the herbicides were potentially bioavailable to soil microorganisms. Nine replicate three-g dry weight (gdw) subsamples of the soil were weighed into glass 25-ml centrifuge tubes. Nine ml of 0.01 M CaCl_2 was added to each tube, the tubes were sealed with parafilm, and shaken for 24 hours on a reciprocating shaker at 90 cycles per minute. The tubes were then centrifuged for 20 min at 5000 X g (6000 rpm) and the supernatant was decanted. The same process was repeated with a second 9 ml of 0.01 M CaCl_2 . Both supernatants were analyzed by HPLC for atrazine, metolachlor, and trifluralin concentrations using a gradient with acetonitrile and Milli-Q water as the mobile phase solvents and a flow rate of 1.8 ml min^{-1} . The gradient started at and maintained 25% ACN / 75% water for 5 minutes, reached 95% ACN / 5% water at 10 minutes, remained at those conditions for 4 minutes, ramped down to 25% ACN / 75%

water by 18 minutes, and stayed at those conditions for 2 minutes. The retention times for atrazine, metolachlor, and trifluralin were 10.4, 12.2 and 13.5 minutes, respectively at UV wavelength 220 nm.

Biostimulation with organic amendments. The amendments chosen for the first biostimulation experiment were municipal compost containing leaf and yard debris (collected from the Ames, IA composting facility), sawdust, aged cattle manure, peat, a corn fermentation byproduct, cornstalks, and an inorganic solution consisting of 0.265 g CaSO_4 , 0.353 g K_2HPO_4 , and 1.785 g NH_4NO_3 in 250 ml Milli-Q water to achieve a 10:1:1:1 ratio of N:P:K:S. The corn fermentation byproduct was supplied by Ali Tabatabai (Agronomy Department, Iowa State University) and had the following characteristics: 40% solids; 6.9% total N; 0.52% P_2O_5 ; a density of 1.19; and a pH of 4.7. The C/N ratios for each of the amendments along with the C/N ratio of the Bravo soil after treatment with each amendment are shown in Table 1. The peat, compost, manure, and sawdust were passed through a 4-mm sieve and air-dried. The cornstalks were ground with a Hamilton Beach blender to a consistency that would pass through a 2-mm sieve. The C/N ratio of all of the amendments was determined on triplicate samples using a Carlo-Erba NA1500 N/C/S analyzer (Milan, Italy). For the first biostimulation experiment, all amendments were added at 0.5% and 5% (w/w). The inorganic solution was added at two different levels, 1 and 2 ml jar⁻¹, to soil without organic amendment, and 1 ml was added to all of the other treatments to alleviate nutrient deficiencies which may arise upon addition of the C amendments. In the second biostimulation experiment, only sawdust or manure (5% w/w) and 1 ml of inorganic solution were added as treatments.

Fifty grams of soil dry weight basis were weighed into 454-ml mason jars. The soil was allowed to equilibrate in the jars for 1 week prior to spiking with the herbicides. Jars were randomly assigned to treatments according to a randomization scheme established by a SAS program (SAS Institute, Gary, NC). The soil was treated with either a mixture containing 200 $\mu\text{g g}^{-1}$ of atrazine, metolachlor, and trifluralin or 200 $\mu\text{g g}^{-1}$ of the individual herbicide, mixed thoroughly by stirring and shaking, and exposed to the ambient air for 30 minutes to allow for evaporation of some of the methanol and

Table 1. C/N ratios for the Bravo soil and organic amendments

Amendment	% C	% N	C/N ratio 1 ^a	C/N ratio 2 ^b
Bravo soil	2.4	0.05	48.0	12.4
Cattle manure	8.4	0.8	10.0	10.8
Peat	10.7	0.7	14.8	14.8
Municipal compost	7.0	0.4	17.6	15.9
Sawdust	45.1	0.2	225.5	123.0
Cornstalk	37.1	0.8	45.2	39.8
Corn fermentation byproduct	13.5	8.3	1.6	1.9

^aC/N ratio 1 is the C/N ratio of the amendment itself and was calculated by the following equation: $C/N \text{ ratio } 1 = \%C \div \%N$.

^bC/N ratio 2 is the C/N ratio in the Bravo soil following treatment with the 5% level of each amendment. Each jar was treated with 1 ml of inorganic solution in an attempt to alleviate any nutrient deficiencies which could be caused by amendment addition. This N was taken into account for the C/N ratio 2 calculation. The Bravo soil C/N ratio 2 is the ratio upon addition of 1 ml of inorganic solution to the Bravo soil.

acetone added with the herbicide solution. A subset of jars, designated QA/QC samples, were immediately placed into the freezer to be extracted with each sampling time to monitor the extraction efficiency throughout both experiments. An additional subset was extracted immediately to determine the actual amount of herbicide initially added to each jar of soil. The soil was incubated at 25°C in the dark until a sampling time. For the first biostimulation experiment, Bravo soil was aged with the herbicide for 63 days prior to addition of the amendments. For the second biostimulation experiment, the herbicides and amendments were added a week after the soil was weighed into the jars. Triplicate samples for each treatment were sacrificed at each sampling point to determine the microbial populations, dehydrogenase enzyme concentrations, respiration rate, and herbicide concentration.

Microbial parameters and herbicide residue analysis. The bacterial and actinomycete populations were estimated by dilution plating on 0.1 strength tryptic soy agar (TSA) supplemented with 50 µg ml⁻¹ cyclohexamide. Fungal populations were estimated on rose bengal agar (RBA) containing 50 µg ml⁻¹ streptomycin sulfate. The plates were incubated at 25°C and the fungal colonies were counted after 3 to 5 days and bacterial and actinomycete colonies were counted after 5 to 7 days. Microbial respiration was measured by opening each jar for approximately 5 minutes to remove residual CO₂ from the soil and then attaching each jar to a CI-301 CO₂ gas analyzer (CID, Inc., Vancouver, WA). The instantaneous respiration rate of each jar was determined by measuring the accumulated CO₂ in 5- or 10-minute intervals. The gas analyzer sampled every 0.05 minutes and the air flow was 1.0 L min⁻¹. The respiration rate was expressed as the amount of CO₂ evolved g⁻¹ dry weight of soil minute⁻¹ and as a percentage of the soil without added herbicides. Dehydrogenase activity was determined using the modified method of Camberdella *et al.* (1994). The amount of 2,3,5-triphenyl formazon (TPF) produced from 2,3,5-triphenyltetrazolium chloride (TTC) by microbial dehydrogenases was measured in triplicate subsamples of each sample.

For herbicide residue analysis, 25 grams dry weight of soil was transferred into a 125-ml bottle, and 50 ml of ethyl acetate was added. Each bottle was sealed with parafilm and stored in the freezer until analysis. Each sample was shaken three times for 20 minutes on a Kahn reciprocating shaker, concentrated by rotary evaporation (686 mm H, 43°C), and rediluted to 10 ml. The samples were injected into a Shimadzu

GC9A gas chromatograph containing a 1.8-m column with OV17 packing and an N-P flame thermionic detector under the following conditions: injector temperature, 250°C; column temperature, 235°C; detector temperature, 250°C; and He carrier gas flow rate, 40 ml min⁻¹. The concentration of herbicide in each sample was determined by constructing standard curves from authentic herbicide standards and comparing the retention times of the analytes with that of the known standards.

Plant bioassay. Corn, soybean, and oat seeds were germinated under moist conditions at 25°C. Twenty-five gdw soil from three separate jars for each treatment was weighed into 50-ml glass test tubes. One of each type of seedling was planted in each tube and covered with an additional five g of soil. The soil was watered with Milli-Q water. Triplicate tubes of Clarion soil from a corn field near Ames, IA were planted and incubated along with the Bravo soils to act as a positive growth control. The soil portion of the tubes was wrapped in aluminum foil to minimize exposure to light. After five days' incubation in the greenhouse, the seedlings were harvested by washing away the soil, and the roots and shoots were cut apart and air-dried for 4 days. The roots were weighed on a Mettler AE166 microbalance to determine if differences among the treatments occurred.

Mineralization experiments. Fifty gdw of soil from the Alpha, Bravo and Echo sites was weighed into biometer flasks. Due to the differences in the texture classification of the soils, the Alpha and Bravo sandy soils were adjusted to 10% moisture, while the two high loam content Echo soils were adjusted to 20% moisture with Milli-Q water. The atrazine-mineralizing population size in the soils was determined by a modified ¹⁴C-atrazine most probable number (MPN) technique (Jayachandran *et al.*, submitted) prior to the start of the experiment. The Alpha, Bravo and Echo soils were screened to determine if the indigenous microbial populations could mineralize atrazine using triplicate samples of 50 g dry weight soil treated with 200,000 dpm of [¹⁴C-UL-ring]-atrazine and additional atrazine to achieve concentrations of 50 and 200 µg g⁻¹ Alpha soil; 5, 50, 100, and 200 µg g⁻¹ Bravo soil; and 5 and 100 µg g⁻¹ in each Echo soil. Three additional flasks of Bravo soil were treated with a combination of 200,000 dpm of [¹⁴C-UL-ring]-atrazine and 200 µg g⁻¹ soil each of atrazine, metolachlor, and trifluralin. Each soil was mixed thoroughly to evenly incorporate the herbicides. Ten ml of 0.5 M sodium hydroxide was added to the biometer flask sidearm to

collect $^{14}\text{CO}_2$, and the samples were incubated at 25°C in the dark. The sodium hydroxide traps were changed periodically to monitor atrazine mineralization. For each treatment, an additional flask was treated with the appropriate concentration of unlabelled atrazine, metolachlor, and trifluralin and incubated along with the [^{14}C]-atrazine-treated soils to measure the atrazine-mineralizing populations at the end of the incubation period.

An additional mineralization experiment was set up to determine how high concentrations of herbicide combinations affect the atrazine mineralization capabilities of the microorganisms in the Bravo soil (biostimulation experiment 3). Each biometer was treated with a solution containing 200,000 dpm of [^{14}C -UL-*ring*]-atrazine and additional atrazine to achieve a concentration of 5 or $200\ \mu\text{g g}^{-1}$ soil. Metolachlor and trifluralin were added to achieve the following treatments: $200\ \mu\text{g g}^{-1}$ atrazine; $200\ \mu\text{g g}^{-1}$ each of atrazine and trifluralin; $200\ \mu\text{g g}^{-1}$ each of atrazine and metolachlor; $200\ \mu\text{g g}^{-1}$ each of metolachlor and trifluralin with $5\ \mu\text{g g}^{-1}$ atrazine added to monitor atrazine mineralization; and $200\ \mu\text{g g}^{-1}$ each of atrazine, metolachlor and trifluralin. Each soil was mixed thoroughly to evenly incorporate the herbicides, incubated at 25°C in the dark, and the sodium hydroxide traps changed periodically. For each treatment, an additional flask was treated with the appropriate concentration of atrazine, metolachlor, and/or trifluralin and incubated along with the ^{14}C -atrazine-treated soils to monitor the atrazine-mineralizing populations.

At day 63 the atrazine-mineralizing populations in each soil treatment were determined by the MPN technique described previously. Additionally, a 10 g subsample from each biometer flask was extracted twice with 30 ml methanol. The methanol was added to the soil, the samples were shaken at 190 rpm on a reciprocating Eberbach shaker for 1 hour, allowed to sit for 24 hours, and shaken again for 30 minutes. The supernatant was removed and centrifuged at 10,000 rpm for 30 minutes to pellet out soil particles. A second 30-ml aliquot of methanol was used to repeat the extraction procedure just described. One ml of each methanol extract was mixed with 10 ml of Ultima Gold cocktail and counted on the LSC. Each extract was analyzed by HPLC using the method described earlier to determine the atrazine, metolachlor, and trifluralin concentrations. The soil was then air-dried, pulverized, and duplicate 1-g aliquots were oxidized (OX500 biological oxidizer, R. J. Harvey Instrument Corporation, Hillsdale, NJ) to determine the radioactivity remaining in the soil after extraction.

The remaining 40 g of soil in each biometer flask was saturated with sterile Milli-Q water and amended with 5% (w/w) cornstalk. The headspace was sparged with N₂ gas, and the flasks were stoppered and sealed with stopcock grease and parafilm to prevent O₂ from entering into the flask headspace. The flasks were incubated in the dark at 25°C, and the sodium hydroxide traps were changed periodically to monitor atrazine mineralization. On day 98, the water was drawn off with a Pasteur pipet, and the soil was stirred and aerated. The concentration of atrazine, metolachlor, and trifluralin in the water that was drawn off was determined using HPLC. The sodium hydroxide traps were monitored for ¹⁴CO₂ production for 197 days.

Statistical analysis. One-way analysis of variance (ANOVA) was used to determine if statistically significant differences at the p≤0.05 significance level were observed among the treatment means. Comparisons between the herbicide-treated, unamended control sample means and the amended sample means were made using Dunnett's and Student's t-tests.

RESULTS

Biostimulation experiment 1 herbicide degradation. Extensive degradation occurred in the herbicide-spiked, unamended Bravo soil with 50% of the atrazine, 33% of the metolachlor, and 44% of the trifluralin degraded over 245 days (Table 2). Herbicide extraction efficiencies were calculated by dividing the average concentration of herbicide present in three samples extracted immediately after spiking and six QA/QC samples by 200 ppm and converting that value to a percentage. The extraction efficiencies were 87.5, 91, and 82.5% for atrazine, metolachlor, and trifluralin, respectively. The 0.5% and 5% manure, 5% peat, and 5% cornstalk treatments yielded significantly more atrazine degradation than the herbicide-spiked, unamended treatment, with only 25, 60, 52, and 36 µg atrazine g⁻¹ soil remaining, respectively. Metolachlor degradation was enhanced over the herbicide-treated, unamended treatment by most of the amendments, with the 5% (w/w) amendment levels resulting in more extensive degradation. The analysis of variance showed that the overall 5% level

Table 2. Herbicide residues remaining in the Bravo soil in the first biostimulation experiment treatments after incubation for 182 days with amendments and 245 days with herbicide treatment

Treatment	Atrazine ^a	Metolachlor ^a	Trifluralin ^a
	(µg herbicide gdw ⁻¹ soil)		
No Amendments			
Bravo soil (Background)	2 (2) c	8 (4) c	2 (3) c
Initial treated concentration ^b	175 (42) a	182 (25) a	165 (23) a
Day 245 unamended ^c	87 (64) b	122 (24) b	92 (13) b
Amendments			
Inorganic			
1 ml	103 (8) b	127 (18) b	99 (5) b
2 ml	93 (7) b	120 (5) b	102 (8) b
Compost			
0.5%	115 (37)	119 (12)	100 (12)
5%	112 (19)	102 (9)	91 (6)
Mean (% of unamended)	130 (30) a	90 (11) ab	104 (11) a
Sawdust			
0.5%	93 (63)	124 (3)	89 (9)
5%	76 (11)	90 (16)	86 (10)
Mean (% of unamended)	97 (48) a	88 (17) abc	95 (9) a
Manure			
0.5%	25 (12)	114 (6)	94 (11)
5%	60 (42)	93 (5)	94 (9)
Mean (% of unamended)	53 (41) b	85 (10) bc	102 (10) a
Peat			
0.5%	109 (33)	126 (8)	108 (9)
5%	52 (38)	111 (13)	95 (18)
Mean (% of unamended)	93 (51) ab	97 (11) a	110 (16) a

^aThe data are expressed as the mean and standard deviation of three replicate jars of soil. Significant differences for amendment types (means averaged over rates, n=6) are indicated by different letters for means within the same column.

^bThe initial concentration treatment is the mean and standard deviation of six time-zero samples and three QA/QC soil samples.

^cThe three-herbicide mixture treatment is unamended Bravo soil spiked with 200 µg g⁻¹ atrazine, metolachlor, and trifluralin and incubated for 182 days.

Table 2. (continued)

Treatment	Atrazine ^a	Metolachlor ^a	Trifluralin ^a
	(µg herbicide gdw ⁻¹ soil)		
CFB			
0.5%	21 (44)	117 (2)	102 (10)
5%	112 (38)	97 (10)	81 (21)
Mean (% of unamended)	107 (57) a	88 (10) abc	100 (20) a
Cornstalk			
0.5%	124 (29)	103 (7)	105 (3)
5%	36 (16)	92 (19)	79 (34)
Mean (% of unamended)	92 (51) ab	80 (11) c	95 (20) a
0.5% mean (% of unamended) ^d	117 (53) A	96 (8) A	108 (10) A
5% mean (% of unamended) ^d	77 (41) B	80 (11) B	95 (18) B

^dMeans for amendment rates averaged over all organic amendment types (n=18). The individual treatment datum was converted to a percent of the unamended, herbicide-spiked treatment prior to analysis. Differences ($p \leq 0.05$) in amendment type and concentration means for each herbicide were determined by ANOVA and a t-test. The Bravo control treatments and the inorganic solution-amended treatments were analyzed separately from the organic-amended treatments.

of amendment resulted in a significantly higher amount ($p \leq 0.05$) of degradation than the overall 0.5% amendment mean for atrazine, metolachlor, and trifluralin. An analysis of variance also showed that the type of amendment resulted in a significant ($p \leq 0.05$) difference in the amount of metolachlor degraded. However, amendment type did not affect the amount of trifluralin or atrazine degraded. All of the amended treatments contained essentially the same amount of trifluralin as the unamended, herbicide-treated soil at the end of the incubation; however, one replicate sample in both the 5% cornstalk and 5% CFB amended treatments had more extensive trifluralin degradation, with only 40 and 57 μg trifluralin g^{-1} soil remaining, respectively.

Biostimulation experiment 1 microbial populations and activity. Microbial populations, dehydrogenase, and respiration varied among the sampling times, amendment concentrations, and types of amendment. We have summarized these results as the mean and standard deviation over all of the sampling time points after amending with the organic and inorganic treatments, i.e. all of the sampling times after day 63 (Table 3). The individual treatment means for each of the microbial parameters at each sampling time are shown in Appendix A. Several treatments, 0.5 and 5% compost, 0.5% sawdust, and 0.5 and 5% manure, increased the bacterial populations to levels greater than the unamended soil ($p \leq 0.05$). All of the amendments at both levels of addition significantly increased actinomycete populations, except the population in the 5% CFB treatment, which remained similar in size to the unspiked Bravo control soil. The fungal populations were dramatically affected by several of the amendments, with the 5% cornstalk treatment increasing the fungal levels 10-fold, the 5% compost and 0.5% sawdust treatments increasing them approximately 100-fold, and the 5% sawdust treatment increasing the populations by an average of 1000 times over all the sampling points, compared to the herbicide-spiked, unamended Bravo soil. However, due to the high standard deviations obtained for the fungal populations, statistically significant differences ($p \leq 0.05$) were not observed. The treatments that significantly increased the dehydrogenase values over the unspiked Bravo background soil were 5% peat, 5% cornstalk, 5% manure, 5% compost, and 0.5% sawdust. Microbial respiration was increased almost five-fold by the 5% manure amendment and nine-fold by the 5% cornstalk over the unamended, spiked soil. None of the other treatments showed more

Table 3. Average microbial populations and activity data over all sampling times for the first biostimulation experiment.

Treatment	Microbial populations (Log colony forming units gdw ⁻¹ soil)			Dehydrogenase ^{ac} (µg TPF gdw ⁻¹ soil day ⁻¹)	Respiration rate ^{ac} % of unspiked control
	Bacteria ^{ab}	Actinomycetes ^{ab}	Fungi ^{ab}		
Background Bravo	4.53 (0.52) a	3.89 (1.11) a	1.73 (1.32) a	36.2 (22.4) a	100.0 (0.0) b
Unamended, spiked	5.63 (0.98) a	4.56 (2.02) a	1.18 (1.05) a	55.0 (25.1) a	175.7 (187.5) b
Compost, 0.5%	5.87 (0.75) a	5.32 (1.18) a	2.15 (0.95) a	32.5 (32.6) a	94.3 (40.8) b
Compost, 5%	6.38 (1.15) b	5.51 (1.35) a	2.56 (1.01) a	102.1 (61.1) b	223.8 (107.0) b
Sawdust, 0.5%	6.12 (1.09) b	5.29 (1.45) a	2.71 (1.71) a	106.7 (90.8) b	114.1 (47.4) b
Sawdust, 5%	5.66 (1.27) a	4.83 (1.76) a	3.43 (2.50) a	71.2 (65.6) a	252.3 (139.4) b
Manure, 0.5%	6.14 (0.81) b	5.61 (1.24) a	1.81 (1.59) a	52.2 (23.5) a	103.4 (61.8) b
Manure, 5%	6.21 (0.84) b	5.26 (0.97) a	2.43 (0.68) a	147.6 (53.0) b	496.4 (372.8) b
Peat, 0.5%	5.18 (1.71) a	5.06 (1.34) a	1.65 (0.95) a	38.1 (36.1) a	138.6 (116.4) b
Peat, 5%	5.57 (1.28) a	5.18 (1.90) a	1.75 (0.90) a	103.6 (75.5) b	255.9 (316.2) b
CFB, 0.5%	5.62 (1.12) a	4.76 (1.57) a	1.29 (1.06) a	57.8 (56.1) a	112.9 (105.5) b
CFB, 5%	5.35 (0.73) a	3.52 (2.39) a	0.77 (0.82) a	66.3 (82.6) a	207.0 (220.0) b
Cornstalk, 0.5%	5.29 (1.77) a	4.34 (1.89) a	2.05 (2.14) a	52.0 (69.0) a	163.9 (150.1) b
Cornstalk, 5%	6.08 (1.59) b	5.21 (2.11) a	2.82 (2.41) a	111.1 (132.9) b	1412.2 (1619.8) c
Inorganic, 1 ml	5.47 (1.00) a	4.78 (1.23) a	1.73 (1.22) a	48.8 (26.6) a	54.7 (28.1) a
Inorganic, 2 ml	5.76 (0.95) a	4.53 (1.04) a	1.70 (1.12) a	39.7 (20.1) a	112.3 (91.1) b

CFB= corn fermentation byproduct

TPF= triphenyl formazon

^aData are expressed as the mean and standard deviation of three replicate samples.

^bFor the bacteria, actinomycete, and fungi populations, a one-way ANOVA and then the Bonferroni t-test for multiple comparisons versus a control group ($p \leq 0.05$) was run in SigmaStat between the unspiked control and the other treatments. Those treatments with a different letter than the control are statistically different at the 95% confidence level.

^cFor the dehydrogenase and respiration data, a one-way ANOVA and then the Student-Newman-Keuls t-test was run in SigmaStat between all treatments. Those treatments with a different letter behind them are statistically different at the 95% confidence level.

than twice the unspiked control respiration rate, however the respiration rate was depressed by inorganic nutrients (1 ml) to only half of the unspiked control soil, but treatment with 2 ml of inorganic nutrients did not result in the same effect. The respiration rate is consistently higher in the higher level of addition for each amendment type.

When the Bravo soil was spiked with a mixture of $200 \mu\text{g g}^{-1}$ atrazine, metolachlor, and trifluralin, the atrazine-mineralizing populations were decreased from 1950 to 110 cells gdw^{-1} soil (Table 4). Addition of the 5% level of all amendments except peat and compost further reduced the atrazine-mineralizing populations, with most of the populations falling to levels below the detection limit of the MPN assay (Table 4).

Mineralization experiment. At the higher initial concentrations of atrazine in the Bravo and control E1 soil, a smaller percentage of the atrazine was mineralized, with greater than 70% of the atrazine mineralized when added at the $5 \mu\text{g g}^{-1}$ level and between 45 and 55% mineralized at the 100 and $200 \mu\text{g g}^{-1}$ level (Figures 2 and 3). If these percentages are expressed as a concentration of atrazine degraded, the concentration of atrazine degraded was higher at greater initial spiking concentrations. When a mixture of $200 \mu\text{g g}^{-1}$ of the herbicides atrazine, metolachlor, and trifluralin were added to the Bravo soil, atrazine mineralization is completely repressed (Figure 2). The atrazine-mineralizing populations in the Bravo soil increased from 1,950 to 13,000 cells gdw^{-1} soil as the amount of atrazine added increased from none to $100 \mu\text{g g}^{-1}$ atrazine (Table 5). However, when the Bravo soil was spiked with $200 \mu\text{g g}^{-1}$ atrazine or with a mixture of the three herbicides at $200 \mu\text{g g}^{-1}$, the atrazine-mineralizing populations declined to levels lower than originally present in the Bravo soil (Table 5). The atrazine-mineralizing populations in the uncontaminated soil from the Echo site increased dramatically at the higher herbicide concentrations (Table 5). The contaminated soil from E2 was unable to mineralize atrazine at either the 5 or $100 \mu\text{g g}^{-1}$ application rate (Figure 3). The populations in the E2 soil dropped below detection limit when spiked with $5 \mu\text{g g}^{-1}$ atrazine and only increased by a factor of 10 when the soil was spiked with $100 \mu\text{g g}^{-1}$ atrazine (Table 5). The Alpha soil microbial populations mineralized the same amount of atrazine, 30%, when it was applied at 50 or $200 \mu\text{g g}^{-1}$ (Figure 4). These percentages correspond to $15 \mu\text{g g}^{-1}$ for the $50 \mu\text{g g}^{-1}$ treatment and to over $60 \mu\text{g g}^{-1}$ degraded in the $200 \mu\text{g g}^{-1}$ treatment. The atrazine-

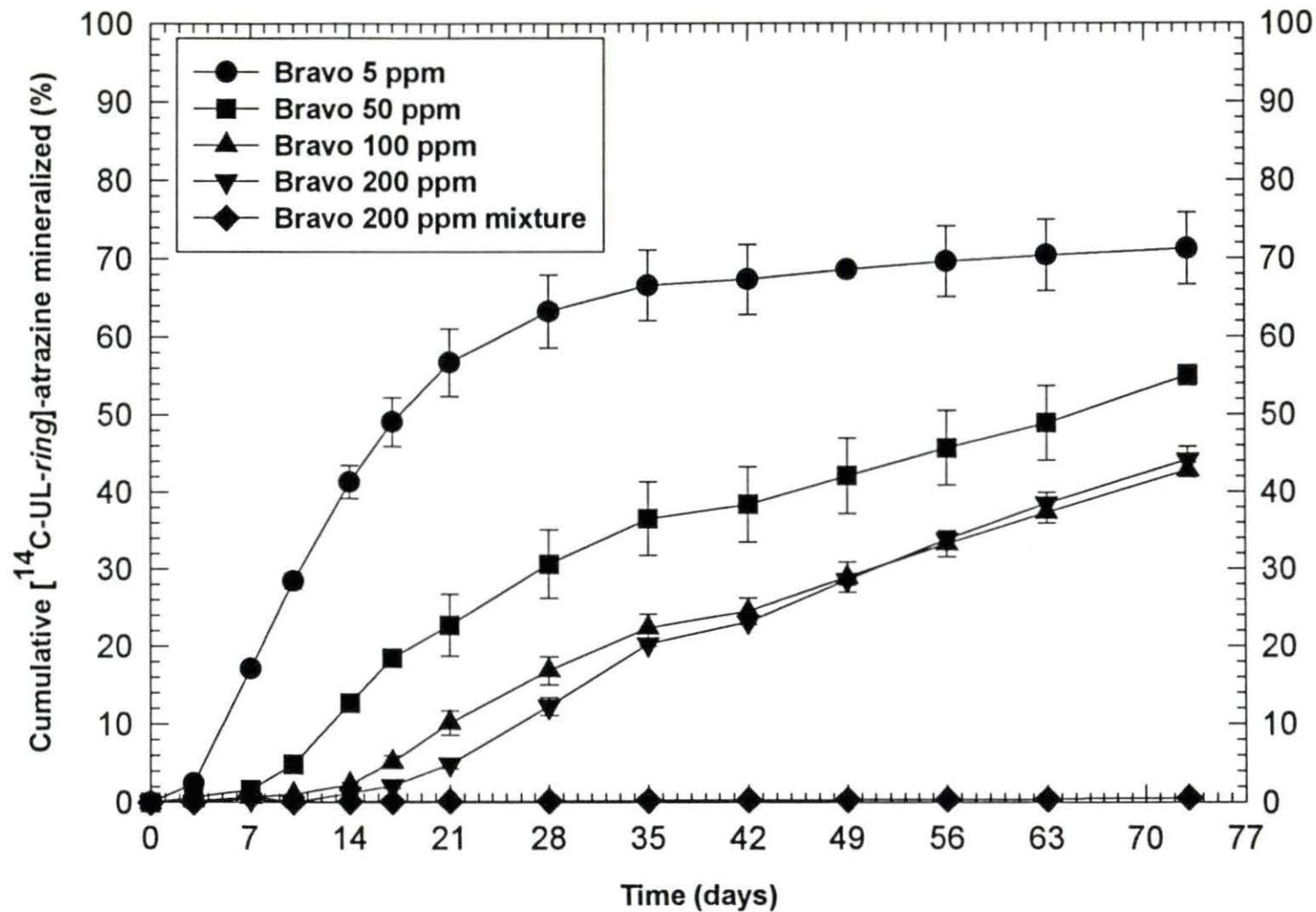


Figure 2. Mineralization of [$^{14}\text{C-UL-ring}$]-atrazine by the Bravo soil when spiked with various concentrations of atrazine and a mixture of 200 $\mu\text{g g}^{-1}$ atrazine, metolachlor, and trifluralin.

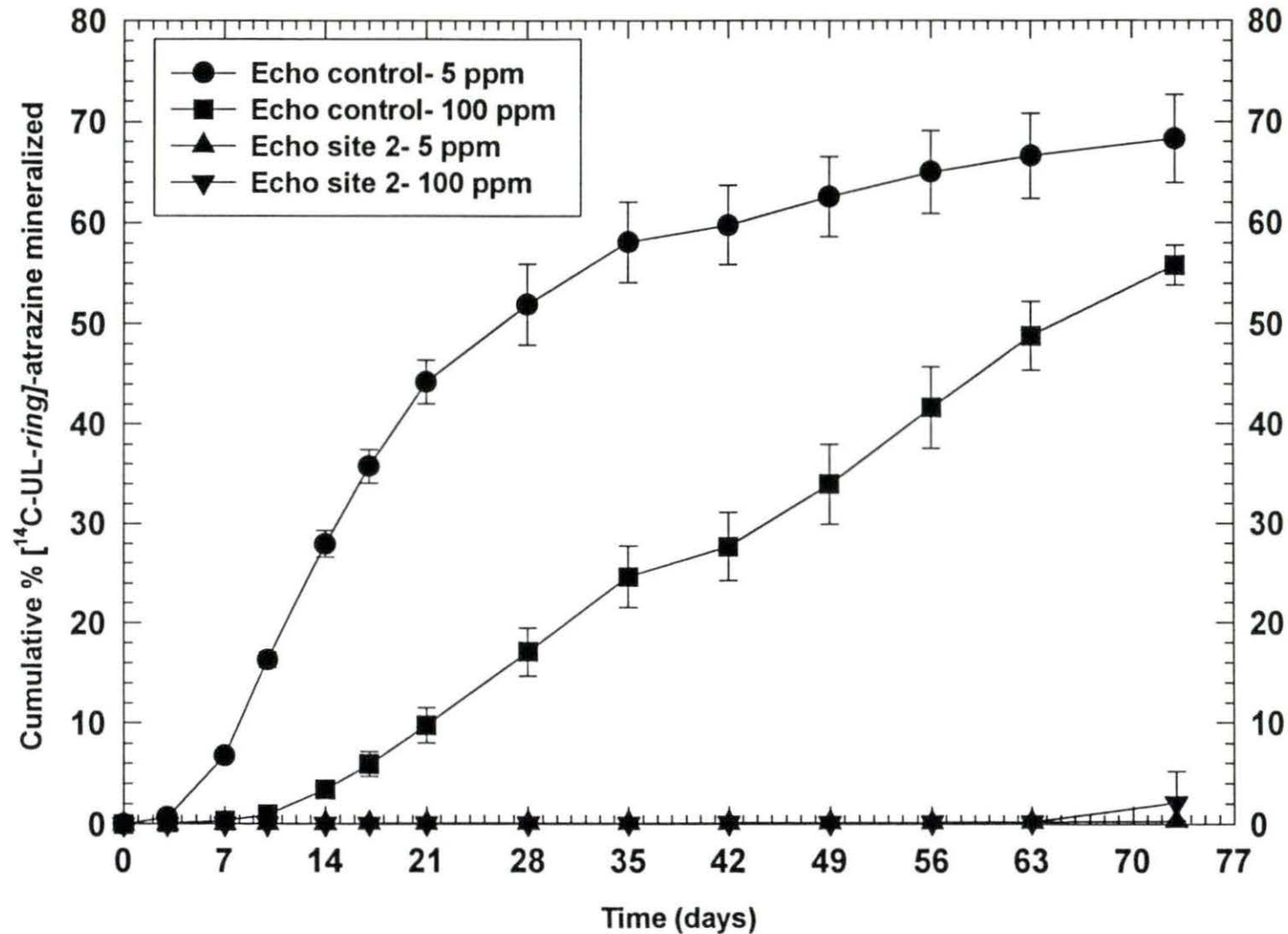


Figure 3. Mineralization of [$^{14}\text{C-UL-ring}$]-atrazine in the Echo control site 1 (E1) soil and the Echo site 2 (E2) soil. E2 was highly contaminated with a mixture of herbicides, while E1 contained only trace amounts of herbicide residues.

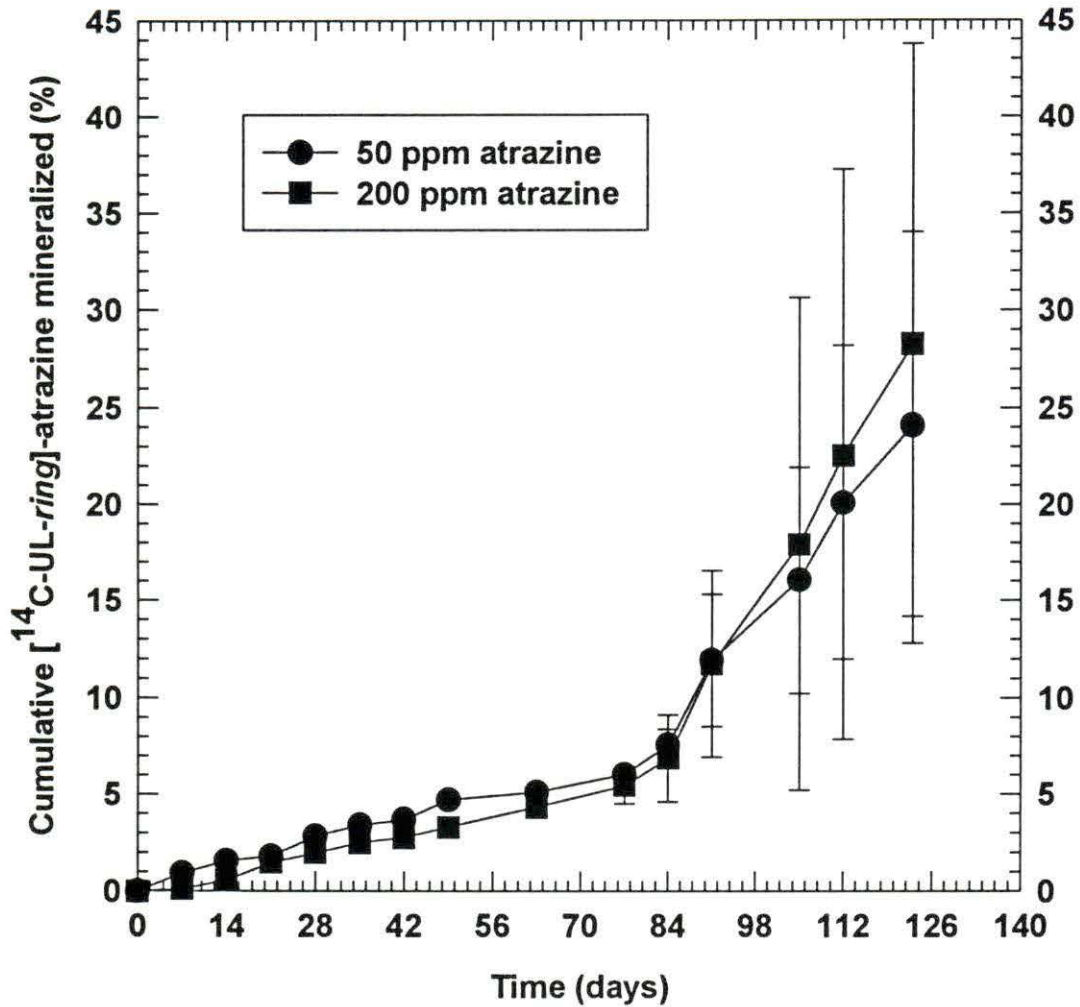


Figure 4. Mineralization of 50 and 200 ppm ^{14}C -atrazine in the Alpha soil. All flasks were treated with 2 ml of Milli-Q water at day 63 to maintain the initial 10% moisture conditions in all the soil samples.

Table 4. Atrazine-degrading populations present in Bravo soil following addition of different amendments (5% w/w) at day 119 of the first biostimulation experiment

Amendment	Atrazine degraders (cells gdw ⁻¹ soil)
Initial Bravo soil- unspiked	1950
Bravo soil- spiked, unamended	110
Cattle manure, 5%	BD
Peat, 5%	790
Municipal compost, 5%	230
Sawdust, 5%	BD
Cornstalk, 5%	BD
Corn fermentation byproduct, 5%	BD
Inorganic, 2 ml	18

BD= populations are below the detection limit of the MPN assay (18 cells gdw⁻¹ soil)

Table 5. Microbial populations in Alpha, Bravo and Echo soils treated with different concentrations of atrazine after incubation for 63 (Alpha) or 73 days (Bravo and Echo)

Atrazine concentration ($\mu\text{g atrazine g}^{-1}$ soil)	MPN populations (cells gdw^{-1} soil)	Bacteria ^a	Actinomycetes ^a	Fungi ^a
		(Log CFU gdw^{-1} soil)		
Alpha (initial)	420			
Alpha 50 $\mu\text{g g}^{-1}$	20			
Alpha 200 $\mu\text{g g}^{-1}$	20			
Bravo (initial)	1950	6.4 (4.9)	6.1 (5.0)	4.8 (3.3)
Bravo 5 $\mu\text{g g}^{-1}$	2300			
Bravo 50 $\mu\text{g g}^{-1}$	4900			
Bravo 100 $\mu\text{g g}^{-1}$	13,000			
Bravo 200 $\mu\text{g g}^{-1}$	450			
Bravo 200 $\mu\text{g g}^{-1}$ plus metolachlor and trifluralin each at 200 $\mu\text{g g}^{-1}$ soil	110			
Echo control (initial)	350	7.3 (6.4)	7.3 (6.7)	3.1 (2.5)
Echo control- 5 $\mu\text{g g}^{-1}$	17,000			
Echo control- 100 $\mu\text{g g}^{-1}$	33,000			
Echo site 2 (initial)	38	6.9 (5.9)	4.3 (3.9)	3.3 (2.3)
Echo site 2- 5 $\mu\text{g g}^{-1}$	BD			
Echo site 2- 100 $\mu\text{g g}^{-1}$	200			

BD= below detection limit of assay (18 cells gdw^{-1} soil)

^aThe microbial populations are expressed as the mean and standard deviation of three replicate samples.

mineralizing populations in the atrazine-treated soil decreased ten-fold from the background populations in the Alpha soil.

Biostimulation experiment two herbicide degradation. The second biostimulation experiment examined the persistence of these three herbicides singly and in combination. Extraction efficiencies of 89, 68, and 84% were obtained for atrazine, metolachlor, and trifluralin, respectively. Sawdust amendment repressed atrazine degradation in atrazine-spiked soil, while manure addition allowed for the same amount of degradation as in the unamended, herbicide-treated soil. Both amendments enhanced degradation of metolachlor over the unamended metolachlor-spiked soil, with 120, 89, and 70 μg metolachlor g^{-1} soil remaining in the unamended, sawdust, and manure treatments, respectively. Sawdust-amendment of the Bravo soil enhanced the degradation of trifluralin, resulting in the degradation of 64% of the trifluralin versus only 46% degradation in the unamended, herbicide-spiked soil (Table 6). Manure addition did not increase degradation of trifluralin.

When the Bravo soil was spiked with all three herbicides, the amount of degradation of the herbicides was the same or reduced from the largest amount of degradation achieved when the herbicide was applied individually for all the amendment treatments. Variation in the amount of atrazine degradation in atrazine-treated soils resulted from a significant interaction ($p \leq 0.05$ level of significance) between the herbicides applied to the soil and the type of amendment added to the soil. Statistically different atrazine degradation resulted (at $p \leq 0.05$) when the soil treated with atrazine alone or in a mixture of the three herbicides. An average of 110 μg g^{-1} atrazine remained in soil treated with a mixture of the three herbicides and 31 μg g^{-1} atrazine remained in the soil treated with atrazine individually. The type of amendment applied to atrazine-treated soils made a statistically significant difference in the amount of atrazine degradation, with the unamended and sawdust atrazine-concentration means being almost twice as high as the mean of manure-amended soil, with 83, 82, and 47 μg g^{-1} atrazine remaining, respectively.

For the metolachlor-spiked soils, both the herbicide treatment and the type of amendment resulted in statistically significant differences in the amount of metolachlor degraded ($p \leq 0.05$). However, interaction between these two factors was not observed,

Table 6. Herbicide residues remaining in the second biostimulation experiment treatments after incubation for 182 days with the amendments

Herbicide	Treatment ^d	Atrazine ^{ab}	Metolachlor ^{ab}	Trifluralin ^{ab}
		(µg herbicide gdw ⁻¹ soil)		
Initial concentration ^c		178 (26) a	135 (18) a	168 (11) a
Background Bravo soil		0.3 (0) c	5 (1) c	0.1 (0.2) c
Solvent spiked Bravo soil		0.3 (0.1) c	5 (1) c	1 (0) c
Atrazine	None	12 (5) c^e	7 (2) c	2 (2) c
Atrazine	Sawdust	75 (5) b	5 (1) c	0.2 (0.4) c
Atrazine	Manure	5 (3) c	5 (1) c	0.1 (0.2) c
Metolachlor	None	0 (0) c	120 (11) a	1 (1) c
Metolachlor	Sawdust	0.2 (0.1) c	89 (16) b	0.2 (0.2) c
Metolachlor	Manure	1 (1) c	70 (16) b	0.2 (0.4) c
Trifluralin	None	2 (3) c	8 (4) c	84 (24) b
Trifluralin	Sawdust	0.4 (0.5) c	4 (1) c	60 (8) b
Trifluralin	Manure	0 (0) c	4 (1) c	87 (7) b
3-Mixture	None	154 (10) a	137 (9) a	90 (18) b
3-Mixture	Sawdust	137 (23) a	87 (8) b	88 (9) b
3-Mixture	Manure	78 (54) b	105 (17) a	89 (22) b
ANOVA means analysis (expressed as µg herbicide gdw ⁻¹ soil)				
	Sawdust amended mean (n=6)	82 A	88 B	74 A
	Manure amended mean (n=6)	47 B	88 B	88 A
	Unamended mean (n=6)	83 A	130 A	87 A
	Herbicide mixture mean (n=9)	110 A	110 A	89 A
	Single herbicide mean (n=9)	31 B	94 B	77 A

^aThe data are expressed as the mean and standard deviation of three replicate jars of soil.

^bTreatment means with different lowercase letters following the standard deviation are considered statistically different from each other at the $p \leq 0.05$ level of significance as determined by a One-way ANOVA and Dunnett's method of multiple comparison pairwise t-tests measured using SigmaStat. The overall herbicide and amendment means were determined by analyzing the data by ANOVA with t-test comparison at the $p \leq 0.05$ level of significance in SAS. The analysis of variance means which are statistically different are indicated by different capital letters following the standard deviation.

^cThe initial concentration of herbicide value is the mean and standard deviation of the three time zero samples and the three QA/QC samples from day 182.

^dAll of the amendments were added at the 5% level.

^eThe values shown in bold face are those samples that were spiked with 200 ppm of the herbicide at day zero. All values shown in the same rows of the table are the Bravo background concentrations for the respective herbicides.

as was for the atrazine-spiked soil. Spiking with a mixture of the herbicides resulted in less metolachlor degradation (averaged over all amendments) than the soil treated with only metolachlor, with 110 and 94 $\mu\text{g g}^{-1}$ remaining, respectively. Amending with either manure or sawdust resulted in the same mean amount of metolachlor degradation (88 $\mu\text{g g}^{-1}$ remaining) and significantly more degradation than in the unamended soil which contained 130 $\mu\text{g g}^{-1}$. Trifluralin degradation was not affected by either the combination of herbicide or the type of amendment applied to the soil.

Biostimulation experiment two microbial populations and activity. Microbial populations, dehydrogenase activity, and respiration rates are expressed as the average and standard deviation over all of the sampling time points after amendment addition in the second biostimulation experiment (Table 7). The individual treatment means for these parameters at each of the sampling times are shown in Appendix B. The sawdust amendment consistently increased the bacteria, actinomycete, and fungi populations, while the manure amendment consistently enhanced the growth of the bacterial populations. The herbicide mixture amended with sawdust treatment caused extreme repression of the dehydrogenase activity, while the unamended, herbicide-treated soil, solvent-spiked control soil, and atrazine treated soils amended with sawdust caused a slight decrease from the background Bravo soil. The atrazine- and metolachlor-treated treatments showed similar values to the herbicide-treated, unamended soil. The trifluralin-treated, sawdust-amended metolachlor, and manure-amended, herbicide-mixture treatments produced values that were significantly higher than the herbicide-untreated control, while the manure-amended atrazine, sawdust and manure-amended trifluralin and manure-amended metolachlor treatments were much higher than the soil not treated with herbicides. Several of the treatments that had high dehydrogenase activity also had respiration values that were significantly higher than the unspiked control, the manure-amended atrazine, the sawdust-amended trifluralin, and the manure-amended metolachlor treatments. The additional treatments with respiration values significantly higher than the control soil are the manure-amended herbicide mixture and the sawdust-amended metolachlor treatments.

Table 7. Average microbial populations and activity data over all sampling times for the second biostimulation experiment.

Treatment	Microbial populations (Log colony forming units gdw^{-1} soil)			Dehydrogenase ^{bc} ($\mu\text{g TPF } \text{gdw}^{-1} \text{ soil day}^{-1}$)	Respiration rate ^{bc} ($\mu\text{g CO}_2 \text{gdw}^{-1} \text{ soil min}^{-1}$)
	Bacteria ^{ab}	Actinomycetes ^{ab}	Fungi ^{ab}		
Background Bravo	6.2 (0.2) a	6.0 (0.2) a	3.6 (0.3) a	77.5 (48.1) c	3.7 (2.0) a
Solvent-treated	6.9 (1.2) a	6.3 (0.8) a	2.8 (0.8) a	39.7 (34.1) b	6.3 (4.9) a
Atrazine	6.2 (0.7) a	6.3 (1.0) a	3.2 (0.9) a	72.0 (31.9) c	3.4 (2.1) a
Sawdust	7.6 (1.1) b	7.3 (1.0) b	5.0 (1.4) b	35.0 (42.1) b	12.0 (11.2) a
Manure	8.0 (0.4) b	7.6 (0.6) b	4.0 (0.6) a	152.4 (49.1) e	27.8 (41.5) b
Metolachlor	6.6 (1.0) a	6.0 (0.7) a	2.9 (0.5) a	129.3 (38.2) d	7.7 (2.6) a
Sawdust	7.6 (1.1) b	7.6 (0.3) b	5.6 (0.5) b	168.6 (80.3) e	24.2 (11.1) b
Manure	7.3 (0.8) b	6.3 (0.2) a	4.6 (0.4) a	146.1 (37.9) e	8.0 (5.8) a
Trifluralin	6.5 (0.6) a	6.2 (0.6) a	5.0 (0.5) b	82.9 (33.5) c	5.0 (5.5) a
Sawdust	8.2 (0.4) b	7.5 (1.1) b	5.7 (1.5) b	113.5 (109.1) d	23.0 (14.1) b
Manure	7.2 (1.0) a	7.4 (0.5) b	4.2 (0.8) a	178.0 (71.1) e	16.8 (24.0) b
Mixture of atrazine, metolachlor, and trifluralin					
Unamended	6.7 (1.0) a	6.3 (0.7) a	3.0 (1.0) a	44.7 (35.8) b	5.2 (5.8) a
Sawdust	6.3 (1.3) a	6.0 (0.9) a	3.9 (1.9) a	10.0 (13.4) a	10.8 (9.8) a
Manure	7.7 (0.5) b	6.7 (0.7) a	3.9 (0.6) a	116.3 (71.5) d	20.9 (24.9) b

^aData is expressed as the mean and standard deviation of three replicate samples.

^bFor the bacterial, actinomycetal, and fungal populations, a one-way ANOVA and then the Bonferroni t-test for multiple comparisons versus a control group ($p \leq 0.05$) was run in SigmaStat between the unspiked control and the other treatments. Those treatments with a different letter than the control are statistically different at the 95% confidence level.

^cFor the dehydrogenase and respiration data, a one-way ANOVA and then the Student-Newman-Keuls t-test was run in SigmaStat between all treatments. Those treatments with a different letter behind them are statistically different at the 95% confidence level.

Plant bioassay. In the plant bioassay, atrazine residues affected soybeans and oats, metolachlor residues affected the oats, and the trifluralin residues affected the corn and the oats. Corn formed “club roots” where the root tips became enlarged in all of the trifluralin treatments, indicating phytotoxic levels of the herbicide or its metabolites. This effect was particularly pronounced in the unamended soil treated with the mixture of herbicides. Atrazine residue damage was observed on the leaves of the soybeans in the sawdust-amended treatments, causing them to crinkle, resulting in necrotic leaf sections. Plants in unspiked Bravo soil were similar in weight to those in the Clarion control soil (Table 8). The unamended, herbicide-treated Bravo soil showed the most pronounced phytotoxic effects. All of the plant responses for the other treatments were intermediate between these two extreme treatments. However, it appeared that the plants in the three treatments spiked with atrazine and/or metolachlor, manure treatment was less phytotoxic, with the next highest weights behind the unspiked Bravo soil. Even though the atrazine was shown to be degraded to 12 and 5 times the normal usage rate in the atrazine alone and the manure-amended, atrazine treatments, respectively, the plants still showed some toxicity effects, indicated by lower plant weights than the unspiked Bravo and Clarion soil treatments. The plants are an alternative measure to GC analysis of the amount of bioremediation that occurred in the soil, because they are sensitive to the presence of phytotoxic compounds.

Biostimulation three atrazine mineralization experiment. Mineralization of atrazine was monitored in Bravo soil upon addition of combinations of trifluralin, atrazine, and metolachlor (Figure 5). Addition of $200 \mu\text{g g}^{-1}$ atrazine alone resulted in approximately 50% mineralization after 63 days incubation and growth of a large atrazine-mineralizing population of $35,000 \text{ cells gdw}^{-1}$ soil, compared to a population of $1950 \text{ cells gdw}^{-1}$ in untreated soil (Table 9). When the Bravo soil was spiked with $200 \mu\text{g g}^{-1}$ trifluralin and atrazine, a similar amount of atrazine was mineralized and a similar-sized population size developed (Table 9). However, addition of $200 \mu\text{g g}^{-1}$ metolachlor to the atrazine spiked soil completely repressed atrazine mineralization through day 112 of the experiment. The same effect was observed when all 3 herbicides were applied to the soil (Figure 5). Metolachlor and trifluralin were applied with $5 \mu\text{g g}^{-1}$ [^{14}C]-atrazine to monitor the effects of these two herbicides at high concentrations on the atrazine-mineralizing populations. The $5 \mu\text{g g}^{-1}$ atrazine mineralization curves and atrazine-

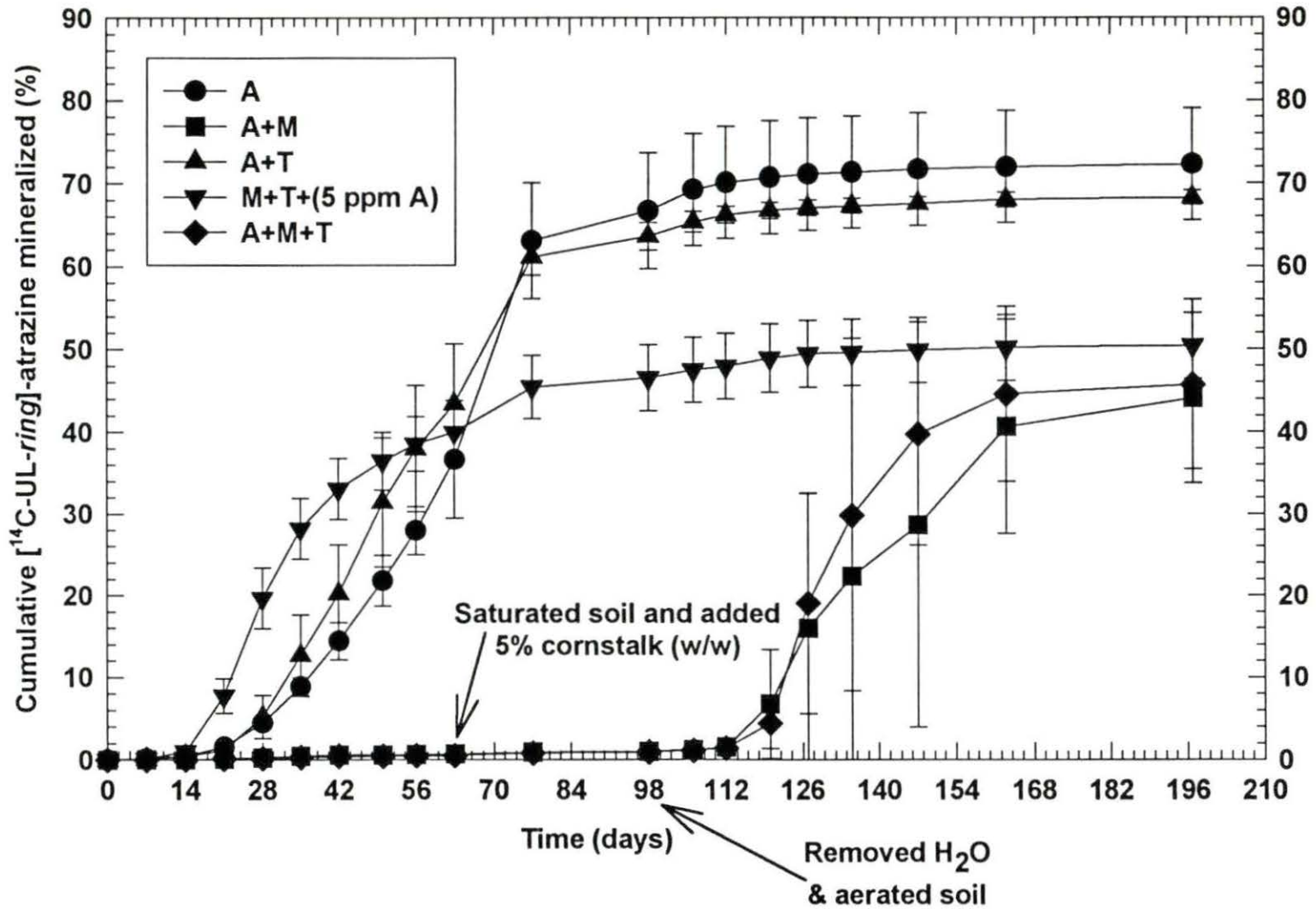


Figure 5. [¹⁴C-UL-ring]-atrazine mineralization in Bravo soil treated with 200 ppm combinations of the herbicides atrazine, metolachlor, and trifluralin. The metolachlor+trifluralin treatment contains 5 ppm atrazine to act as a toxicity indicator.

Table 8. Root weights for the corn, soybean, and oat plants grown in soil treated with atrazine, metolachlor, and trifluralin for the second biostimulation experiment

Treatment	Corn weight ^{ab} (mg)	Soybean weight ^{ab} (mg)	Oat weight ^{ab} (mg)
Clarion control	81.6 (16.9) a	20.8 (11.1) a	7.2 (1.4) a
Background Bravo	85.3 (26.0) a	8.7 (1.4) b	5.8 (0.8) a
Atrazine, unamended	56.3 (9.7) a	14.3 (3.2) b	3.1 (3.2) b
Atrazine, sawdust	47.5 (13.4) a	11.2 (11.3) b	3.6 (2.8) b
Atrazine, manure	41.6 (3.3) a	16.5 (16.1) b	3.1 (3.8) b
Metolachlor, unamended	48.9 (15.6) a	16.2 (15.1) b	2.0 (1.6) b
Metolachlor, sawdust	77.5 (28.2) a	8.1 (12.9) b	0.8 (0.9) c
Metolachlor, manure	52.8 (22.4) a	23.8 (16.1) a	3.3 (1.9) b
Trifluralin, unamended	32.5 (20.4) a	4.8 (9.1) c	1.9 (2.5) b
Trifluralin, sawdust	13.6 (16.5) b	11.0 (14.3) b	3.8 (3.6) b
Trifluralin, manure	19.5 (13.1) b	8.6 (12.6) b	1.7 (3.7) b
3-Mixture, unamended	3.5 (4.1) b	2.7 (2.7) c	0.3 (0.5) c
3-Mixture, sawdust	25.4 (26.1) b	10.8 (6.1) b	2.5 (0.9) b
3-Mixture, manure	25.9 (12.8) b	10.3 (11.4) b	1.0 (0.8) c

^aThe data was expressed as the mean and standard deviation of three replicate jars of soil.

^bTreatment means with different letters following the standard deviation are considered statistically different from each other at the $p \leq 0.05$ level of significance as determined by a One-way ANOVA and Dunnett's method of multiple comparison pairwise t-tests measured using SigmaStat.

Table 9. Microbial populations in the Bravo soil 63 days after treatment with various combinations of 200 $\mu\text{g g}^{-1}$ each of atrazine, metolachlor, and trifluralin

Treatment ^a	Atrazine degraders (cells gdw^{-1} soil)	Bacteria	Actinomycetes (log CFU gdw^{-1} soil)	Fungi
Background Bravo soil	1950	6.4	6.1	4.8
Atrazine	35,000	7.4	7.6	3.8
Atrazine + metolachlor	340	8.3	7.1	2.3
Atrazine + trifluralin	28,000	7.9	7.8	2.9
Metolachlor + trifluralin + 5 $\mu\text{g g}^{-1}$ atrazine	370	8.1	7.3	3.1
Atrazine + metolachlor + trifluralin	78	5.4	5.1	BD

BD= population is below detection limit of dilution plating procedure.

^aUnless otherwise stated, all of the herbicides were applied at a rate of 200 $\mu\text{g g}^{-1}$ soil.

mineralizing population sizes were compared. A significant decrease in the extent of atrazine mineralization was observed in the soil amended with trifluralin and metolachlor compared to the mineralization observed in soil treated only with $5 \mu\text{g g}^{-1}$ atrazine (Figure 3). At day 14, 40% of the atrazine was mineralized in the $5 \mu\text{g g}^{-1}$ atrazine alone and <1% in the trifluralin+metolachlor treatment and at day 56, 70% mineralization in the atrazine-alone treatment compared to only about 45% mineralized in the trifluralin+metolachlor treatment. The atrazine-mineralizing populations at day 63 showed a 10-fold decrease in size from the $5 \mu\text{g g}^{-1}$ atrazine alone treatment (Table 5) to the trifluralin+metolachlor ($+5 \mu\text{g g}^{-1}$ atrazine) treatment (Table 9), from 2300 to 370 cells gdw^{-1} soil. All three treatments containing metolachlor had much smaller atrazine-mineralizing populations at day 63, with 340, 370, and 78 cells gdw^{-1} soil in the atrazine+metolachlor, metolachlor+trifluralin, and the 3-mixture treatments, respectively (Table 9).

Saturation, oxygen removal, and amendment of the soil with cornstalks at day 63 resulted in increased mineralization rates in the atrazine, atrazine+trifluralin, and metolachlor+trifluralin treatments. However, mineralization still did not occur in the atrazine+metolachlor and the three-mixture treatments. Mineralization of atrazine began after a 14-day lag period in these two treatments upon removal of the water and aeration of the soil. An aliquot of the water removed from each sample was analyzed for herbicide residues by HPLC. Based on the amount of metolachlor applied, approximately 4% of the metolachlor was removed in the water in the atrazine+metolachlor, trifluralin+metolachlor, and 3-mixture treatments. Thus, removal of herbicide in the water is not a primary mechanism of herbicide loss.

DISCUSSION

Organic amendment effects on herbicide degradation. In soils contaminated with a mixture of herbicide residues at high concentrations, biostimulation may be necessary due to toxic effects of the herbicides on the herbicide-degrading populations. A readily available C or energy source may be necessary to increase the biomass size or stimulate cometabolic activity. Amendments consistently enhanced metolachlor degradation, showed

inconsistent effects on atrazine degradation, and no effect on trifluralin degradation. The high level of amendment appear to be more effective overall at remediation than the low level of amendment, especially for enhancing metolachlor degradation. This may be because metolachlor is degraded cometabolically and therefore addition of a high level of a potential cometabolic substrate would help to increase biodegradation. Another explanation for enhanced degradation after higher rates of amendments is that a large dose of C allows for growth of a greater biomass size and potentially greater degrading population size.

The two amendments with the highest N content (lowest C/N ratios), corn fermentation byproduct and manure, resulted in the greatest mean atrazine degradation. This result is somewhat surprising considering the reports showing that many microorganisms degrade atrazine and utilize the N in the side-chains and s-triazine ring (Alvey and Crowley, 1995; Assaf and Turco, 1994; and Mandelbaum *et al.*, 1995). However, when these two amendments were applied at the high amendment level, less degradation occurred than at the lower amendment level. This indicates that the N present in the amendments applied at the 5% level may be repressing some atrazine-degrading microorganisms that are utilizing atrazine as a N source or that the two amendments may contain some toxic products that are inhibiting the atrazine-mineralizers. The cornstalk amendment was also effective in enhancing atrazine degradation, with the high level of amendment resulting in much more degradation than the low level of amendment, with 36 and 124 $\mu\text{g g}^{-1}$ atrazine remaining, respectively. The cornstalk had the second highest C/N ratio, but probably contained more easily utilizable C than sawdust, the highest C/N ratio amendment, sawdust. No trends between the C/N ratio and herbicide degradation were observed for metolachlor or trifluralin.

In some cases, it appeared that addition of organic and inorganic amendments actually decreased atrazine and metolachlor degradation. The 2 ml inorganic, compost (both levels), 5% CFB, and 0.5% peat and cornstalk amendments repressed atrazine degradation below the amount occurring in the herbicide mixture, unamended Bravo soil. The 2 ml inorganic solution and 0.5% peat treatments also appeared to repress metolachlor degradation. Of these treatments, only the 5% compost contained higher microbial populations or activity over the unamended, spiked Bravo soil, with a bacterial

population increase from a log value of 5.6 to 6.4. Otherwise, the remaining microbial parameters showed a decrease or only minimal growth from the value obtained for the unamended, spiked and background Bravo soil values. The addition of a variety of organic amendments (rice hulls, starch, compost, glucose, Sudan hay, or sodium citrate) to a soil spiked with $100 \mu\text{g g}^{-1}$ atrazine, respectively, resulted in 88, 75, 59, <2, 9, and <2% mineralization of atrazine versus 73% mineralization in the unamended soil, indicating that addition of all of the amendments, except rice hulls and starch, reduced mineralization (Alvey and Crowley, 1995).

Amendment effects on microbial populations and activity. Although amendment additions stimulated some microbial populations and activity, amendments may not stimulate specific herbicide-degrading populations. Microbial populations stimulated by amendments may outcompete the herbicide degraders for the inorganic nutrients and C sources added with the amendments. All of the metolachlor treatments show 10- to 100-fold higher bacterial and actinomycete populations than the background Bravo soil, but do not result in a proportionally higher amount of metolachlor degradation than the unamended Bravo soil, indicating that the metolachlor-degrading populations did not increase. In the second biostimulation experiment, the microbial populations, respiration rates, and dehydrogenase activities were consistently larger in amended versus the unamended, trifluralin-treated soils, but no enhanced degradation of trifluralin occurred. Davidson *et al.* (1980) monitored respiration in two soils that were treated with atrazine or trifluralin and observed that the respiration rates increased at higher concentrations of herbicide up to $20,000 \mu\text{g g}^{-1}$, but that the herbicide biodegradation rates decreased concomitantly.

Inorganic amendment effects. Inorganic nutrient addition did not appear to be an effective remediation treatment. Addition of N, P, and K in ratios optimal for microbial growth into the Bravo soil resulted in slight decrease in the amount of degradation of all three herbicides. This may indicate that bioavailable C or energy sources may be the major limiting factor in herbicide degradation. It also indicates that the Bravo soil is not nutrient limited, although based on the soil C to N ratio it would be predicted that this soil should be N-limited.

Biostimulation remediation effectiveness. None of the soil treatments were completely remediated, although the half-lives of the herbicides were reduced upon

addition of the best amendment over the unamended Bravo soil. Assuming first order degradation kinetics for all three herbicides in the first biostimulation experiment, the 0.5% corn fermentation byproduct amendment decreased the atrazine half-life from 180 to 58 days. For the metolachlor, addition of 5% sawdust reduced the half-life from 315 days to 178 days. Trifluralin showed a smaller change in half-life with a decrease from 216 to 171 days upon addition of the 5% cornstalk amendment. In the second biostimulation experiment, amending atrazine-spiked soils with manure resulted in decreased half-lives from 165 days in the unamended and sawdust-amended soil to only 95 days. When amended with sawdust or manure the half-life of metolachlor was reduced to 295 days. However, the trifluralin-spiked amended and unamended samples have essentially the same half-lives of 88 and 74 days, respectively.

It also appears that soil microorganisms are incapable of reducing the herbicide residues to concentrations below the GC detection limits of 300, 1200, and 300 $\mu\text{g kg}^{-1}$ for atrazine, metolachlor, and trifluralin, respectively. The Bravo background soil contains residual levels of all three herbicides: atrazine levels from 300 to 2,000 $\mu\text{g kg}^{-1}$ soil; metolachlor from 4,000 to 10,000 $\mu\text{g kg}^{-1}$ soil, and trifluralin from 100 to 5,000 $\mu\text{g kg}^{-1}$ soil. This is a concern because these residue levels are all at least 10 times the US EPA MCL levels in public drinking water established for atrazine, trifluralin, and metolachlor of 3, 2, and 100 $\mu\text{g L}^{-1}$, respectively. The atrazine residue levels for the atrazine and atrazine, manure-amended treatments in the second biostimulation experiment were reduced from 200 $\mu\text{g g}^{-1}$ to 12 and 5 $\mu\text{g g}^{-1}$, respectively, at the end of the incubation period, but also showed slight repression in the plant bioassay weights. This may indicate that atrazine metabolites, such as DEA and DIA, which can be phytotoxic when present at high enough levels, could still be present at levels high enough to effect the seedlings.

Indigenous microbial herbicide degradation. Previous studies at these sites and others established that pesticide-contaminated soils contain microorganisms capable of degrading the contaminating herbicides. The rate of degradation is variable depending upon the pesticide, its concentration, and soil factors. Studies by Kruger (1996) with the Alpha (rhizosphere and nonrhizosphere), Bravo (rhizosphere and nonrhizosphere) and Echo (E1 and E2) soils showed that both the Bravo and Echo control soils were effective at degrading atrazine. When spiked with 50 $\mu\text{g g}^{-1}$ soil [^{14}C]-

atrazine, the Bravo soils and Echo control soil mineralized about 30% of the atrazine, while the Alpha soil mineralized approximately 1% of the atrazine during a 63 day incubation period. The Echo control soil mineralized 1% of 50 $\mu\text{g g}^{-1}$ soil, while all of the other soils mineralized $\leq 0.5\%$ of the metolachlor. When 50 $\mu\text{g g}^{-1}$ soil [^{14}C]-pendimethalin, a dinitroaniline herbicide in the same family as trifluralin, was applied to the soils, the Echo control soil mineralized 2%, the Bravo rhizosphere soil mineralized about 1%, while all the other soils mineralized $< 1\%$. Alvey and Crowley (1995) spiked a soil with 100 $\mu\text{g g}^{-1}$ atrazine and observed mineralization of 73% of the added atrazine in an 11-week period by the soil without amendment addition. Field plots were treated with freshly applied or were landfarmed with a soil contaminated with a mixture of moderate levels of aged alachlor, trifluralin, metolachlor, and atrazine residues, ranging from 15 to 180 $\mu\text{g g}^{-1}$ herbicide; they showed essentially the same amount of degradation of each herbicide in unamended plots versus cornstalk- or sewage-amended plots (Dzantor *et al.*, 1993). This indicates that soils with previous exposure to herbicide residues may possess the microorganisms which have adapted to degrade the herbicides. Therefore, biostimulation may not be necessary in a soil contaminated with a single herbicide or with a combination of herbicides at low levels of contamination, such as may occur at a minor spill site.

Our results are similar to those of Kruger (1996), who showed that atrazine was degraded in Alpha, Bravo, and Echo soils. The Bravo and Echo site soils, which contained low concentrations of herbicide residues, contained microorganisms capable of atrazine degradation and mineralization after addition of low to moderate concentrations of atrazine. Growth of the atrazine-mineralizing populations in the Bravo and Echo control soils occurred with increasing atrazine concentration up to 100 $\mu\text{g g}^{-1}$ atrazine.

Degradation in soil contaminated with herbicide mixtures. In our experiments, high levels of added herbicides in the Bravo (200 $\mu\text{g g}^{-1}$ atrazine and mixtures of 200 $\mu\text{g g}^{-1}$ of the 3 herbicides) and the residues in the soil from E2 reduced atrazine mineralization. After treatment with the mixture of herbicides, the Bravo soil atrazine-mineralizing microorganisms declined to levels below those in the untreated soil. This suggests that the mixture was directly toxic to the cells. However, when Kruger (1996) performed a similar experiment with rhizosphere Bravo soil, applying 200

$\mu\text{g g}^{-1}$ soil [^{14}C]-atrazine alone, and in combination with $200 \mu\text{g g}^{-1}$ trifluralin, $200 \mu\text{g g}^{-1}$ metolachlor, and $200 \mu\text{g g}^{-1}$ of both metolachlor and trifluralin, no inhibition of atrazine mineralization was observed. All of the treated soils mineralized between 60 to 80% atrazine after 63 days incubation. Kruger (1996) did not examine the fate of the herbicides that were not mineralized. The difference in results may be due to the fact that the Bravo soil used in these earlier experiments was a rhizosphere soil collected from Bravo one year prior to the non-rhizosphere soil used in our experiment. In other studies, $10,000 \mu\text{g g}^{-1}$ alachlor applied alone or in a combination with metolachlor, atrazine, and trifluralin, only minimal degradation of any of the herbicides was observed (Dzantor and Felsot, 1991). The pesticide concentration was determined to be the single most important factor affecting degradation of six different pesticides, with much slower degradation rates at high ($1000 \mu\text{g g}^{-1}$) levels of contamination than at moderate ($100 \mu\text{g g}^{-1}$) levels (Schoen and Winterlin, 1987).

Metolachlor toxicity or competition with the indigenous microorganisms for the added C was demonstrated by repression of the amount of mineralization in all three combination treatments and reduction in the atrazine-degrader population. The atrazine-mineralizing populations appeared to recover after the saturation portion of the experiment. This recovery may be a result of metolachlor degradation during the anaerobic period, resulting in detoxification of the herbicides or metabolites. Saturation may also have increased the mass of dissolved metolachlor or atrazine as the herbicide precipitate dissolved or the herbicides desorbed from the soil and became more bioavailable to degrading microorganisms. The increase in atrazine mineralization after the saturation period may also be attributed to the cornstalk amendment, which may increase the atrazine-mineralizing or metolachlor-degrading populations.

Herbicide physicochemical characteristics. In these experiments, we used three herbicides with very different physicochemical characteristics, which will cause the herbicides to be present in three different phases within the soil microcosm: (a) sorbed to the soil; (b) dissolved in the soil water; or (c) precipitated in crystalline form. Trifluralin had the highest sorption potential with large $\log K_{oc}$ values ranging from 2.94 to 4.49 and a very low solubility of 0.3 mL g^{-1} , so most of it will be bound to the soil or crystallized. Metolachlor should be the most soluble and bioavailable compound of the three, due to its high water solubility of 530 mL g^{-1} . Since the soil microcosms contained only 10%

moisture (5 ml of water per microcosm), then a maximum of 2650 µg metolachlor, 165 µg atrazine, and 1.5 µg trifluralin will be soluble in the soil water and we applied 10,000 µg of each herbicide to the soil. Therefore, most of the trifluralin and a large portion of the atrazine and metolachlor will be present in the soil in crystalline form. Despite the large differences in chemical behavior and bioavailability of the three herbicides in the soil, it did not appear that this affected the amount of degradation that occurred for the different chemicals.

CONCLUSIONS

These results indicate that in most situations where a soil is contaminated with low to moderate levels of single or mixed herbicide residues, the soil microorganisms most likely possess the enzymes necessary to degrade the herbicide residues and natural attenuation of the herbicide contamination may occur, as was observed in the Bravo and Echo control soils. In the Alpha soil, it appeared that the indigenous microbial populations were able to adapt after a long lag period to slowly degrade atrazine. When a soil is contaminated with high levels of a single or multiple herbicides, it appears that a remediation technology, such as biostimulation or bioaugmentation, may be necessary to increase the rate of degradation or to overcome the toxic effects of the herbicides on the degrading microorganisms. However biostimulation with one of the six organic amendments or the inorganic solution was not effective in completely removing the contamination in the Bravo soil. It appeared that biostimulation was necessary to enhance the degradation of metolachlor, however only a few amendments enhanced atrazine degradation and none of the amendments helped to increase trifluralin degradation over the unamended Bravo soil. Perhaps the most appropriate amendments for enhancing biodegradation of these three herbicides were not chosen for this experiment. It appeared that aerobic-anaerobic cycling was beneficial in reducing the toxic components of the herbicide residues and increasing the solubility of the remaining residues to make them more bioavailable to the soil microorganisms for degradation.

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GENERAL SUMMARY

In this thesis I have investigated the effectiveness of using bioaugmentation and biostimulation technologies to enhance biodegradation of herbicide-contaminated soils. I have isolated a bacterium, *Agrobacterium radiobacter* strain J14a, that is capable of mineralizing 94% of 50 $\mu\text{g ml}^{-1}$ atrazine in 72 hours for utilization as a N source, producing the metabolites hydroxyatrazine (HA), deethylatrazine (DEA), and hydroxy-deethylatrazine (DEHA). Addition of organic N in the form of tryptic soy broth resulted in partially repression of ring mineralization, however exposure to high levels of inorganic NH_4NO_3 did not inhibit atrazine degradation. The bacterium produced the atrazine-degrading enzymes constitutively, and these enzymes remained intracellular during atrazine degradation. J14a also degraded the s-triazine herbicides ametryne, cyanazine, prometon, and simazine in N-limited medium.

Addition of 10^5 J14a cells g^{-1} into a soil with low atrazine-mineralization capabilities, spiked with 50 and 200 $\mu\text{g atrazine g}^{-1}$ soil, resulted in 2- to 5-times greater mineralization than without added cells. Sucrose addition did not result in significantly higher mineralization rates or shorter lag times. However, introduction of J14a (10^5 cells g^{-1}) into another soil with an indigenous atrazine-mineralizing population, reduced atrazine degradation lag times over the indigenous populations in all treatments, but increased mineralization only in the 50 $\mu\text{g atrazine g}^{-1}$ soil supplemented with 1% sucrose.

Atrazine mineralization screenings in soils collected from three agricultural dealership sites showed that atrazine is readily mineralized at concentrations ranging from 5 to 200 $\mu\text{g atrazine g}^{-1}$ soil. However, addition of atrazine to soils contaminated with herbicide mixtures, resulted in repression of atrazine mineralization and reductions in the atrazine-mineralizing populations. The reduction in atrazine mineralization was particularly severe when metolachlor was added in combination with atrazine or atrazine+trifluralin. Addition of two different levels of organic amendments, 0.5% and 5%, to soil contaminated with a 200 $\mu\text{g g}^{-1}$ mixture of atrazine, metolachlor, and trifluralin resulted in enhanced degradation of atrazine and metolachlor, but not trifluralin. The 5% amendment level resulted in an overall increase in the amount of all three herbicides degraded over the 0.5% level of addition. The same soil was spiked with 200 $\mu\text{g g}^{-1}$ of the three herbicides alone or in a three-herbicide combination and were amended with the 5% level of manure or

sawdust. This experiment showed that the type of amendment added and whether the herbicide was applied alone or in a combination affected the amount of biodegradation of atrazine and metolachlor. These two factors did not affect the amount of trifluralin degraded, with essentially the same amount of degradation occurring in all treatments.

Overall, the results indicate that soils contaminated with low levels of herbicides may not need remediation treatments to obtain herbicide biodegradation, as the soil microorganisms appear to possess the ability to degrade contaminants. However, when soils are contaminated with high concentrations of herbicides or in combinations, it appears that microbial toxicity problems result in the need for use of a bioaugmentation agent or biostimulating amendments to obtain degradation of the herbicides.

APPENDIX A**THE MICROBIAL POPULATIONS (BACTERIA, ACTINOMYCETES, AND FUNGI),
MICROBIAL RESPIRATION, AND DEHYDROGENASE RESULTS AT EACH SAMPLING
TIME FOR THE FIRST BIOSTIMULATION EXPERIMENT**

Each data point for the different types of measurements is the average of three replicate jars of Bravo soil for each treatment which were sacrificed at each time point. If error bars are present, they are the standard deviation of three replicate jars of Bravo soil. The methods for determining each of the microbial parameters are shown in the “Materials and Methods” section in Chapter 3 of this thesis.

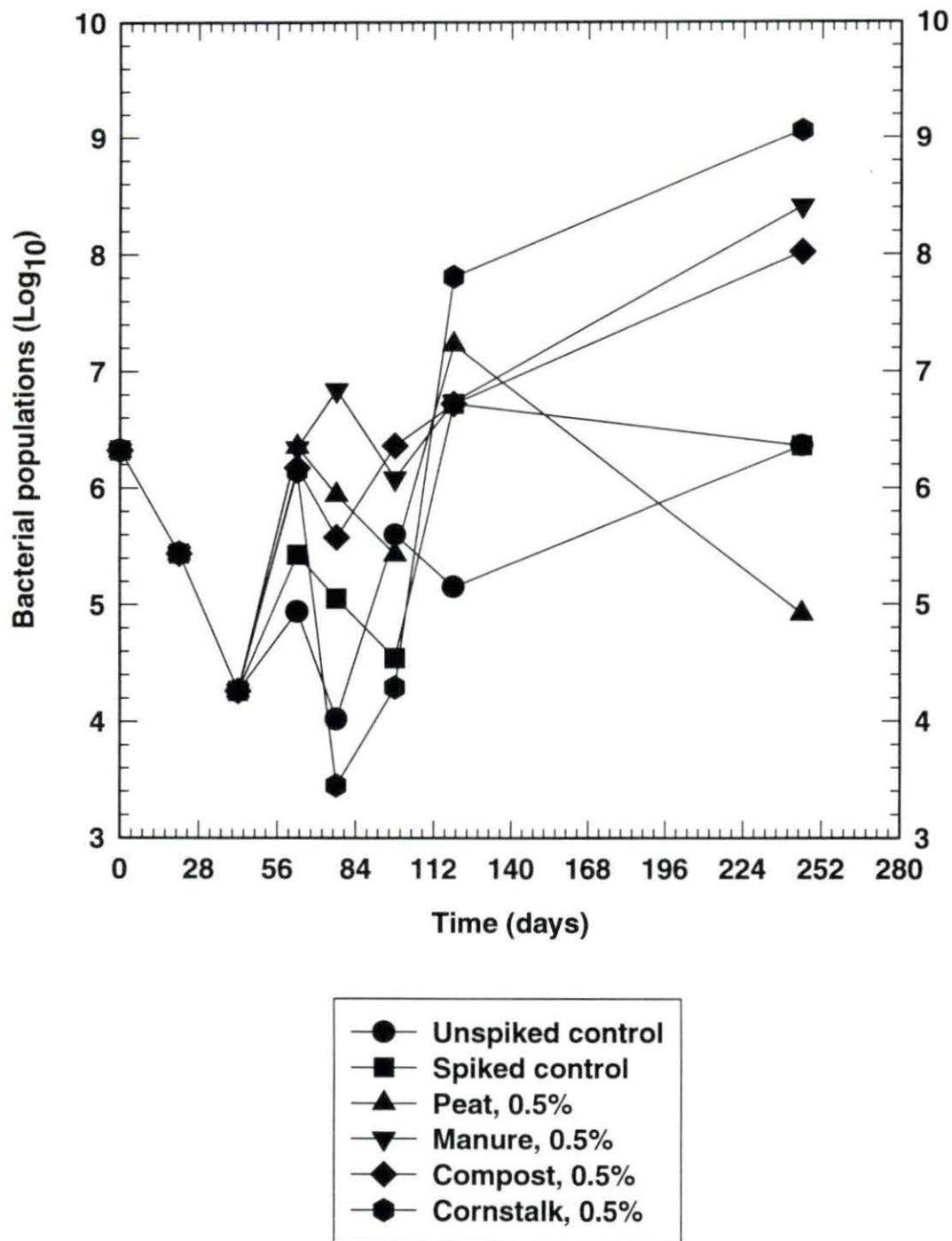


Figure 1. Biostimulation I experiment bacterial populations for the 0.5% amendment rate of peat, manure, compost, and cornstalk as compared to the spiked and unspiked controls.

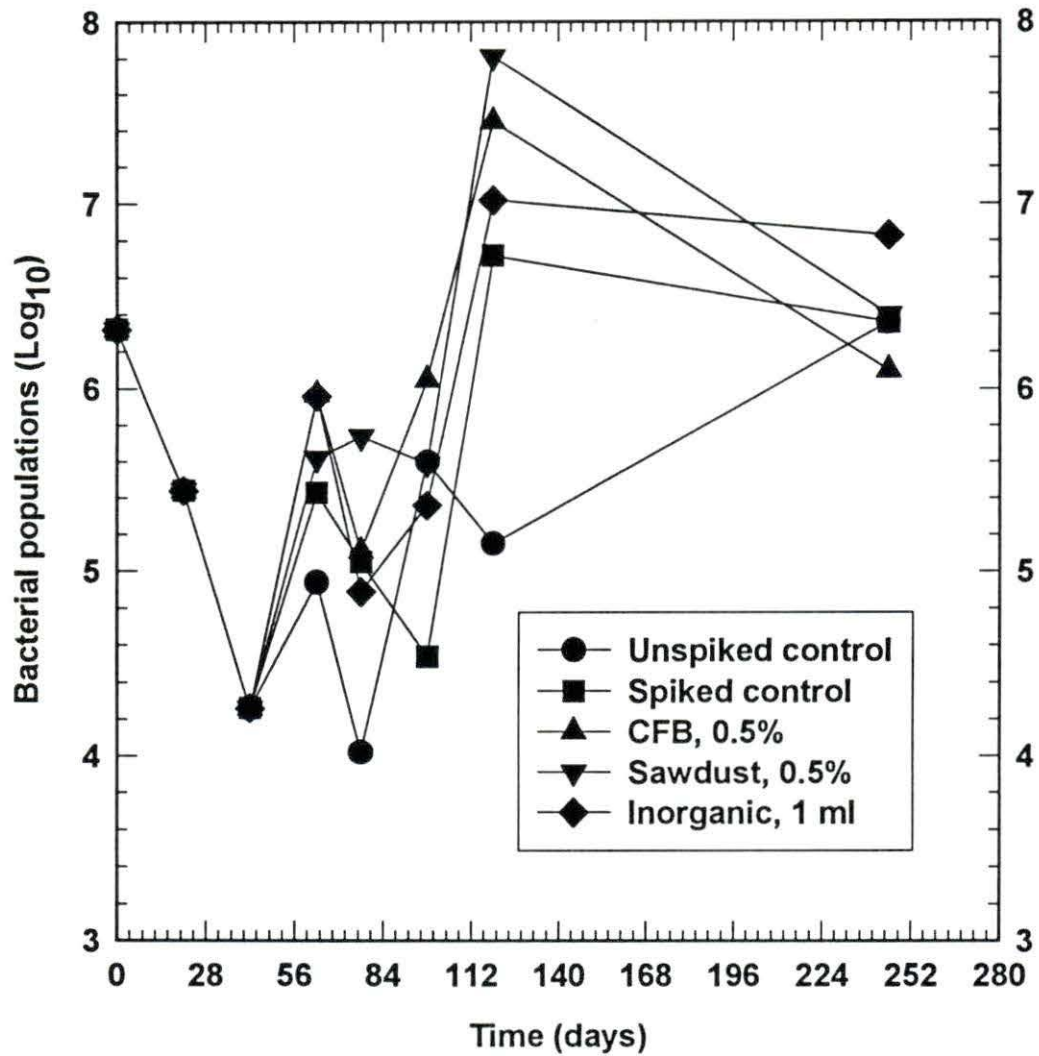


Figure 2. Biostimulation I experiment bacterial populations for the low amendment rate of corn fermentation byproduct (CFB), sawdust, and inorganic solution as compared to the spiked and unspiked controls.

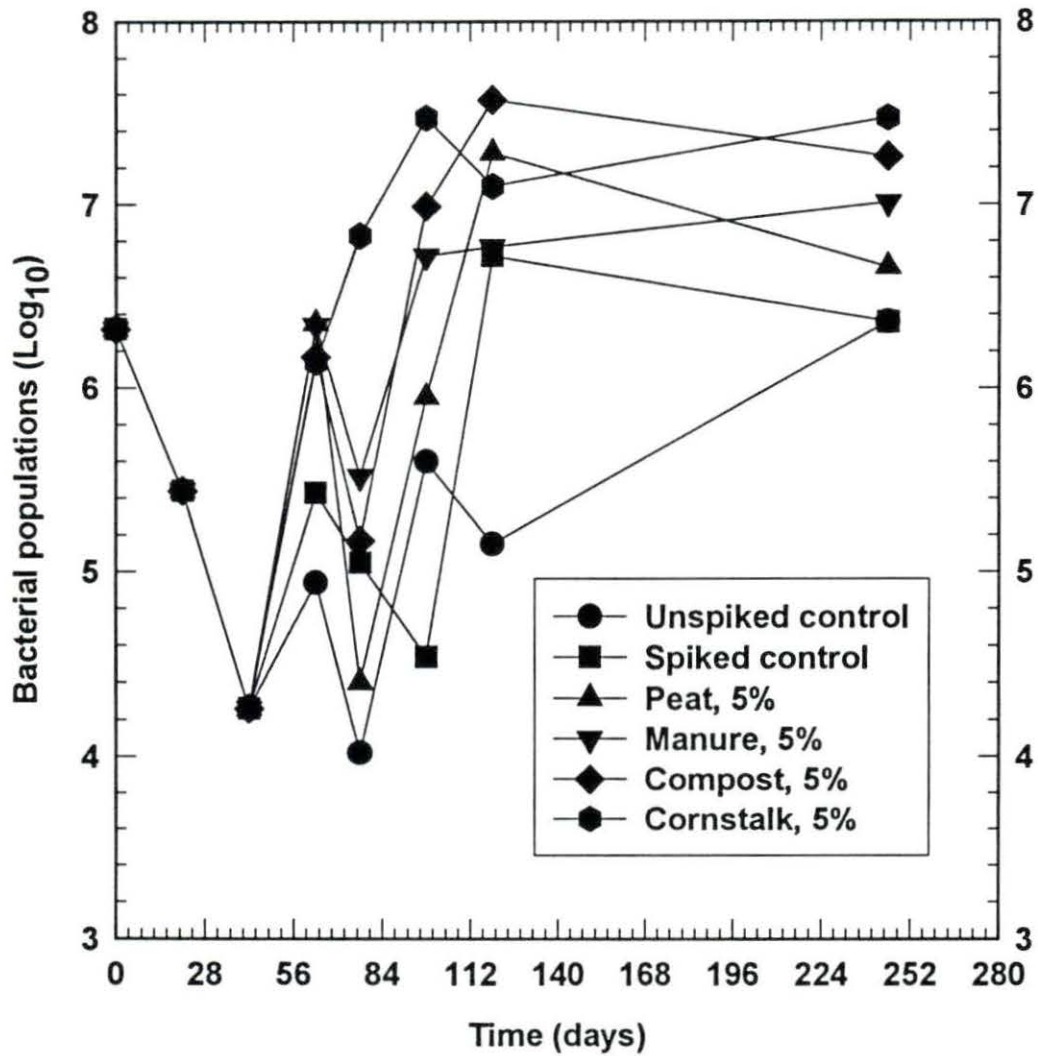


Figure 3. Biostimulation I experiment bacterial populations for the 5% amendment rate of peat, manure, compost, and cornstalk as compared to the spiked and unspiked controls.

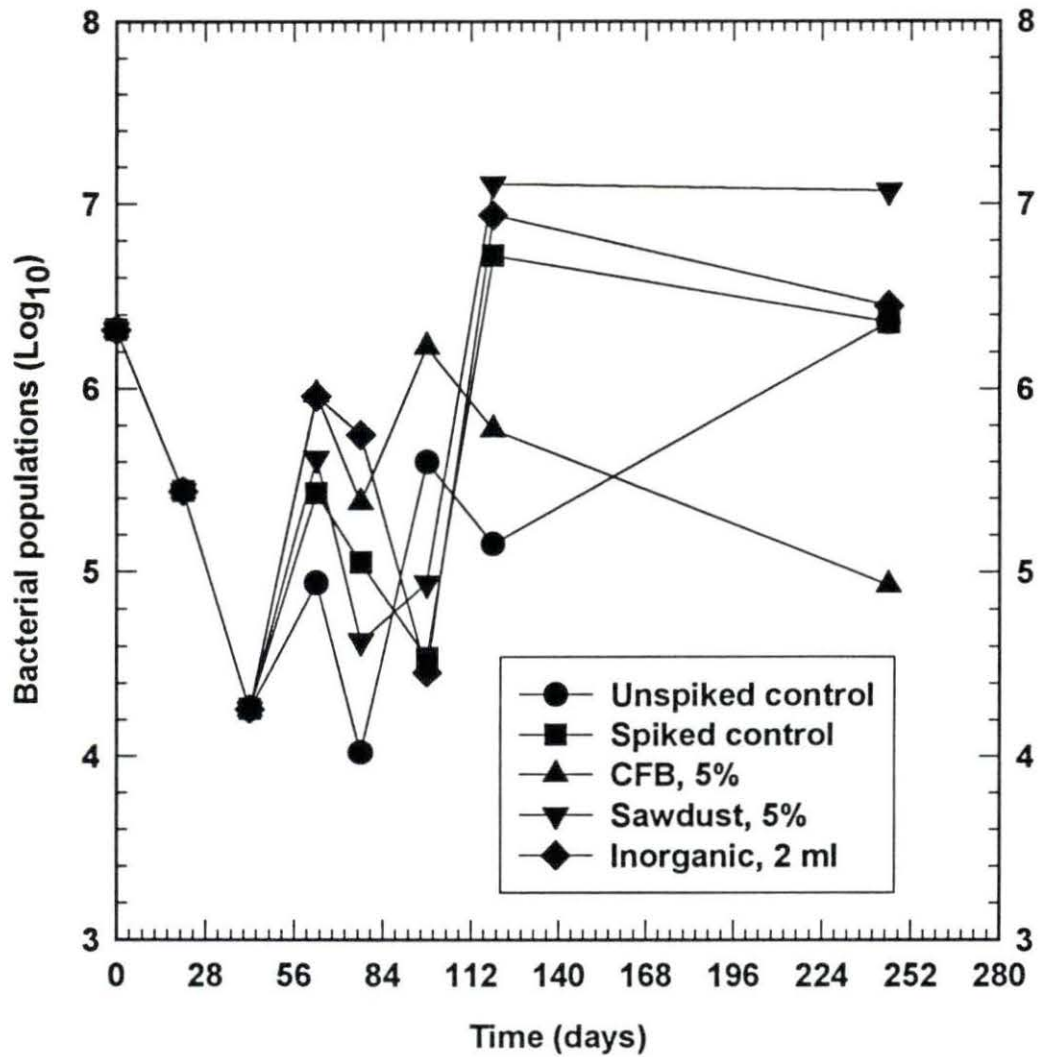


Figure 4. Biostimulation I experiment bacterial populations for the high amendment rate of corn fermentation byproduct (CFB), sawdust, and inorganic solution as compared to the spiked and unspiked controls.

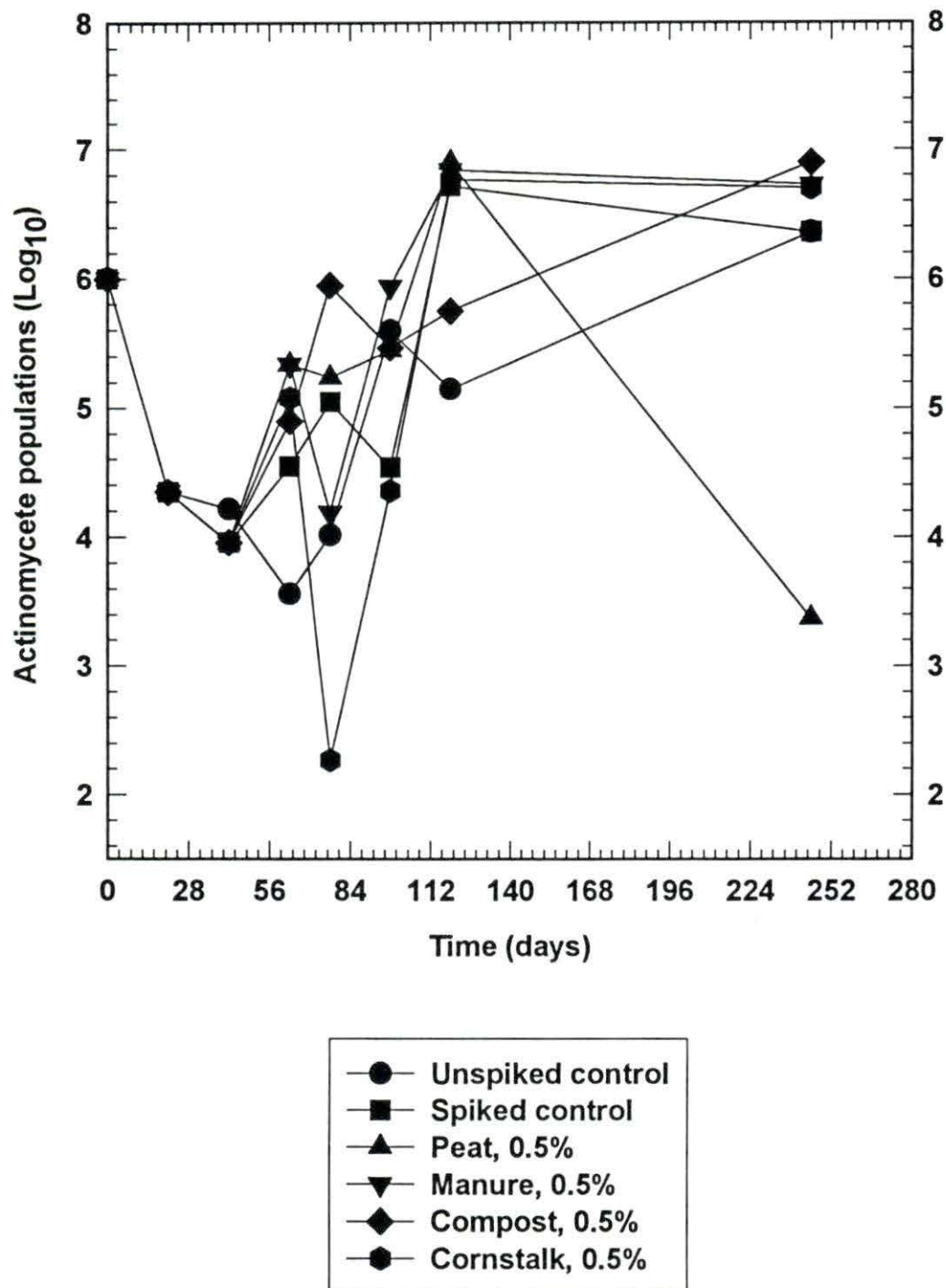


Figure 5. Biostimulation I experiment actinomycete populations for the 0.5% amendment rate of peat, manure, compost, and cornstalk as compared to the spiked and unspiked controls.

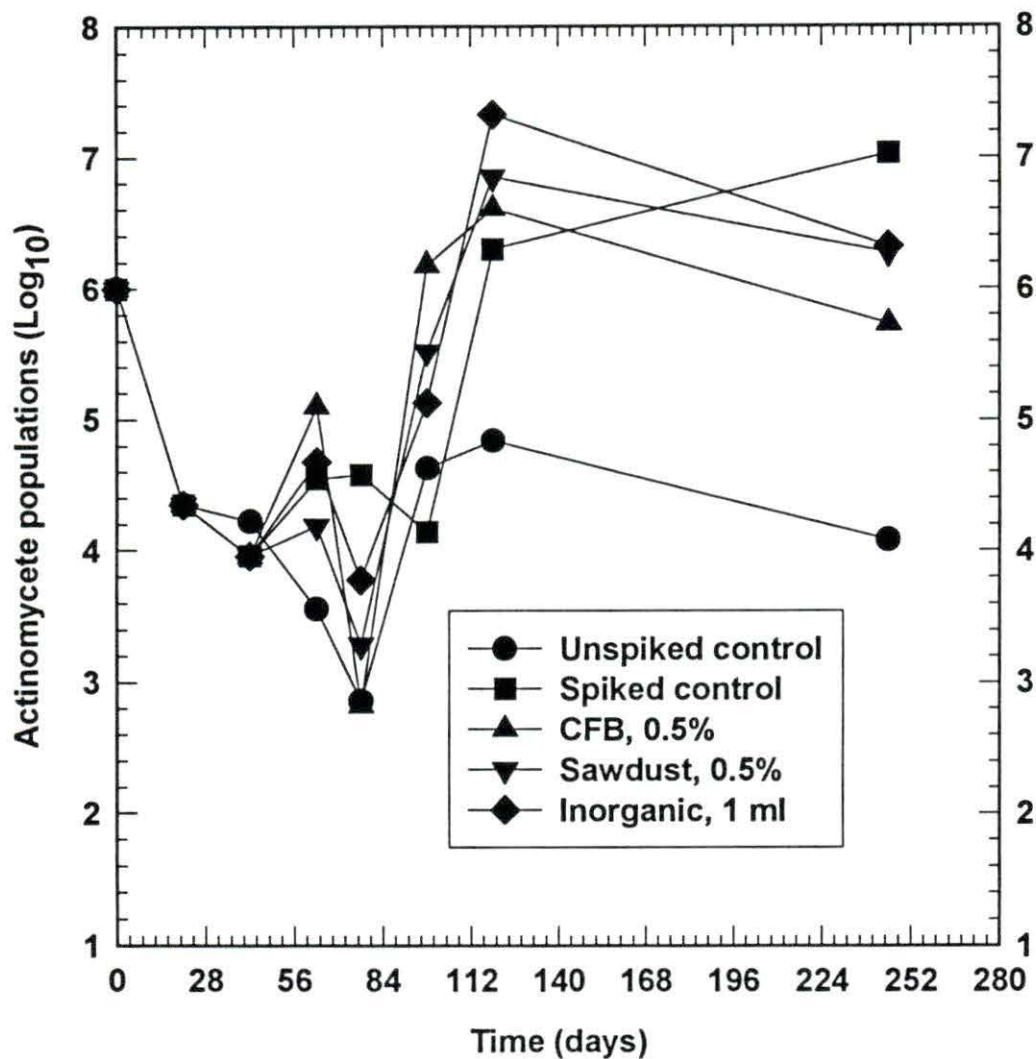


Figure 6. Biostimulation I experiment actinomycete populations for the low amendment rate of corn fermentation byproduct (CFB), sawdust, and inorganic solution as compared to the spiked and unspiked controls.

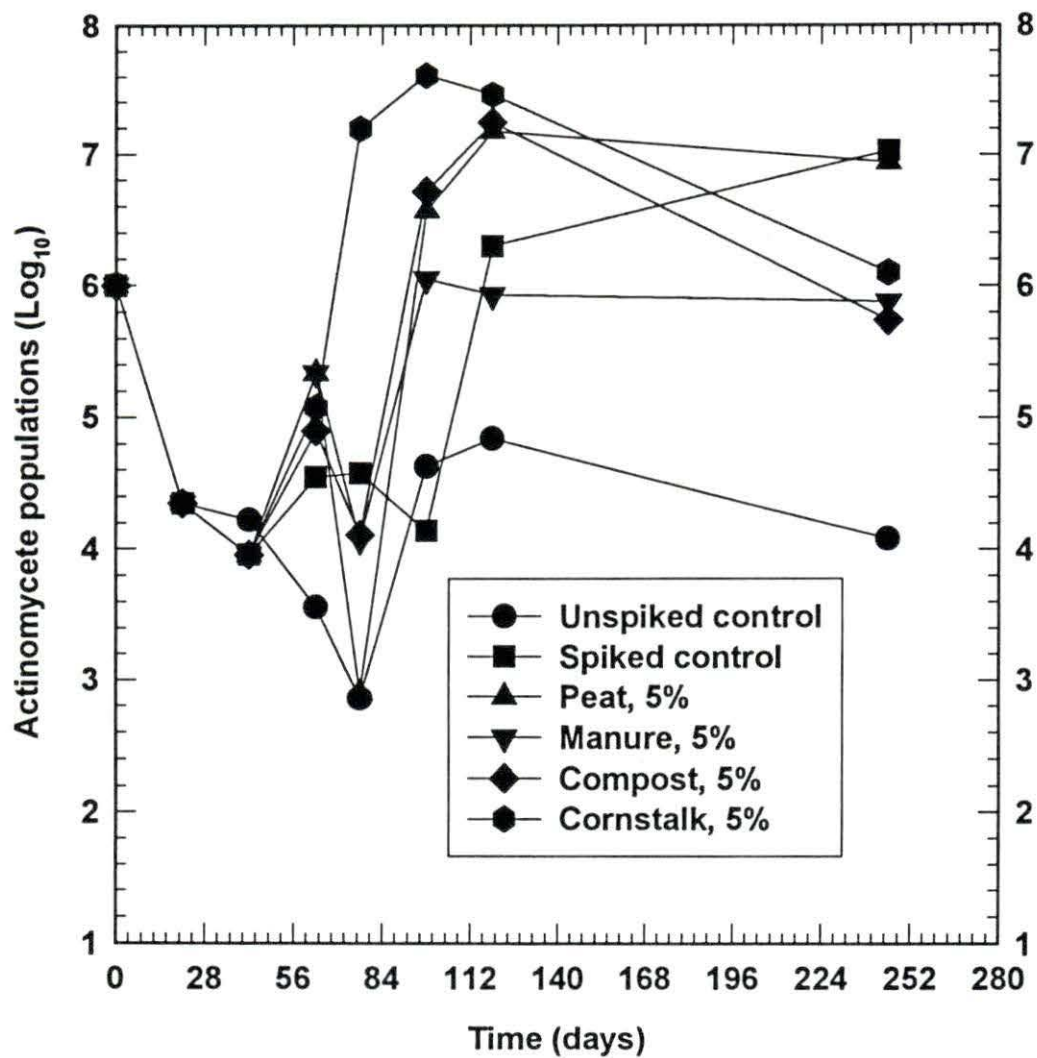


Figure 7. Biostimulation I experiment actinomycete populations for the 5% amendment rate of peat, manure, compost, and cornstalk as compared to the spiked and unspiked controls.

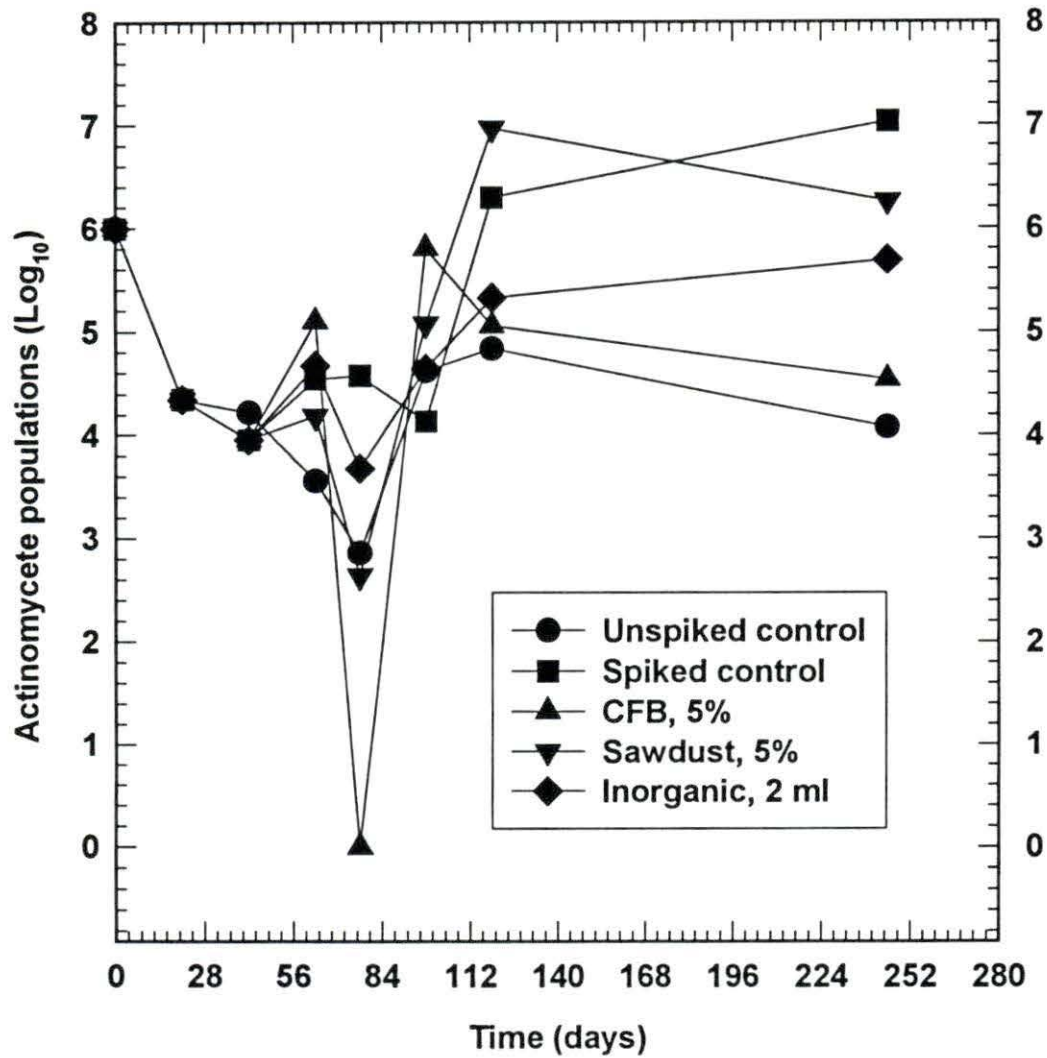


Figure 8. Biostimulation I experiment actinomycete populations for the high amendment rate of corn fermentation byproduct (CFB), sawdust, and inorganic solution as compared to the spiked and unspiked controls.

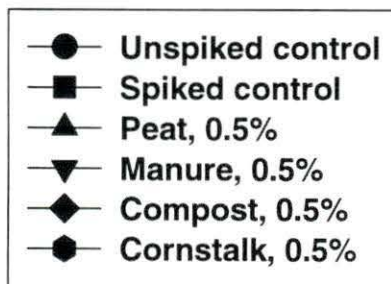
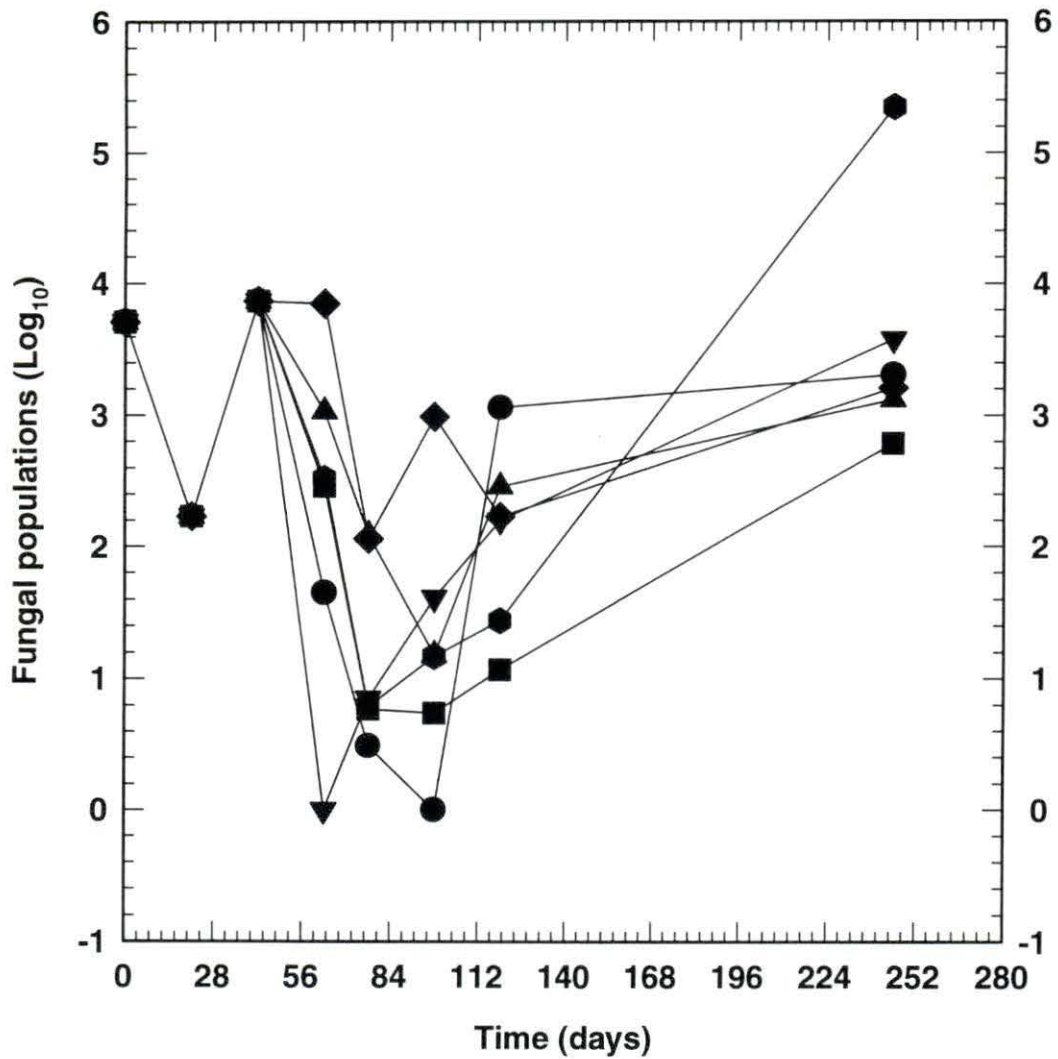


Figure 9. Biostimulation I experiment fungal populations for the 0.5% amendment rate of peat, manure, compost, and cornstalk as compared to the spiked and unspiked controls.

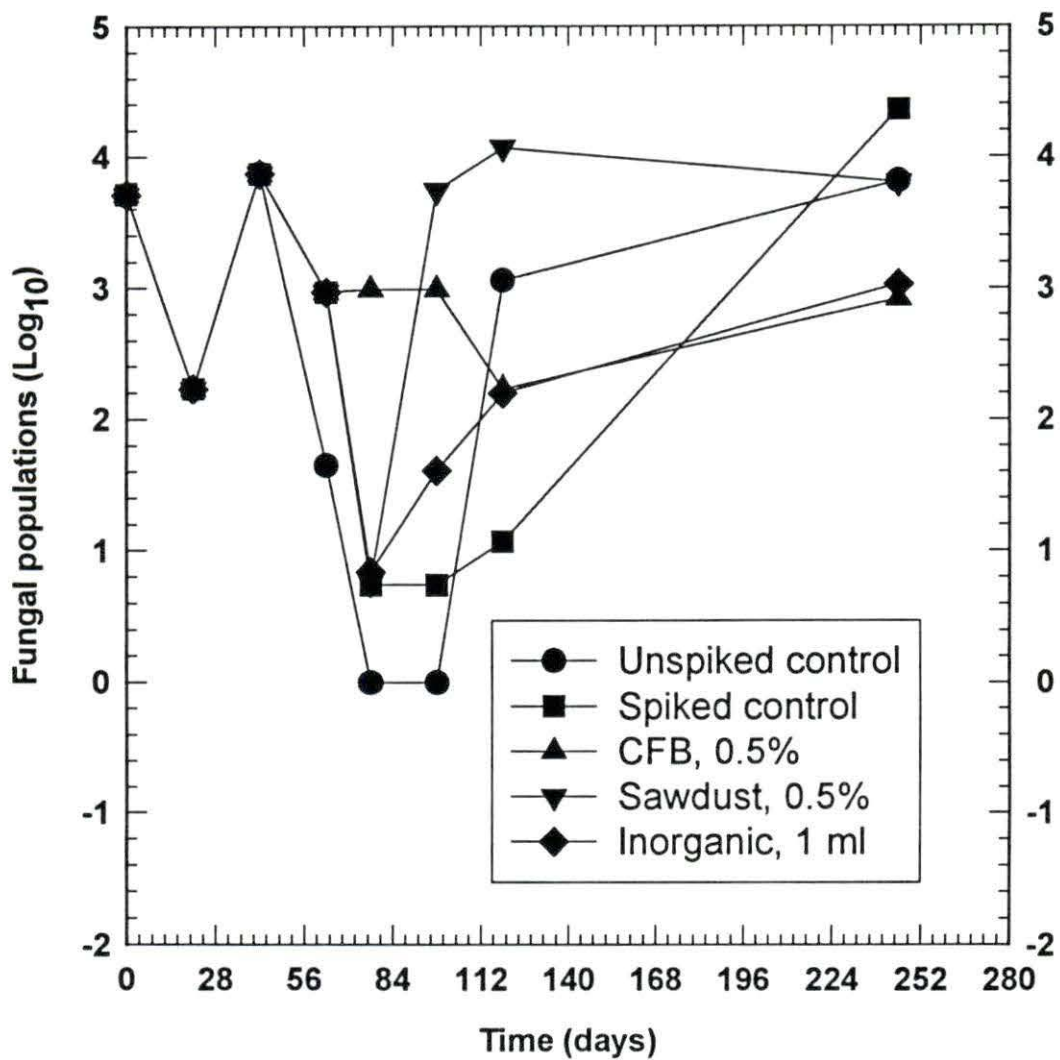


Figure 10. Biostimulation I experiment fungal populations for the low amendment rate of corn fermentation byproduct (CFB), sawdust, and inorganic solution as compared to the spiked and unspiked controls.

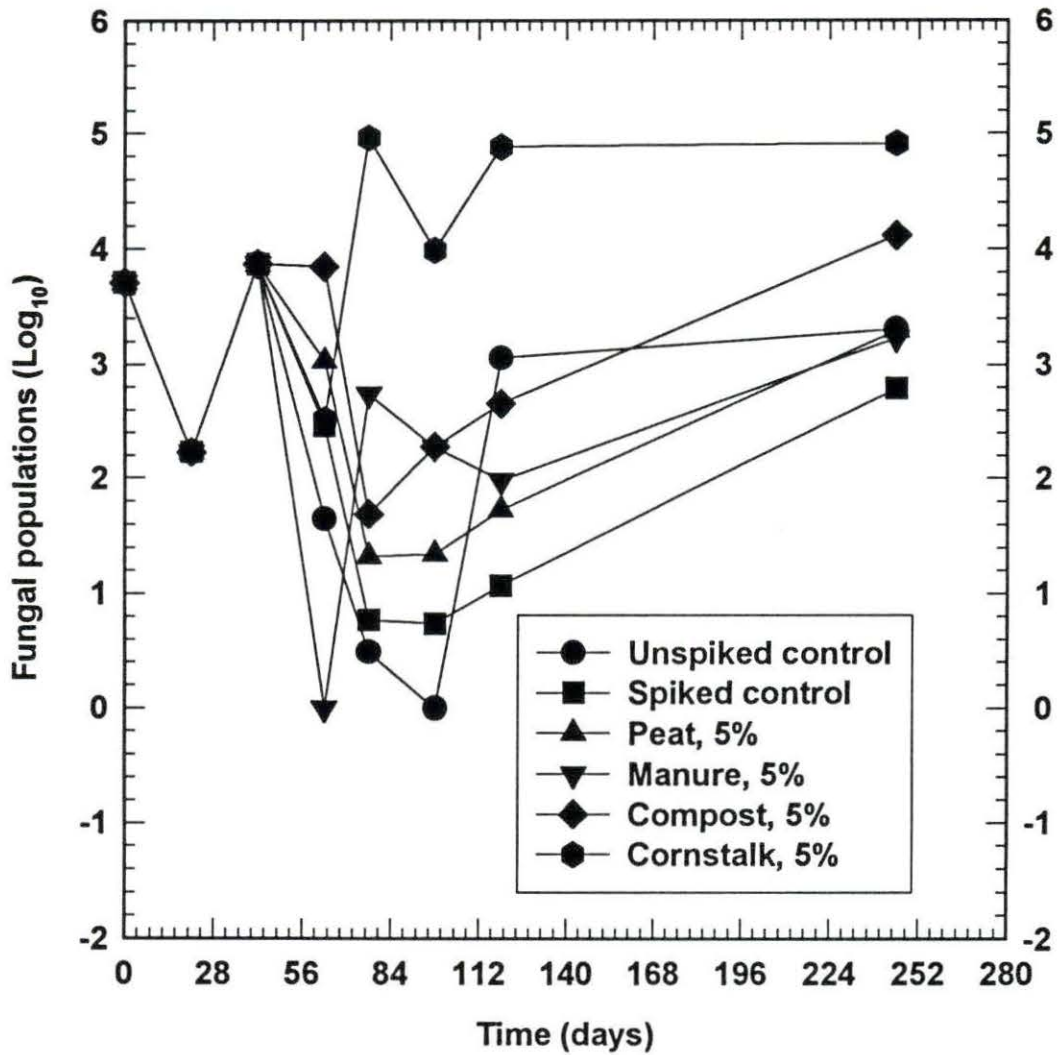


Figure 11. Biostimulation I experiment fungal populations for the 5% amendment rate of peat, manure, compost, and cornstalk as compared to the spiked and unspiked controls.

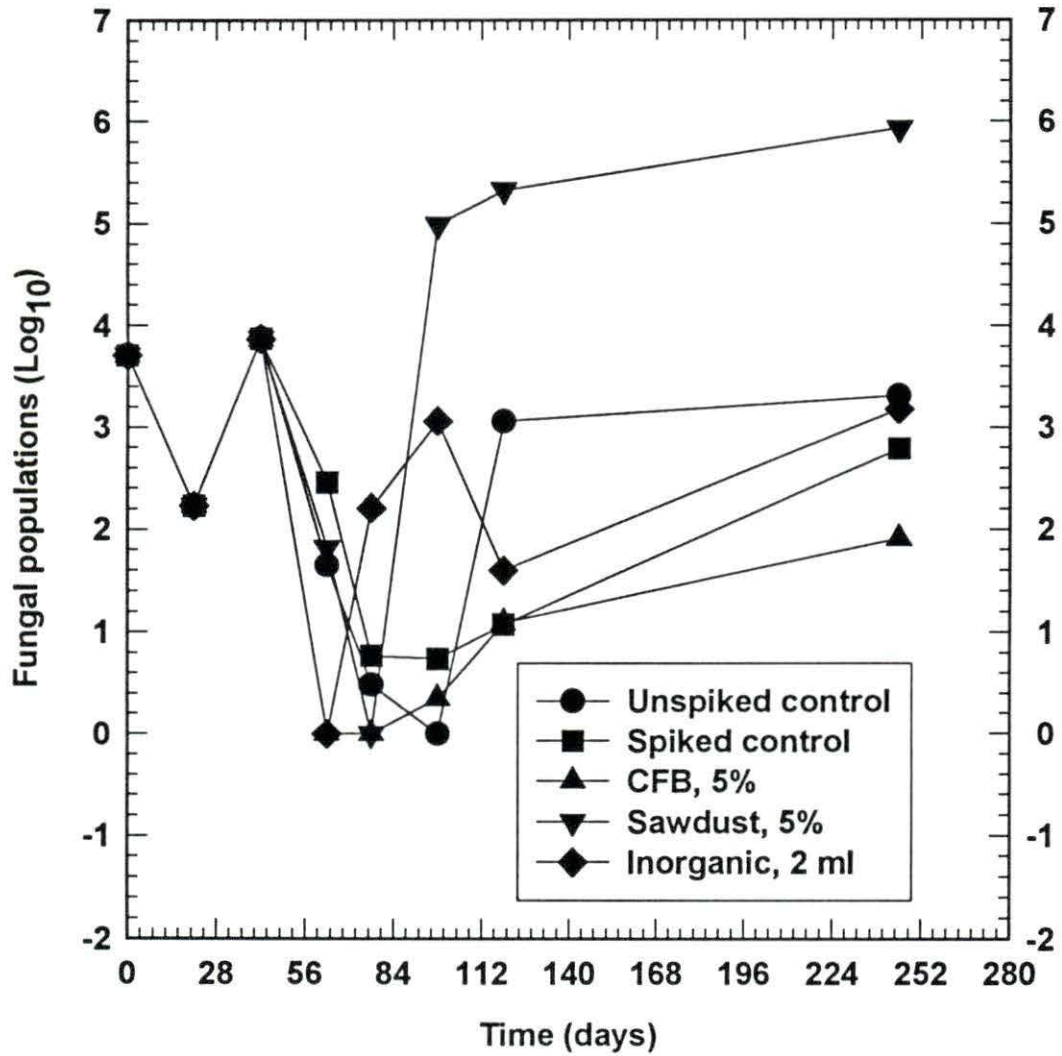


Figure 12. Biostimulation I experiment fungal populations for the high amendment rate of corn fermentation byproduct (CFB), sawdust, and inorganic solution as compared to the spiked and unspiked controls.

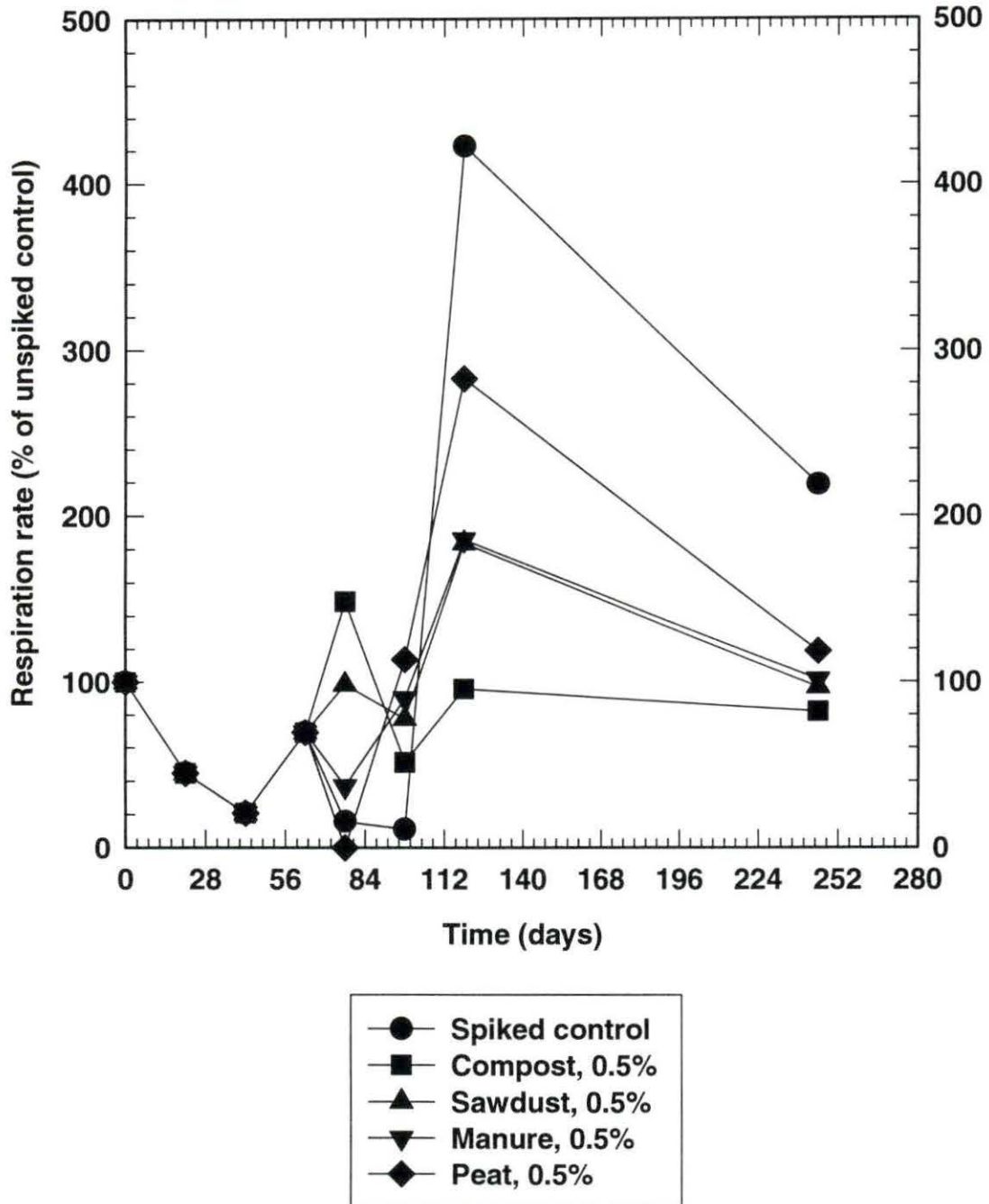


Figure 13. Biostimulation experiment I respiration results for the 0.5% amendment rate of the compost, sawdust, manure, and peat treatments.

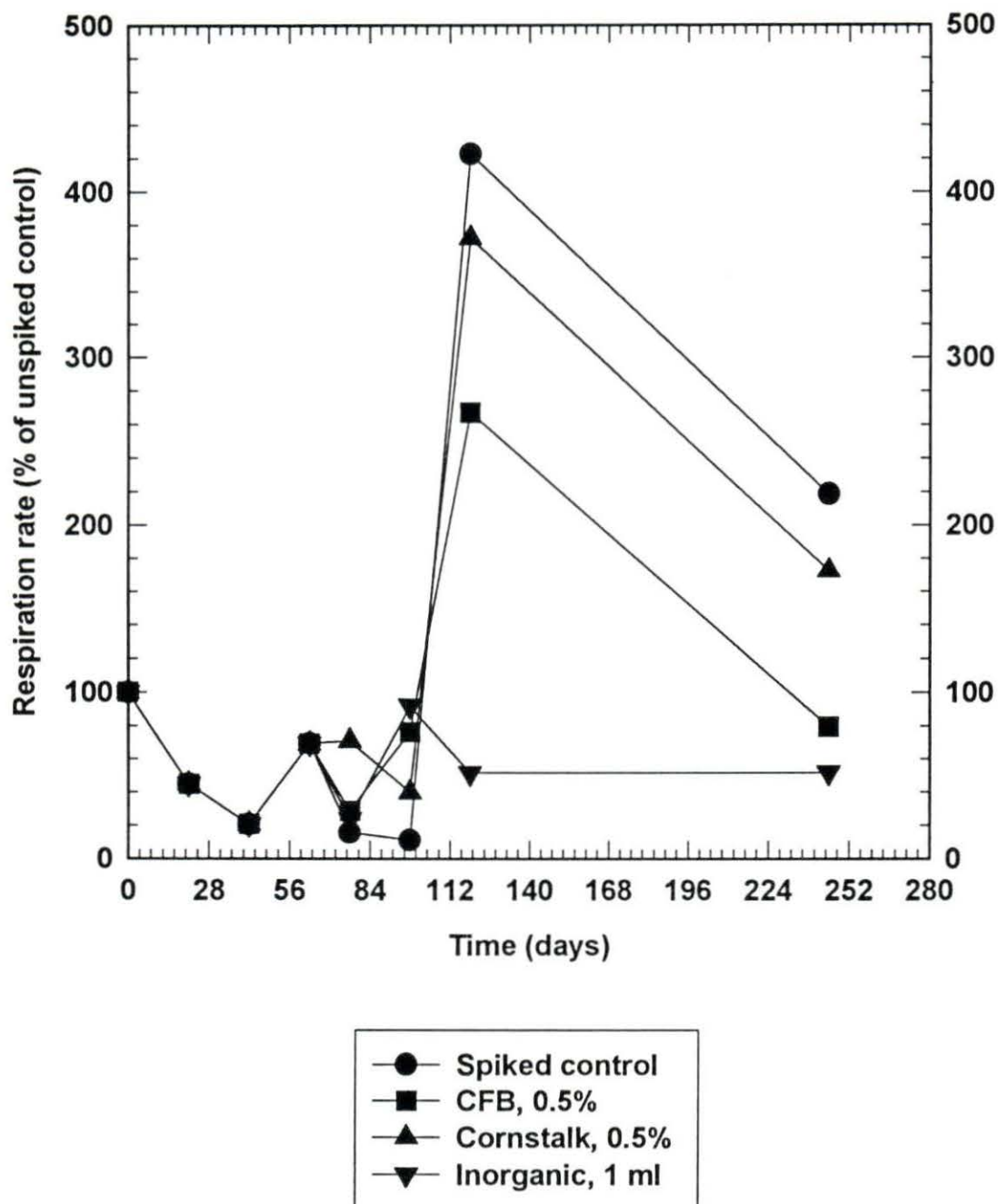


Figure 14. Biostimulation experiment I respiration results for the low amendment rate of the CFB (corn fermentation byproduct), cornstalk, and inorganic nutrient treatments.

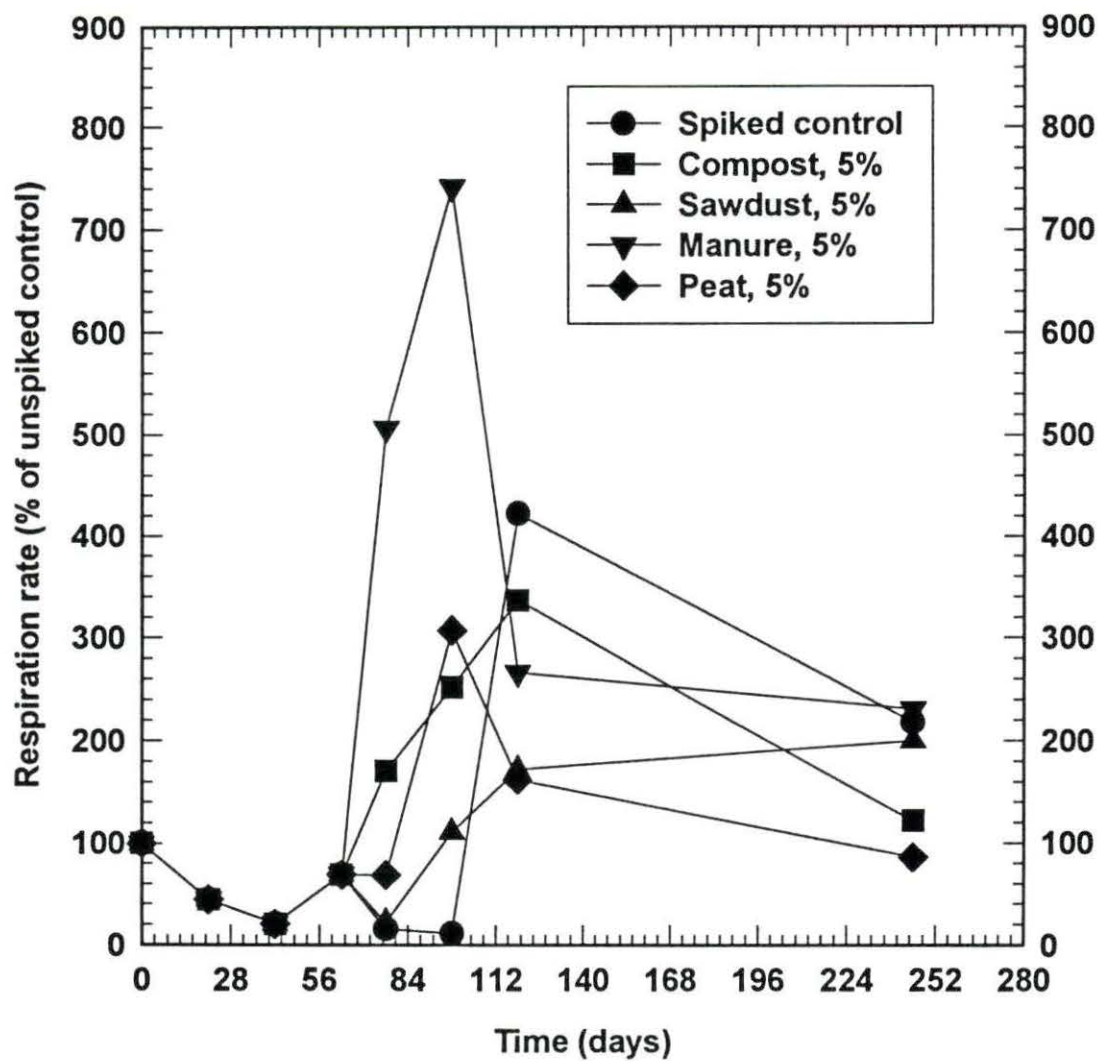


Figure 15. Biostimulation experiment I respiration results for the 5% amendment rate of the compost, sawdust, manure, and peat treatments.

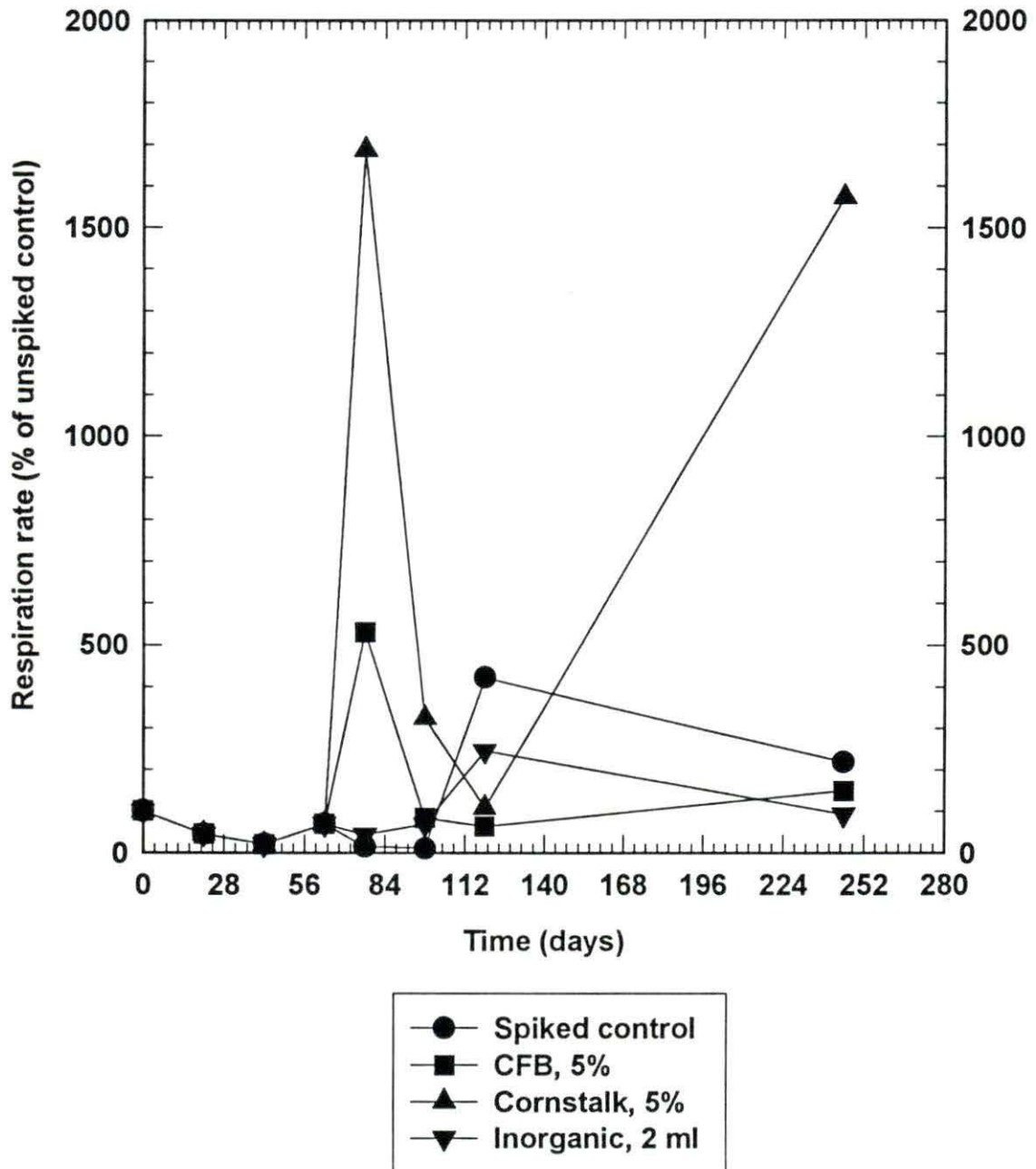


Figure 16. Biostimulation experiment I respiration results for the high amendment rate of the CFB (corn fermentation byproduct), cornstalk, and inorganic nutrient treatments.

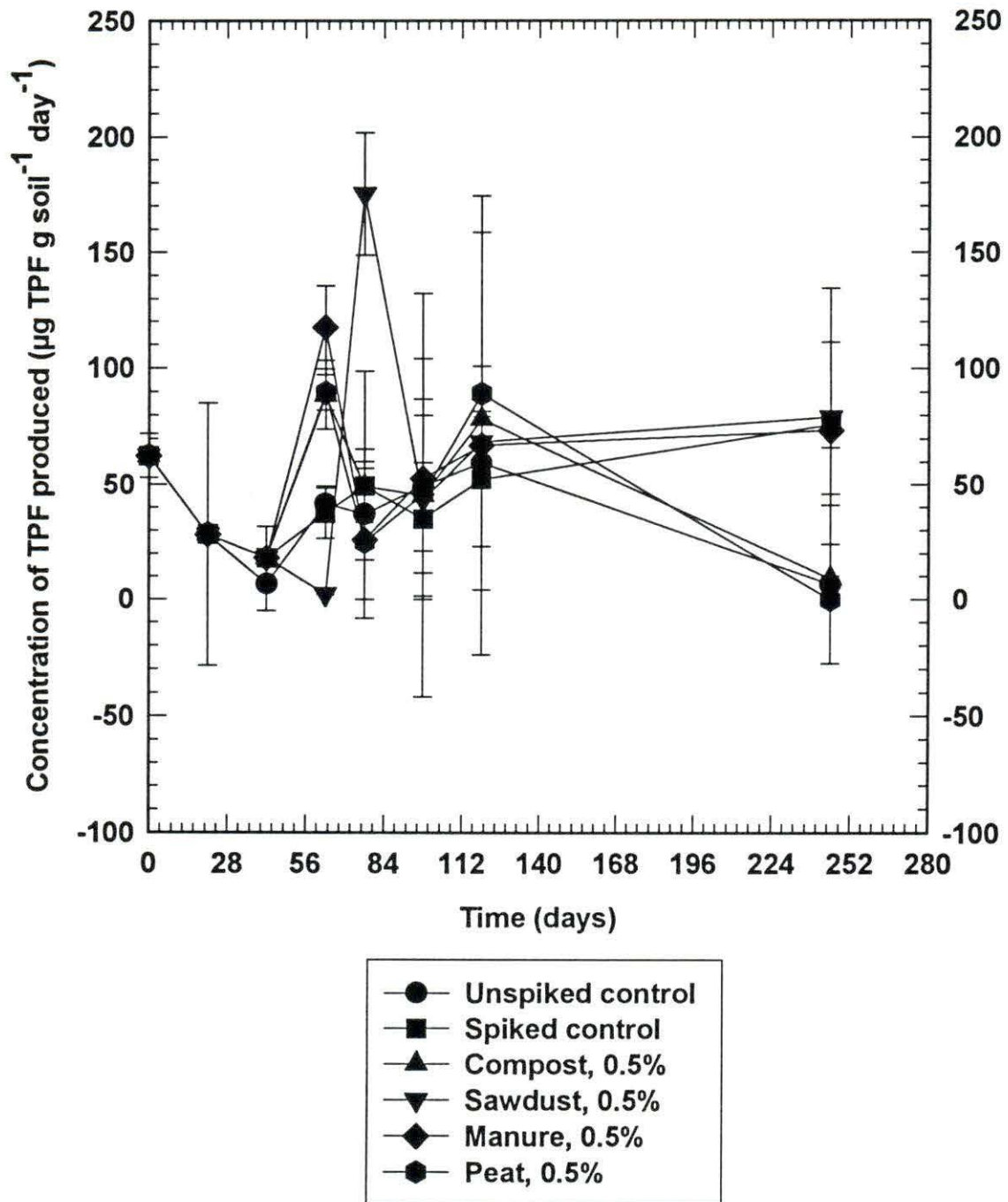


Figure 17. Biostimulation experiment I dehydrogenase results for the low amendment rate of the compost, sawdust, manure, and peat treatments.

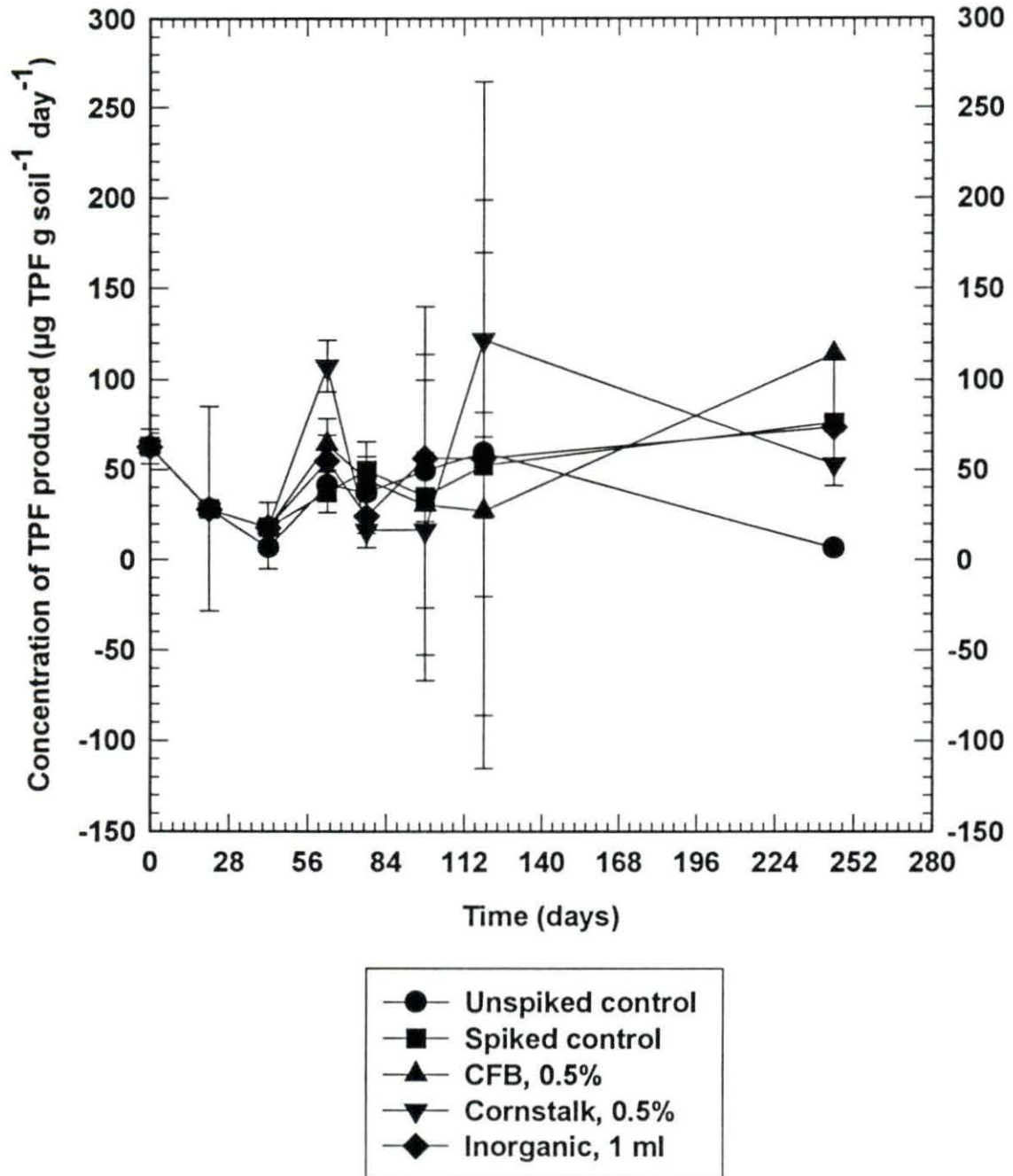


Figure 18. Biostimulation experiment I dehydrogenase results for the low amendment rate of the corn fermentation byproduct (CFB), cornstalk, and inorganic solution treatments.

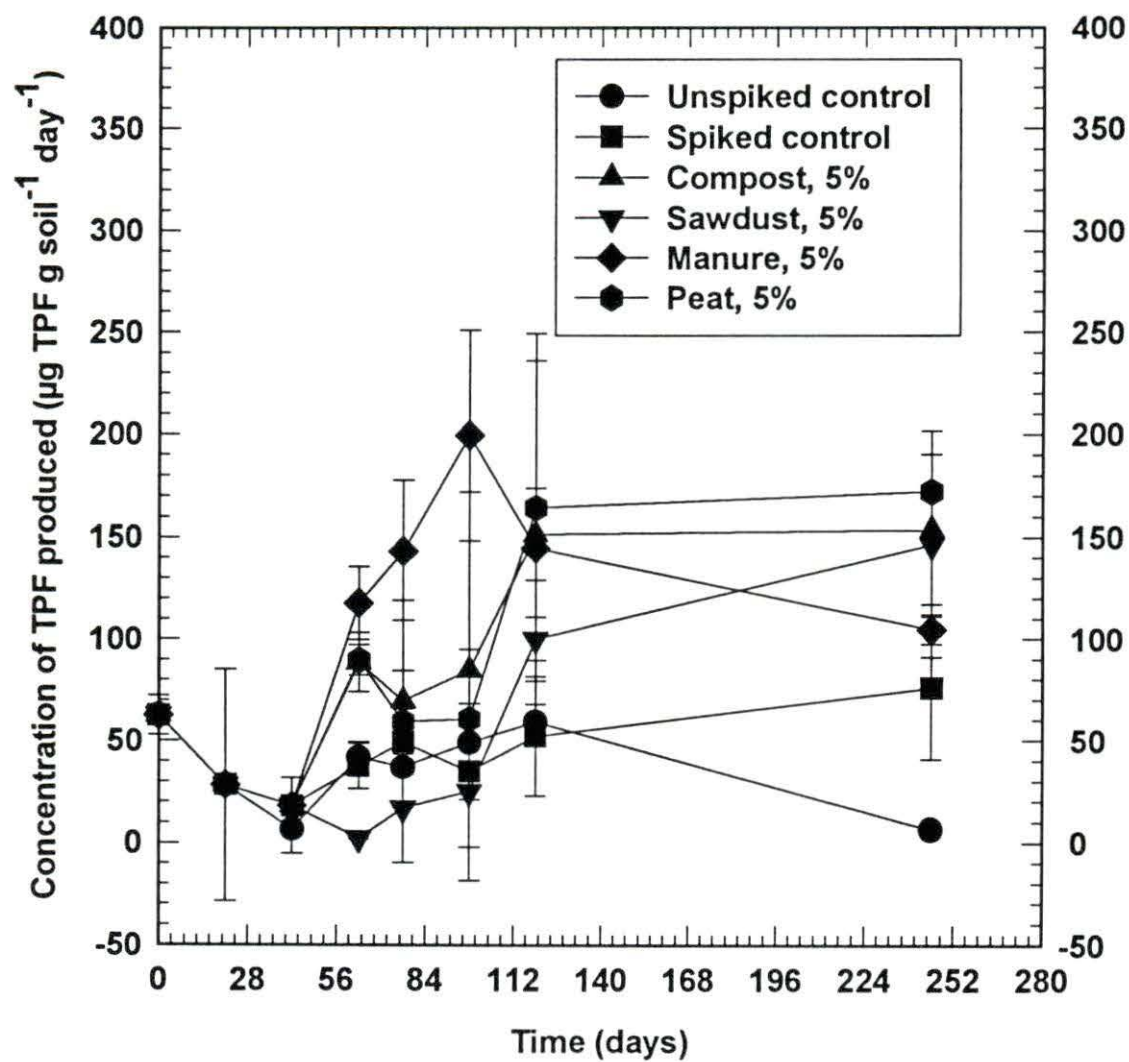


Figure 19. Biostimulation experiment I dehydrogenase results for the 5% amendment rate of the compost, sawdust, manure, and peat treatments.

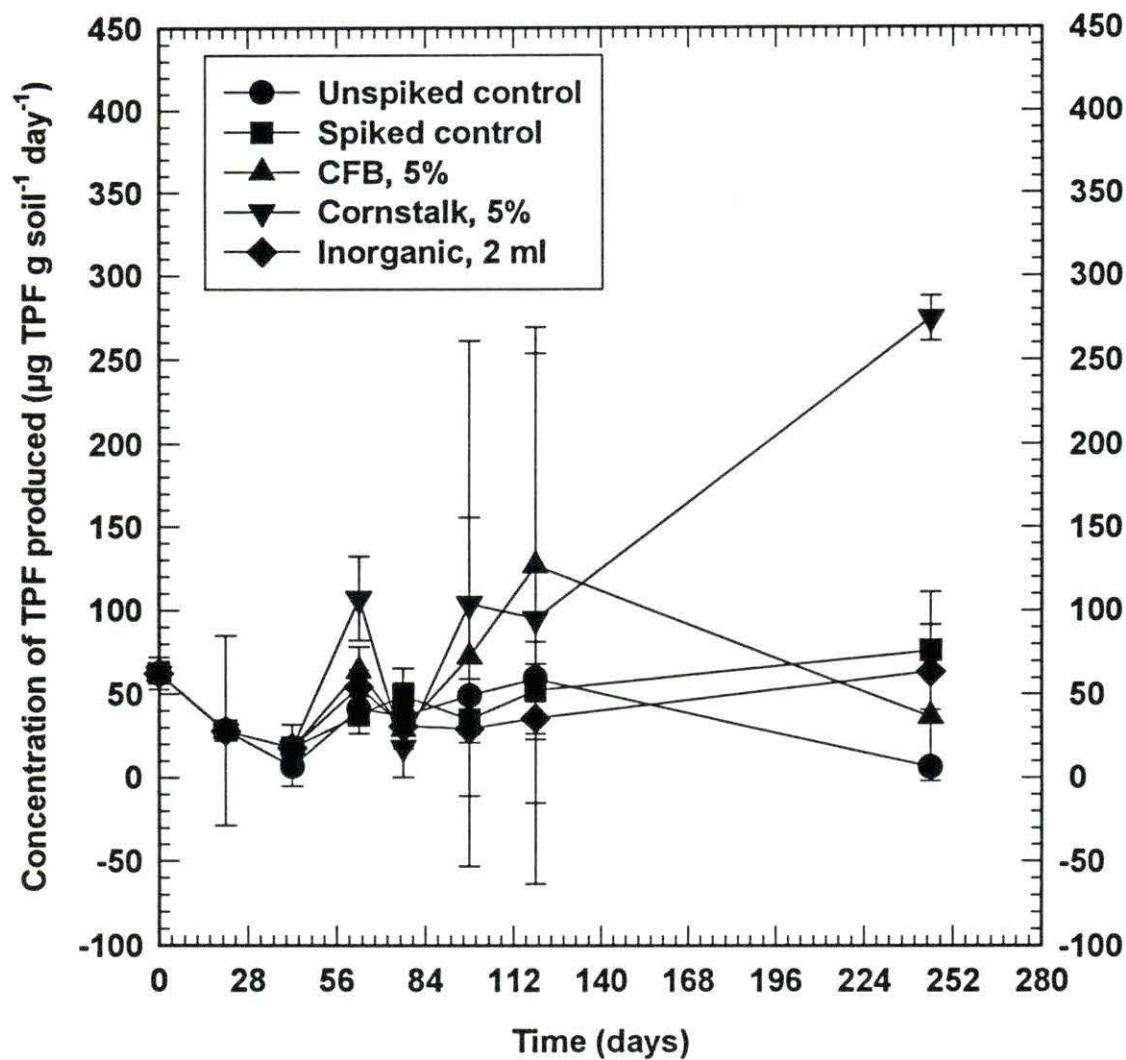


Figure 20. Biostimulation experiment I dehydrogenase results for the high amendment rate for the corn fermentation byproduct (CFB), cornstalk, and inorganic solution treatments.

APPENDIX B

THE MICROBIAL POPULATIONS (BACTERIA, ACTINOMYCETES, AND FUNGI), MICROBIAL RESPIRATION, AND DEHYDROGENASE RESULTS AT EACH SAMPLING TIME FOR THE SECOND BIOSTIMULATION EXPERIMENT

Each data point for the different types of measurements is the average of three replicate jars of Bravo soil for each treatment which were sacrificed at each time point. If error bars are present, they are the standard deviation of three replicate jars of Bravo soil. The methods for determining each of the microbial parameters are shown in the “Materials and Methods” section in Chapter 3 of this thesis.

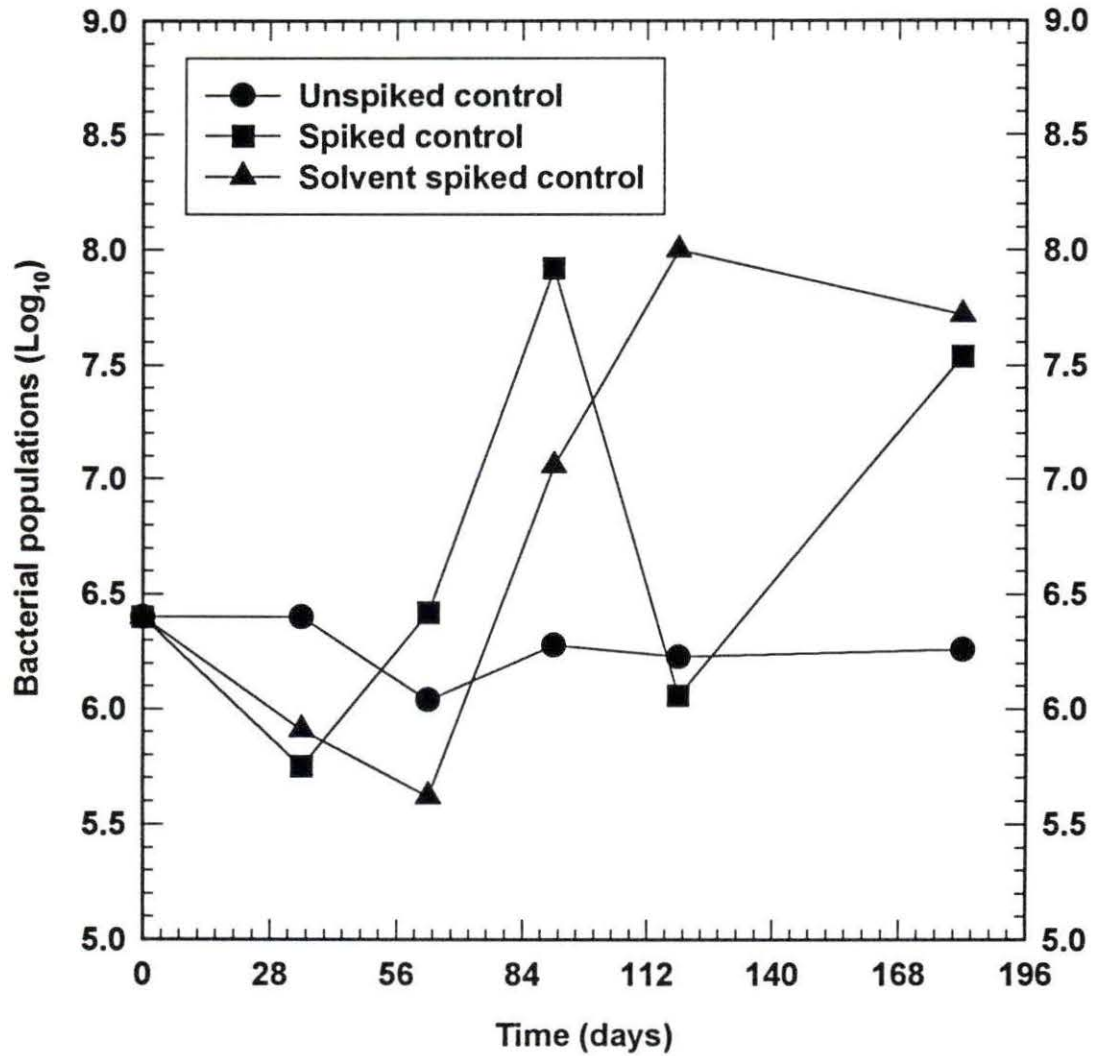


Figure 1. Biostimulation experiment II bacterial population results for the control treatments.

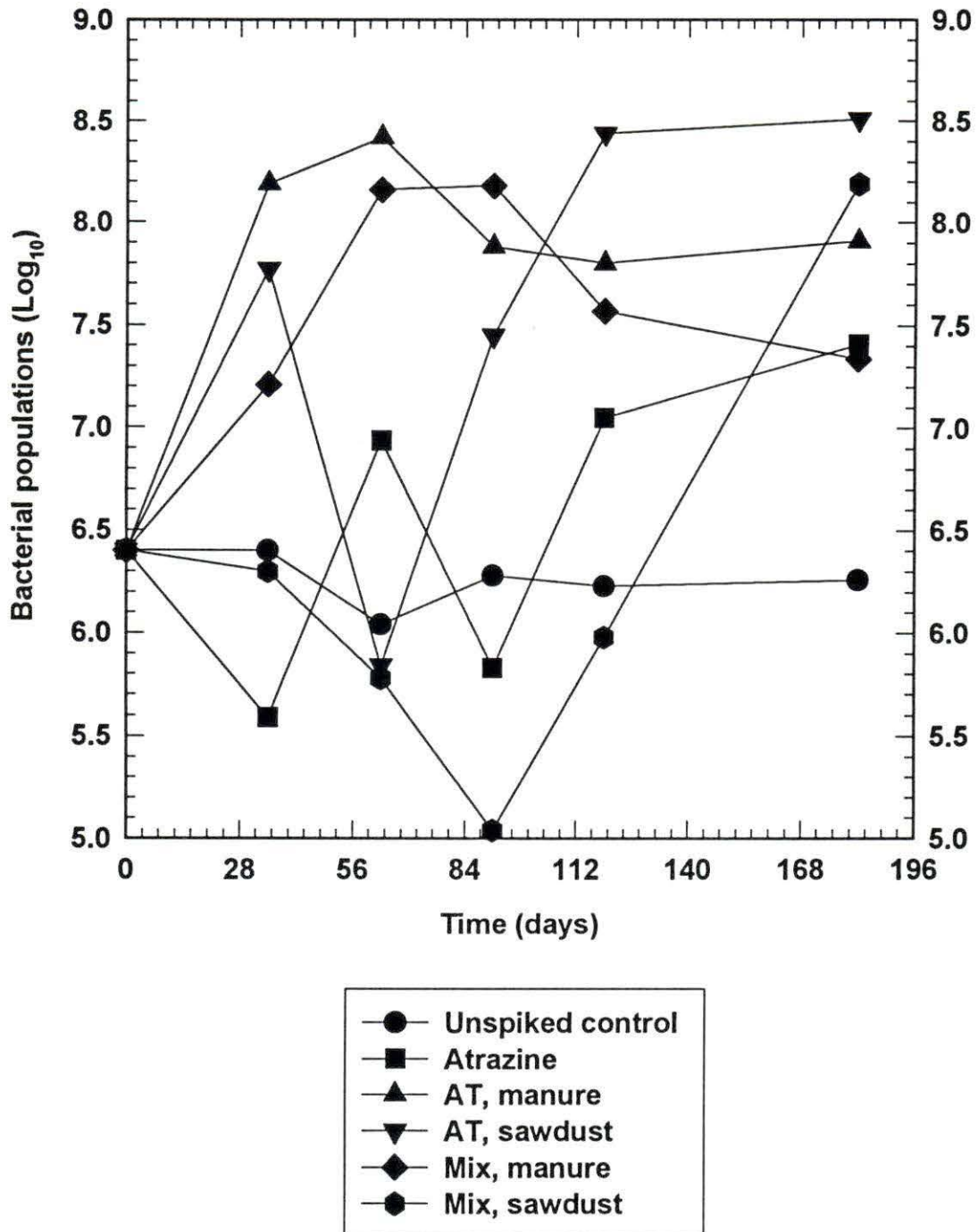


Figure 2. Biostimulation experiment II bacterial population results for the atrazine treatments as compared to the unspiked control treatment.

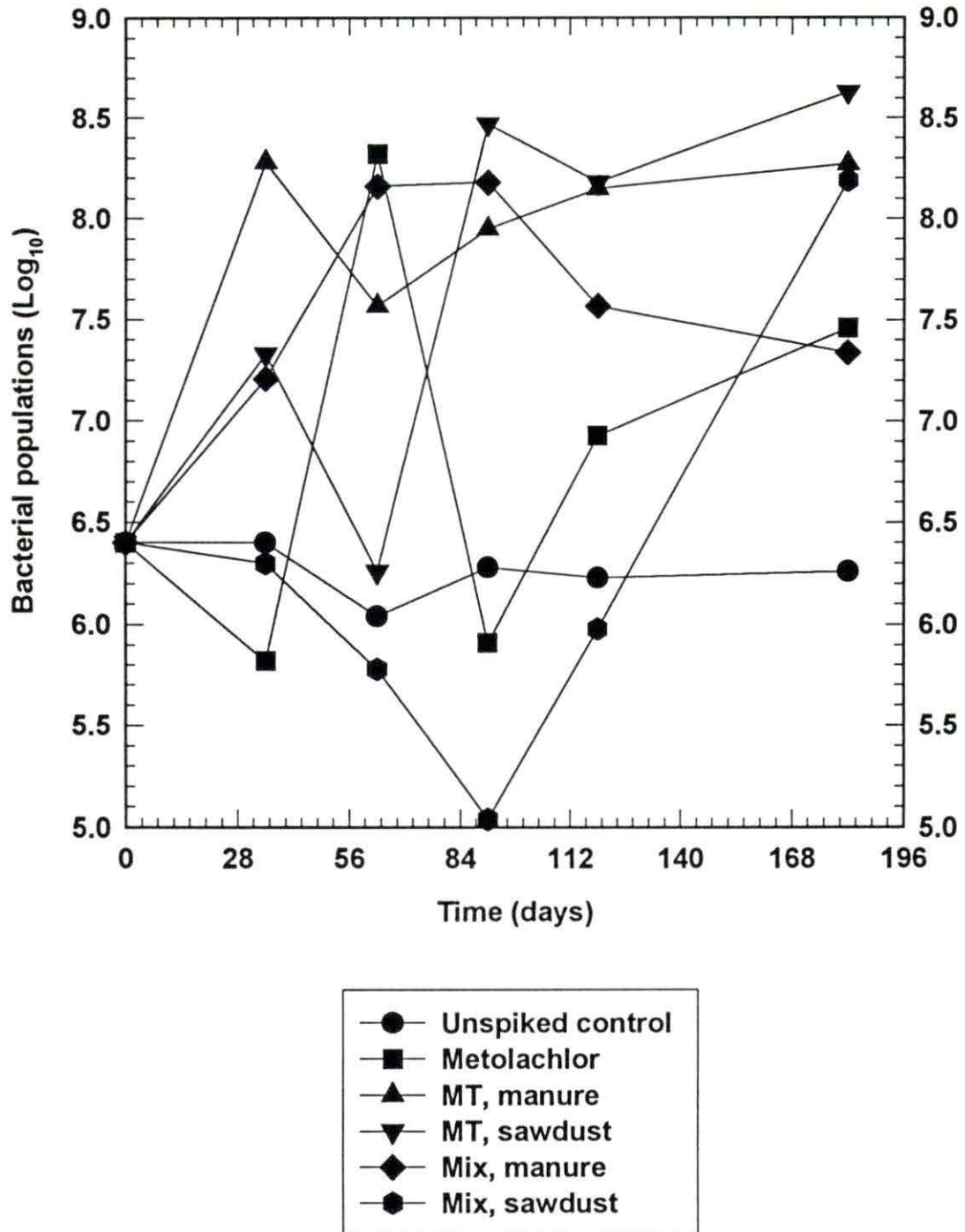


Figure 3. Biostimulation experiment II bacterial population results for the metolachlor treatments as compared to the unspiked control treatment.

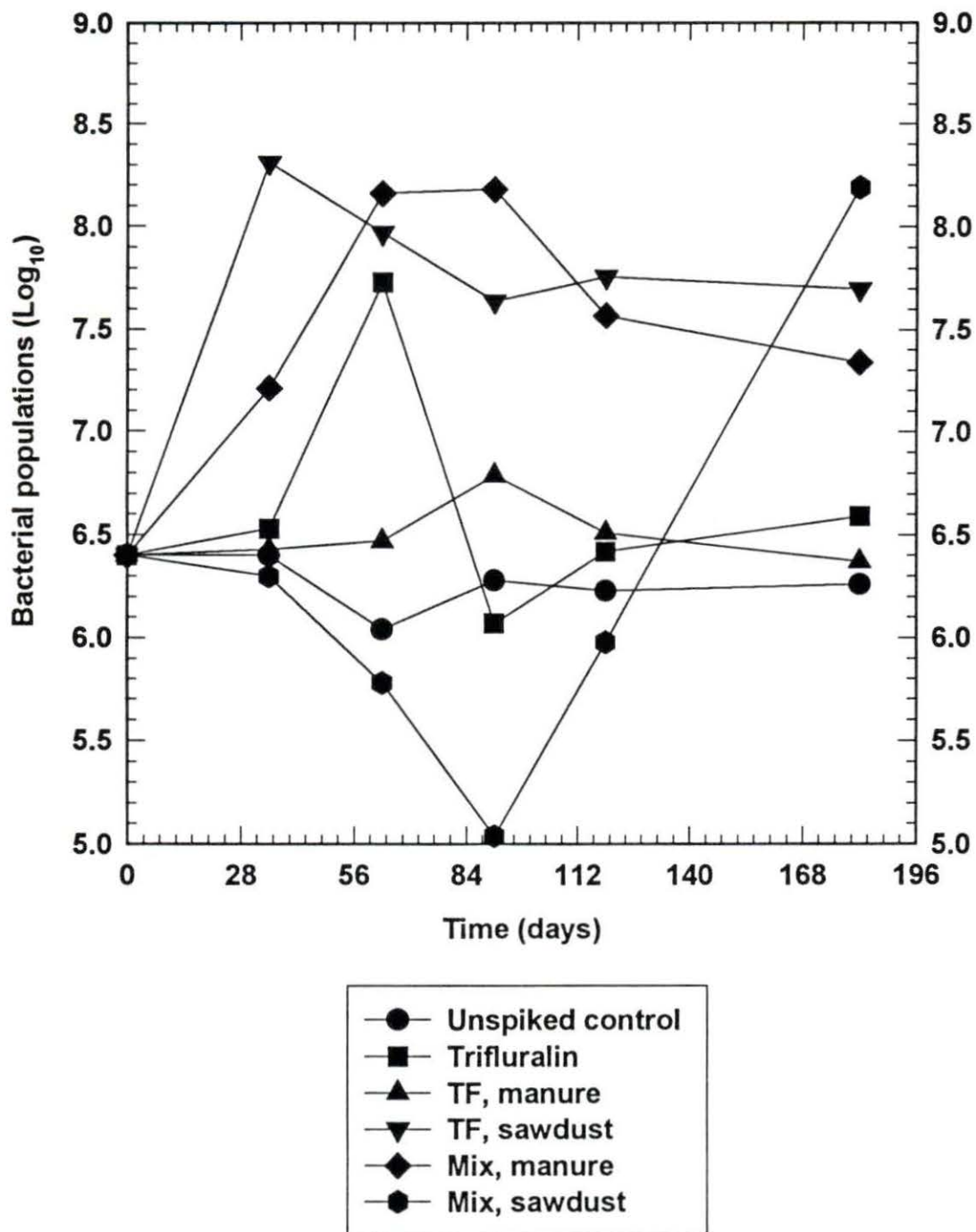


Figure 4. Biostimulation experiment II bacterial population results for the trifluralin treatments as compared to the unspiked control treatment.

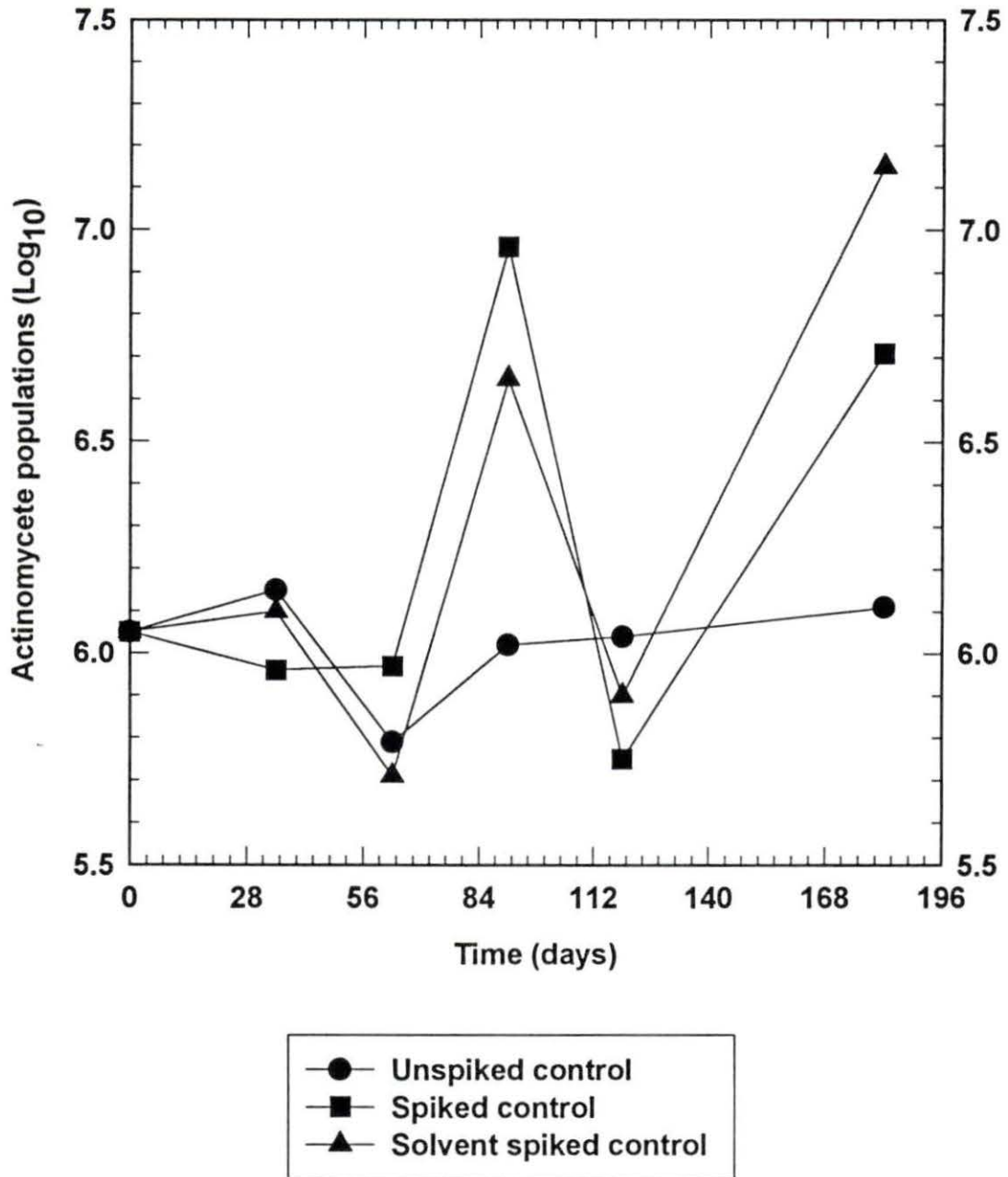


Figure 5. Biostimulation experiment II actinomycete population results for the control treatments.

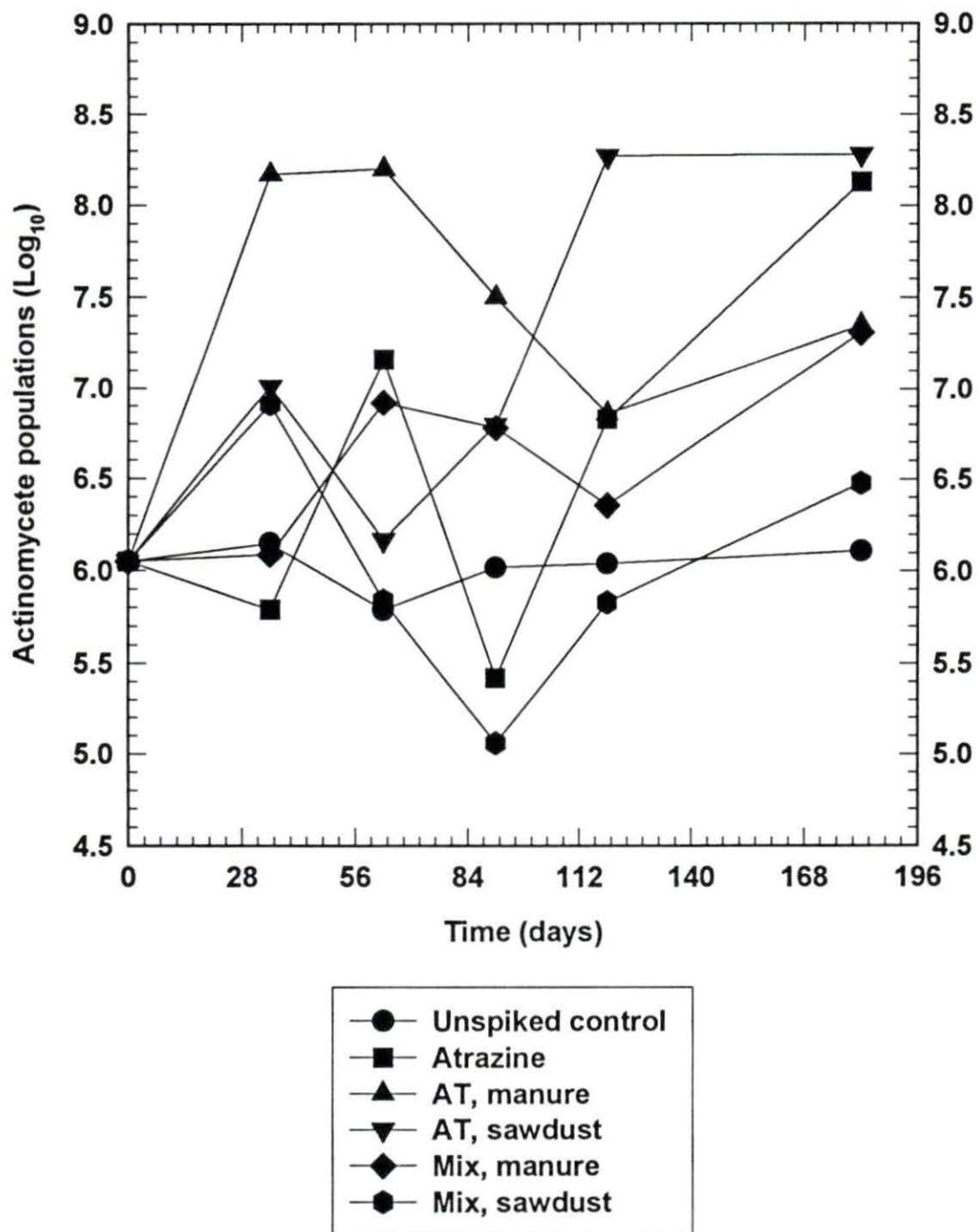


Figure 6. Biostimulation experiment II actinomycete population results for the atrazine treatments as compared to the unspiked control treatment.

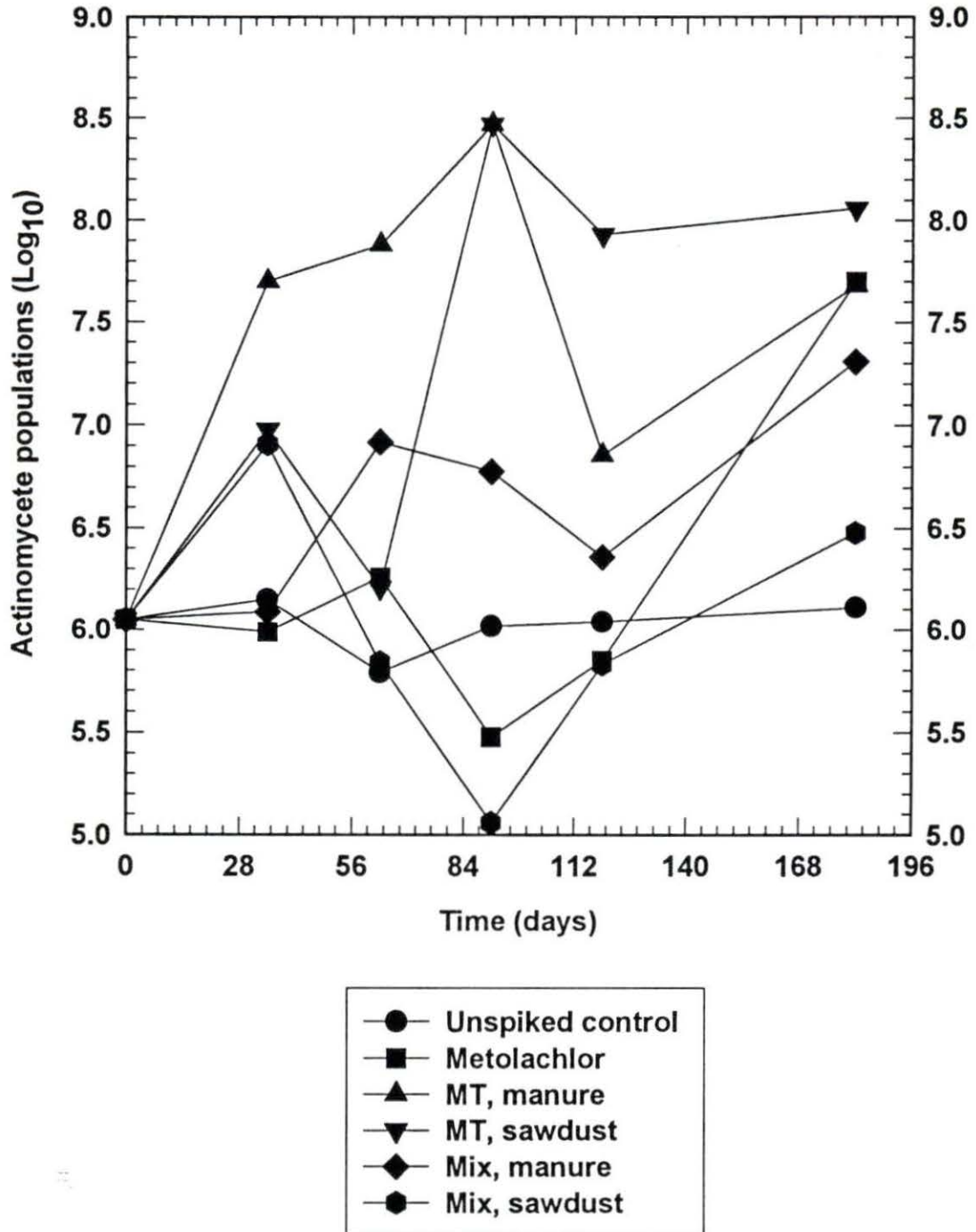


Figure 7. Biostimulation experiment II actinomycete population results for the metolachlor treatments as compared to the unspiked control treatment.

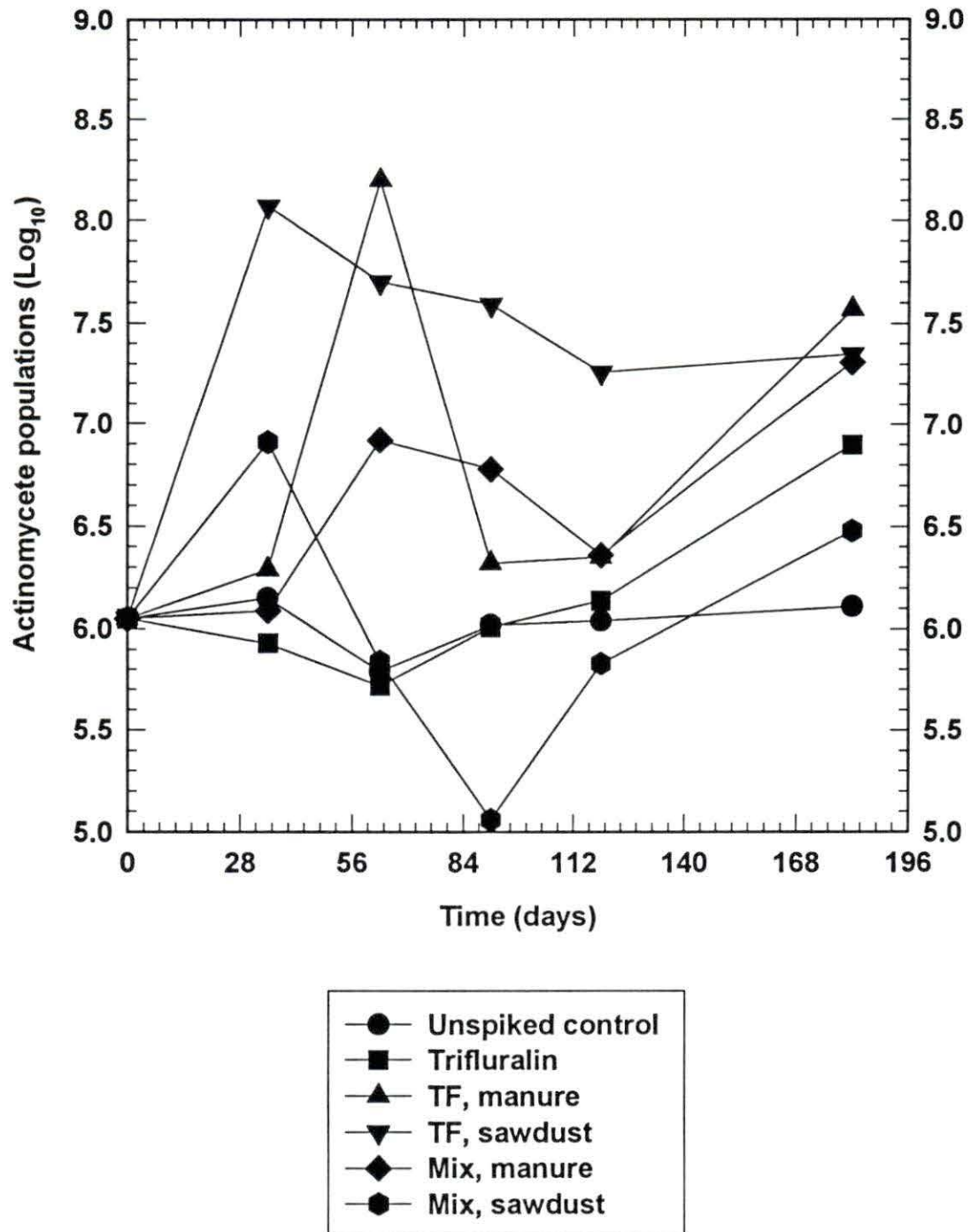


Figure 8. Biostimulation experiment II actinomycete population results for the trifluralin treatments as compared to the unspiked control treatment.

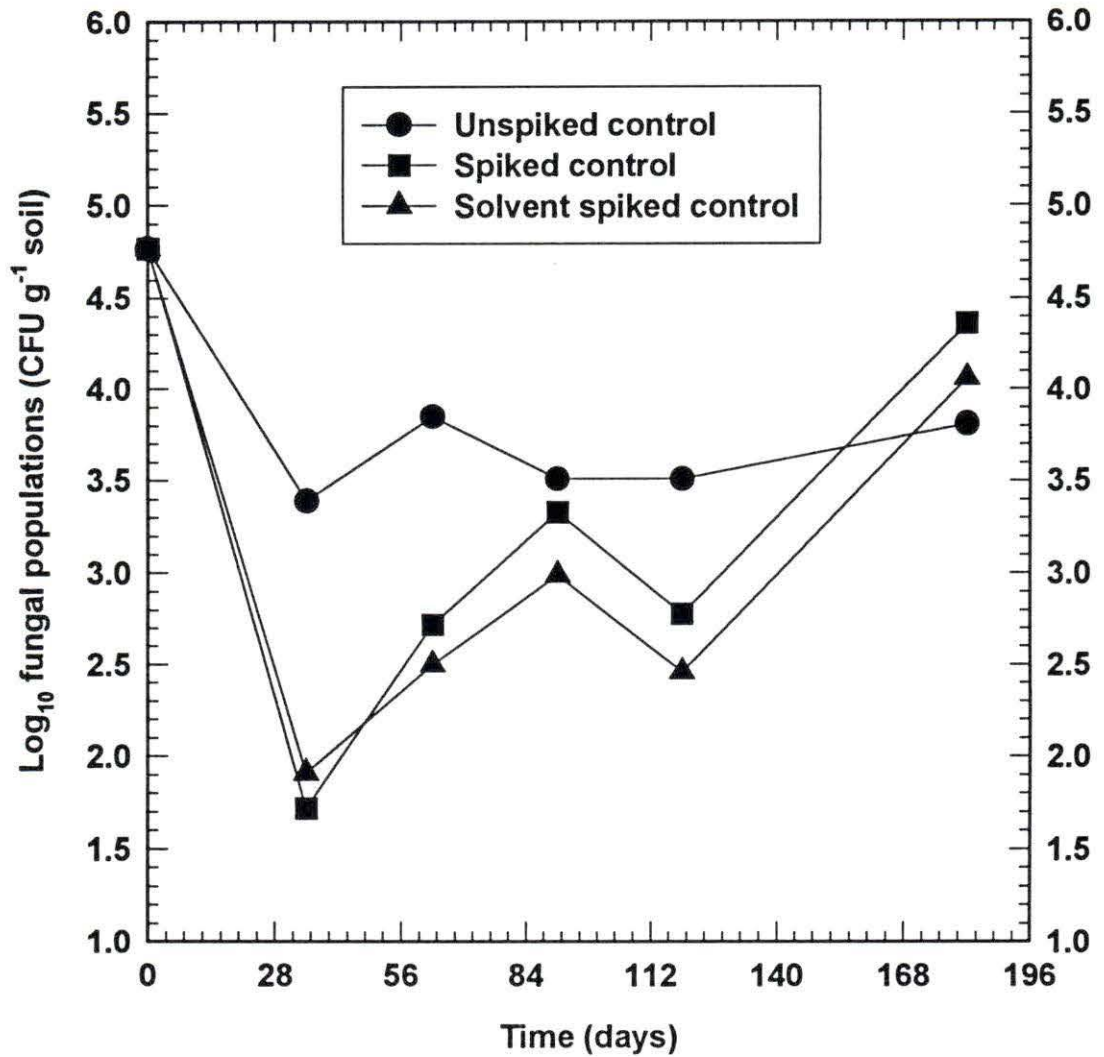


Figure 9. Biostimulation experiment II fungal population results for the control treatments.

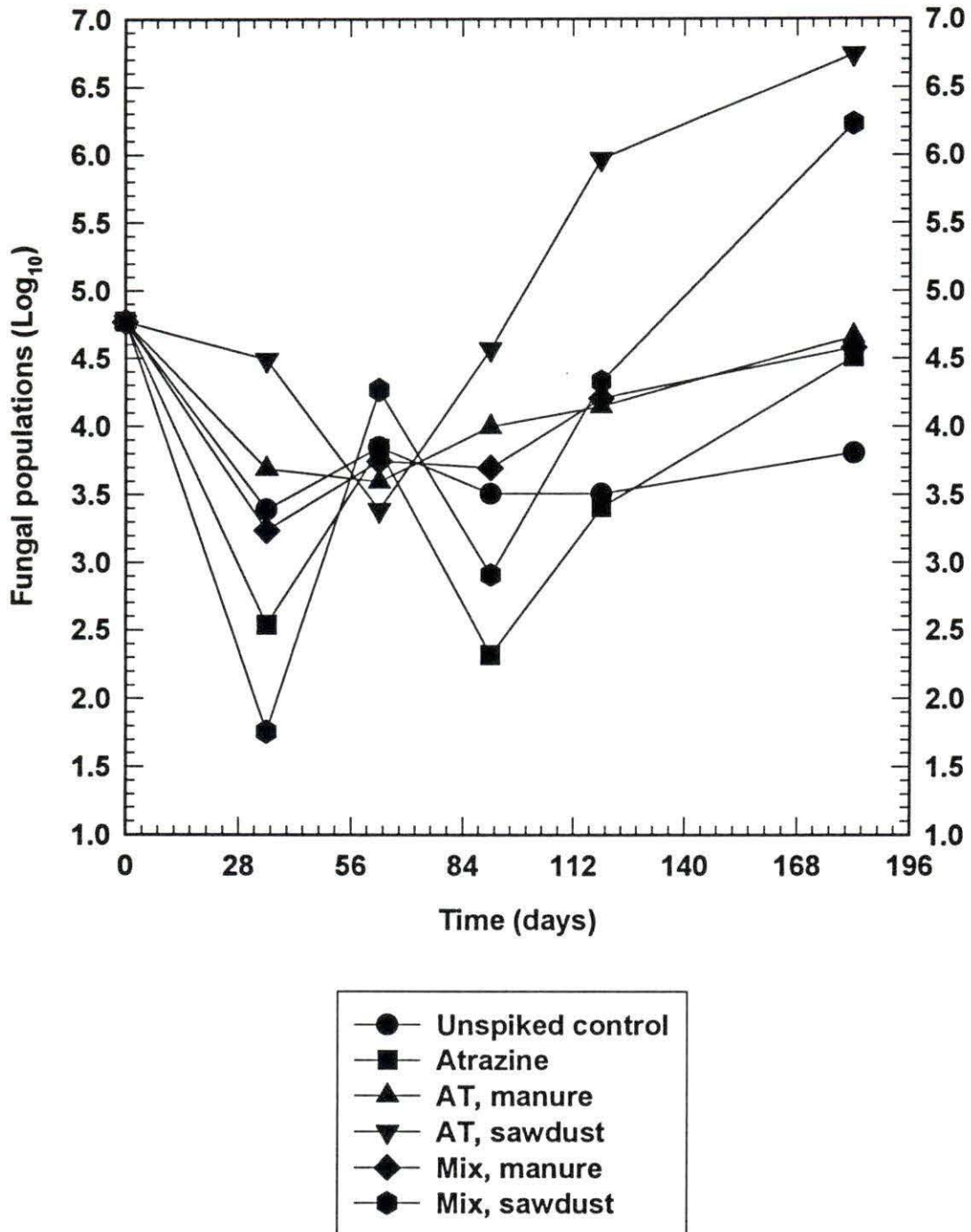


Figure 10. Biostimulation experiment II fungal population results for the atrazine treatments as compared to the unspiked control treatment.

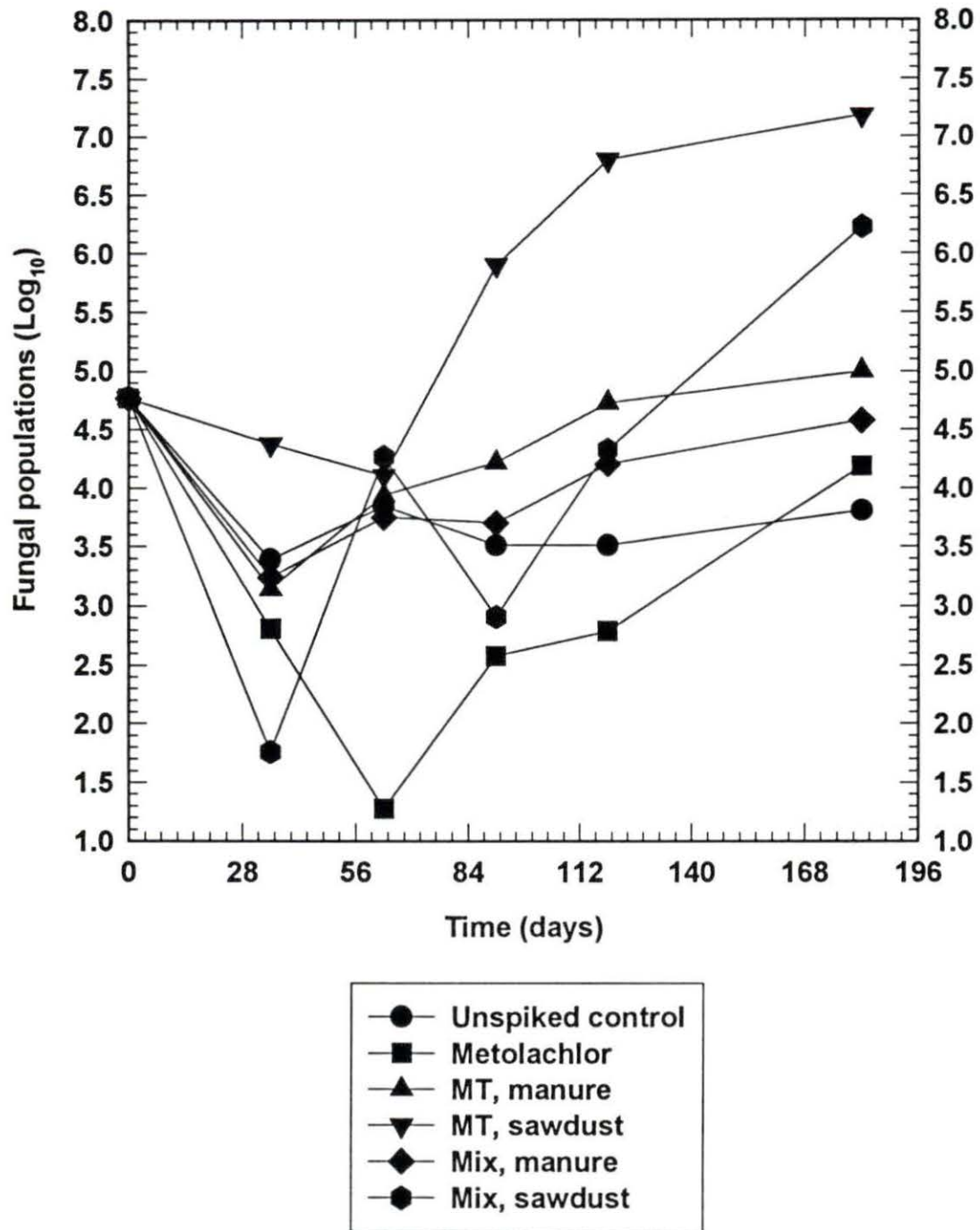


Figure 11. Biostimulation experiment II fungal population results for the metolachlor treatments as compared to the unspiked control treatment.

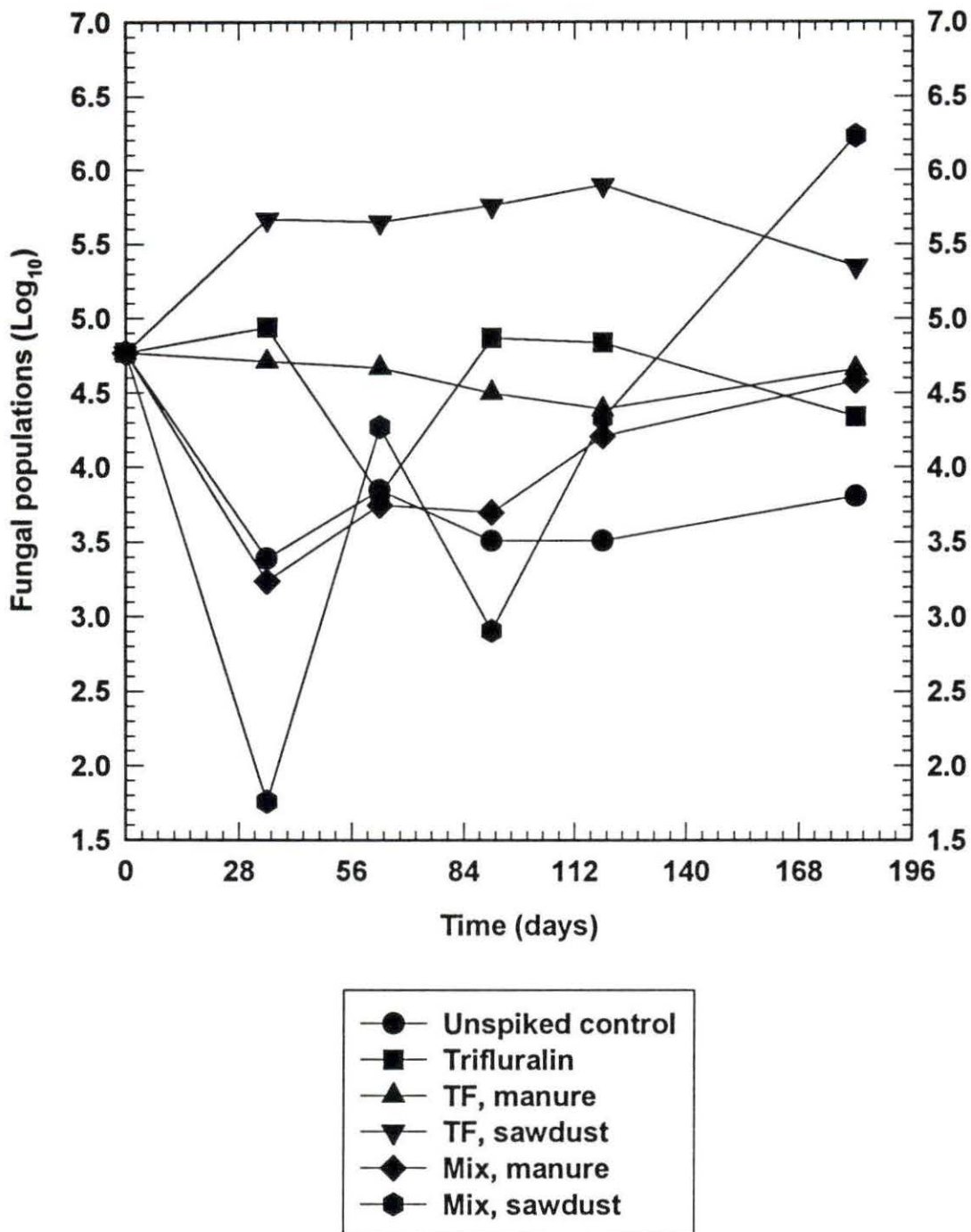


Figure 12. Biostimulation experiment II fungal population results for the trifluralin treatments as compared to the unspiked control treatment.

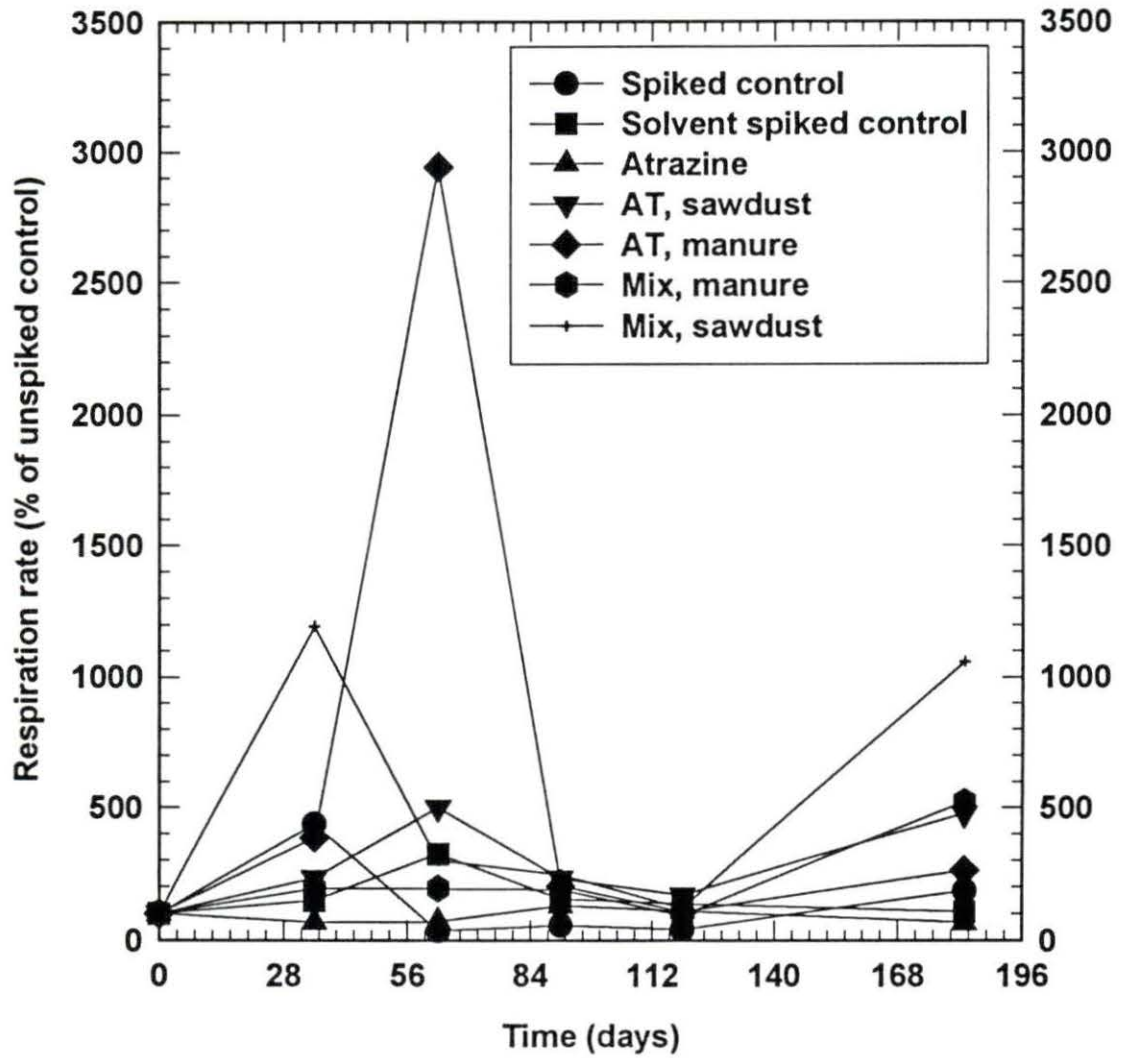


Figure 13. Biostimulation experiment II respiration results for the atrazine treatments expressed as a percentage of the unspiked control treatment.

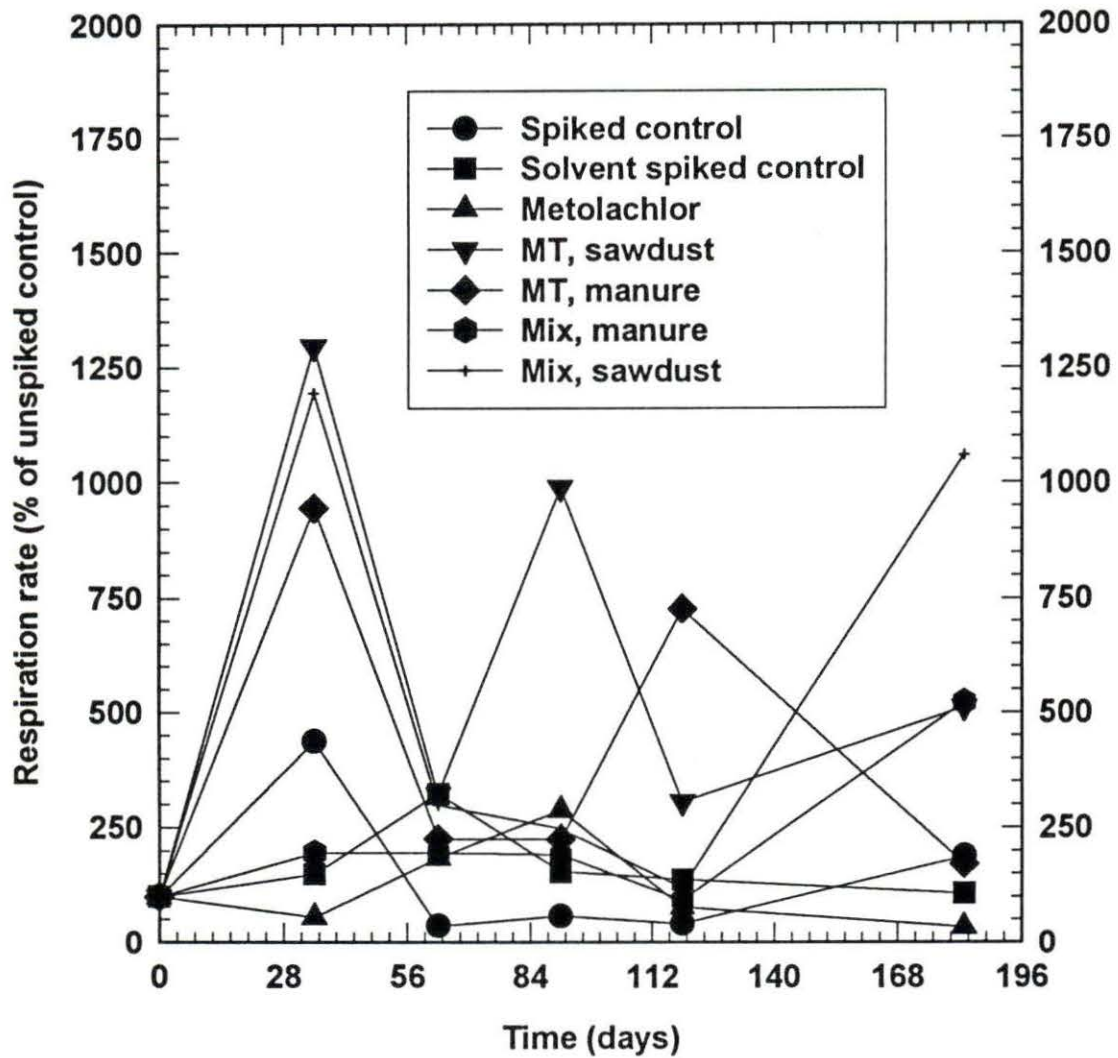


Figure 14. Biostimulation experiment II respiration results for the metolachlor treatments expressed as a percentage of the unspiked control treatment.

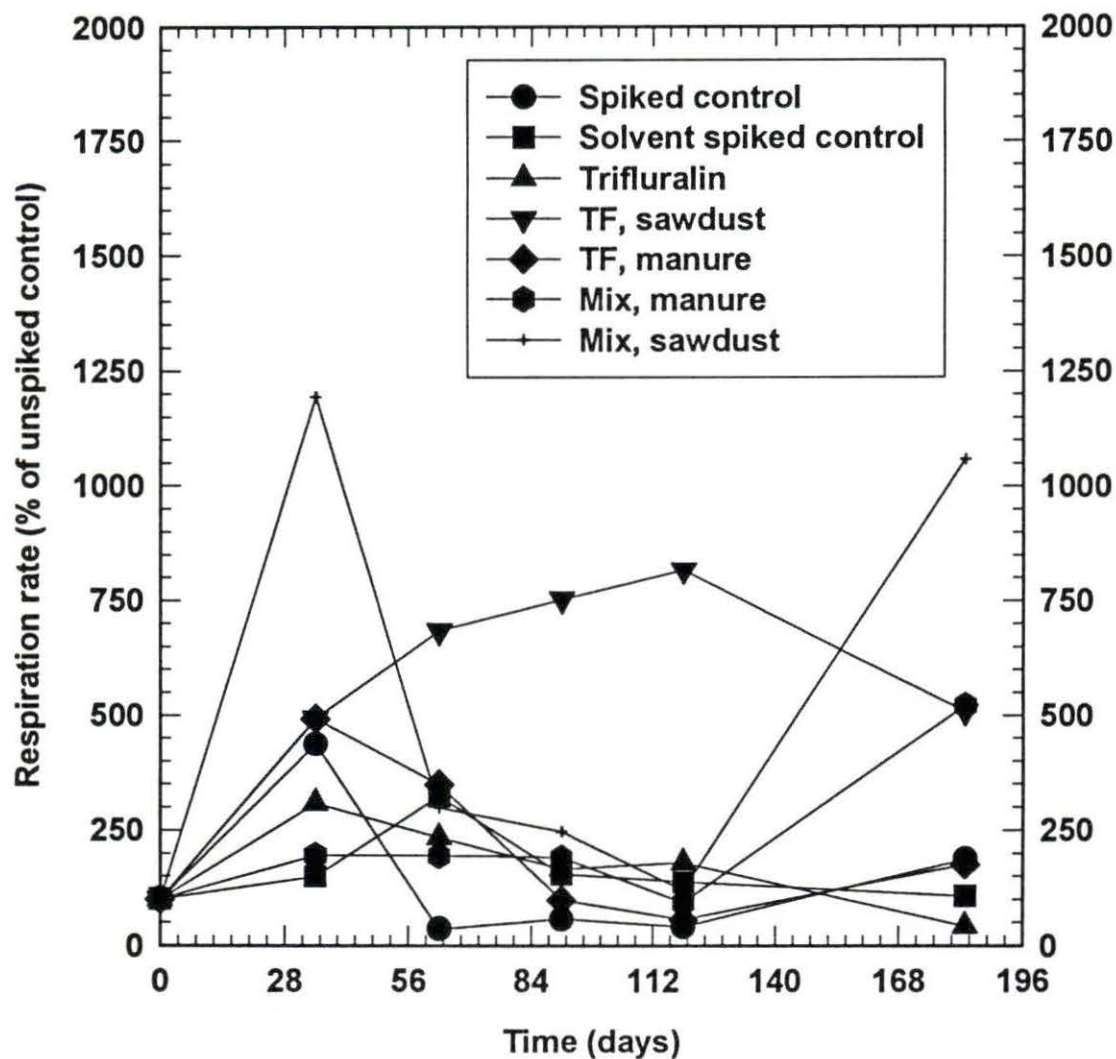


Figure 15. Biostimulation experiment II respiration results for the trifluralin treatments expressed as a percentage of the unspiked control treatment.

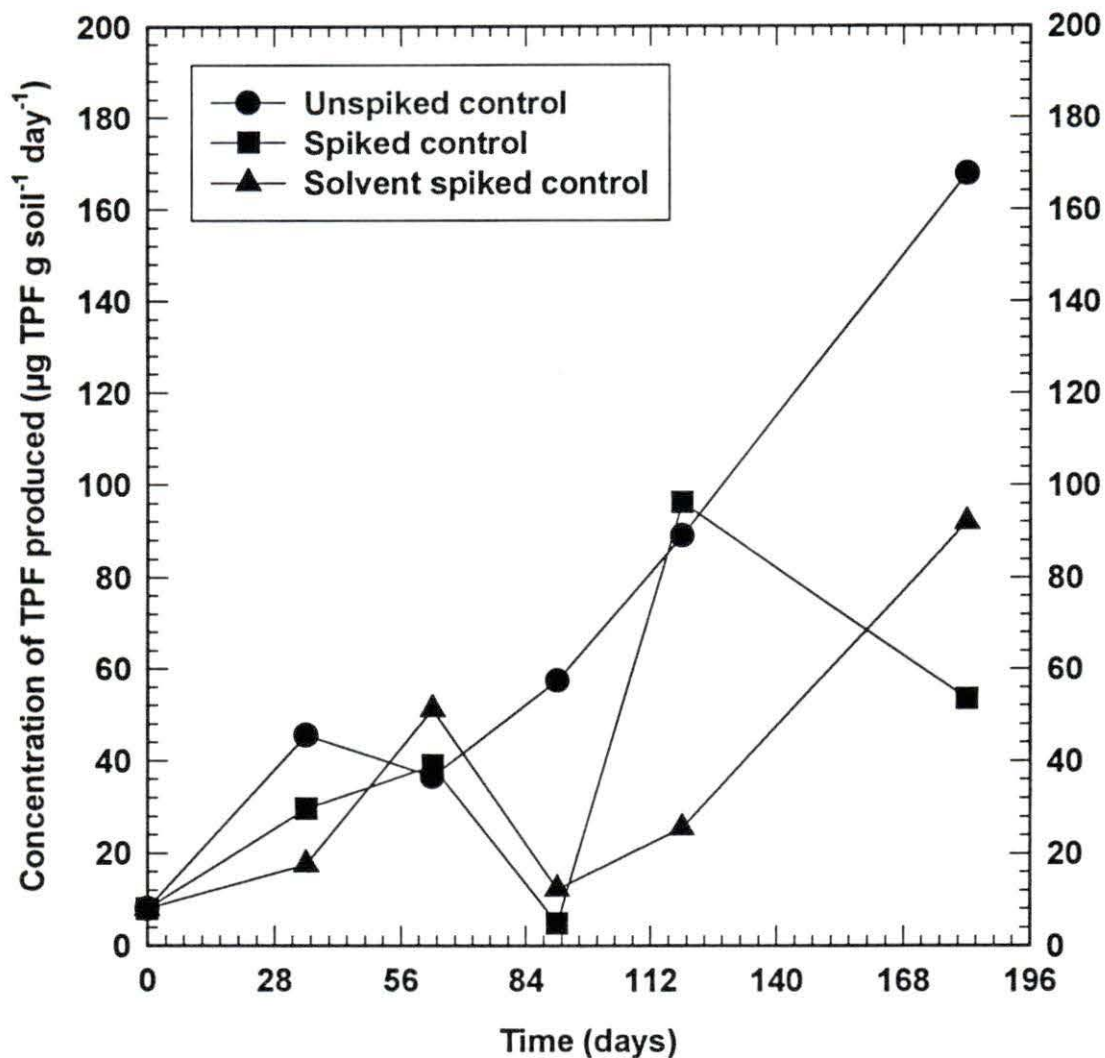


Figure 16. Biostimulation experiment II dehydrogenase results for the control treatments. The solvent spiked control was treated with 2.1 ml of methanol to determine the effect of solvent addition on the soil microbial populations.

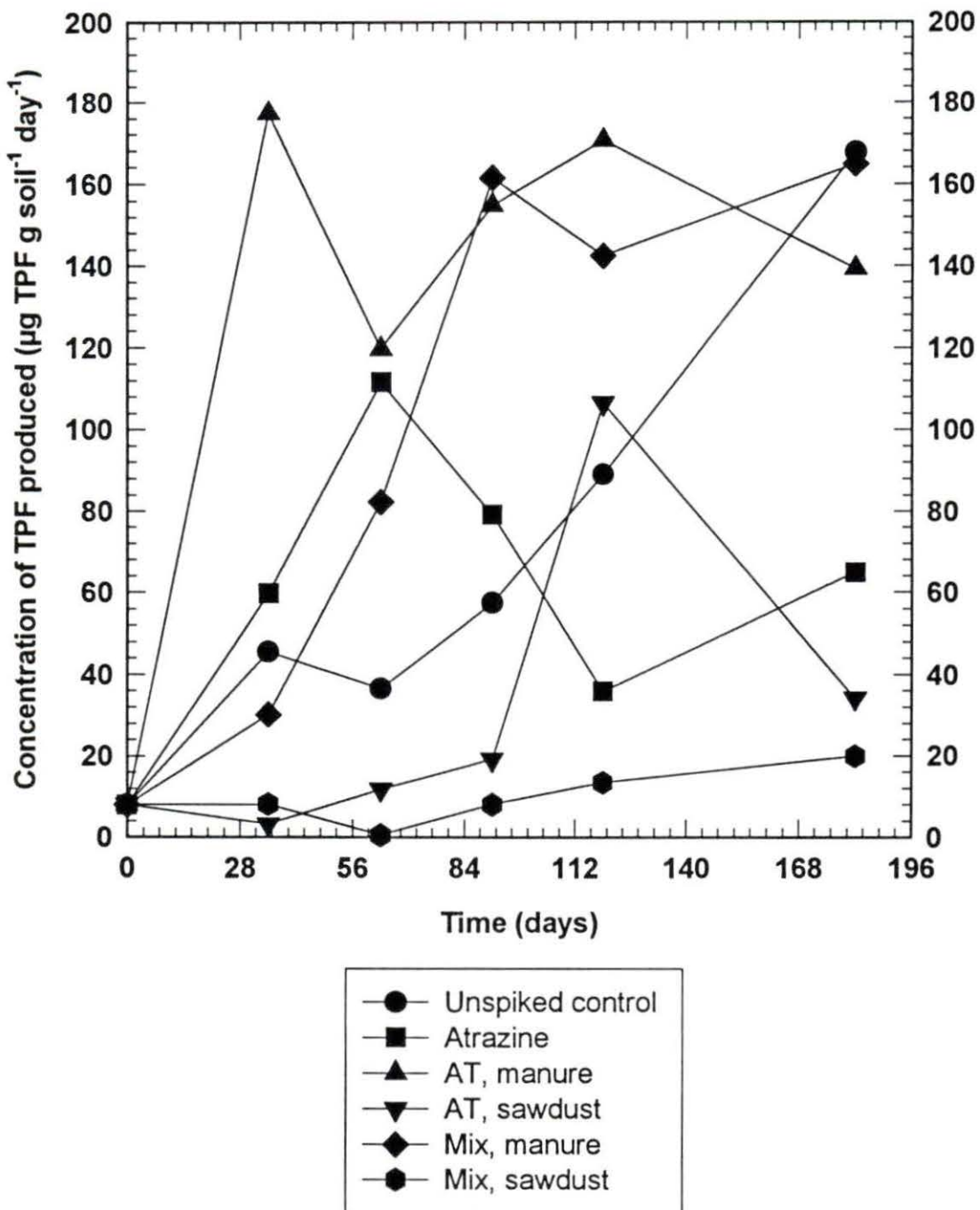


Figure 17. Biostimulation experiment II dehydrogenase results for the atrazine treatments.

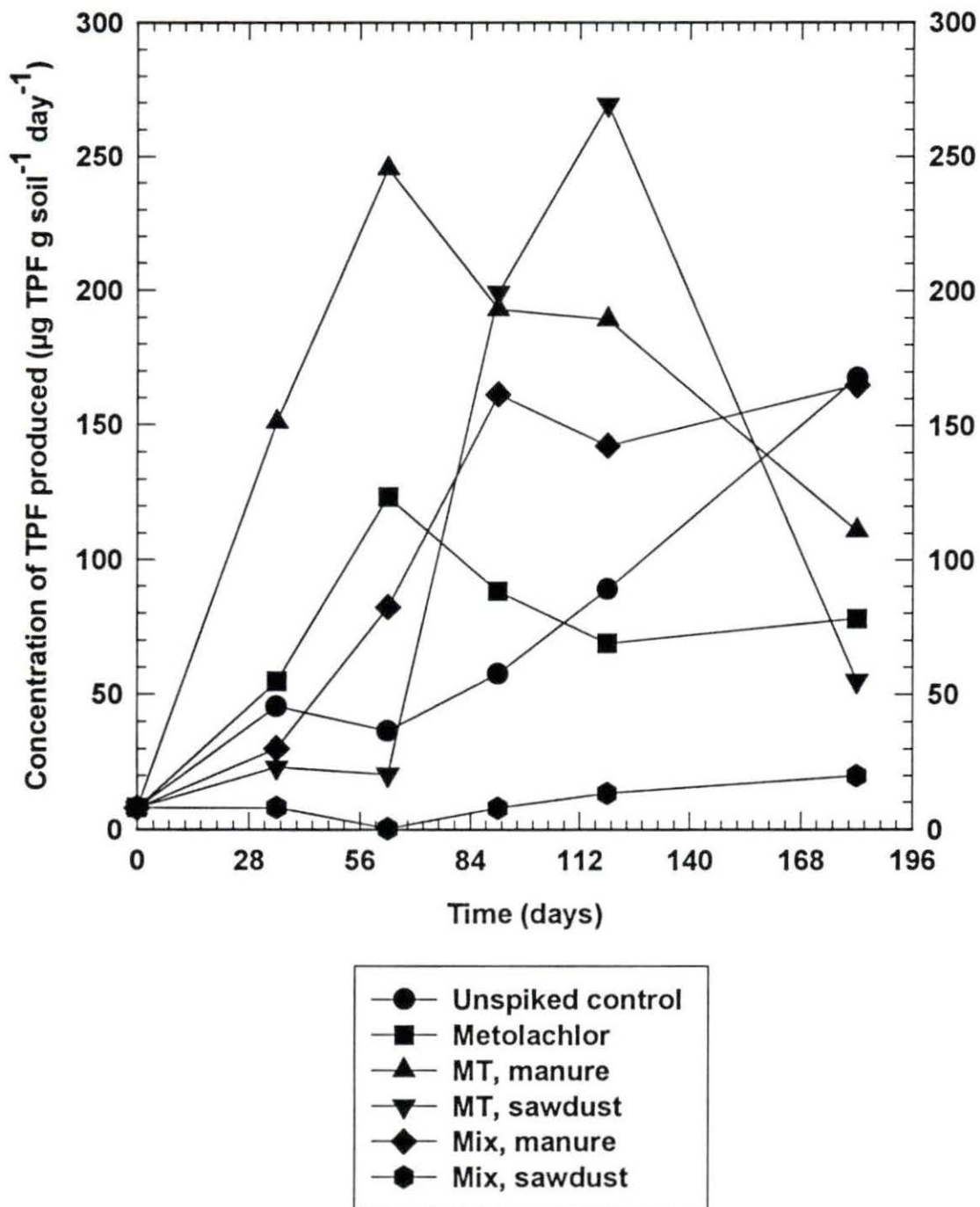


Figure 18. Biostimulation experiment II dehydrogenase results for the metolachlor treatments as compared to the unspiked control treatment.

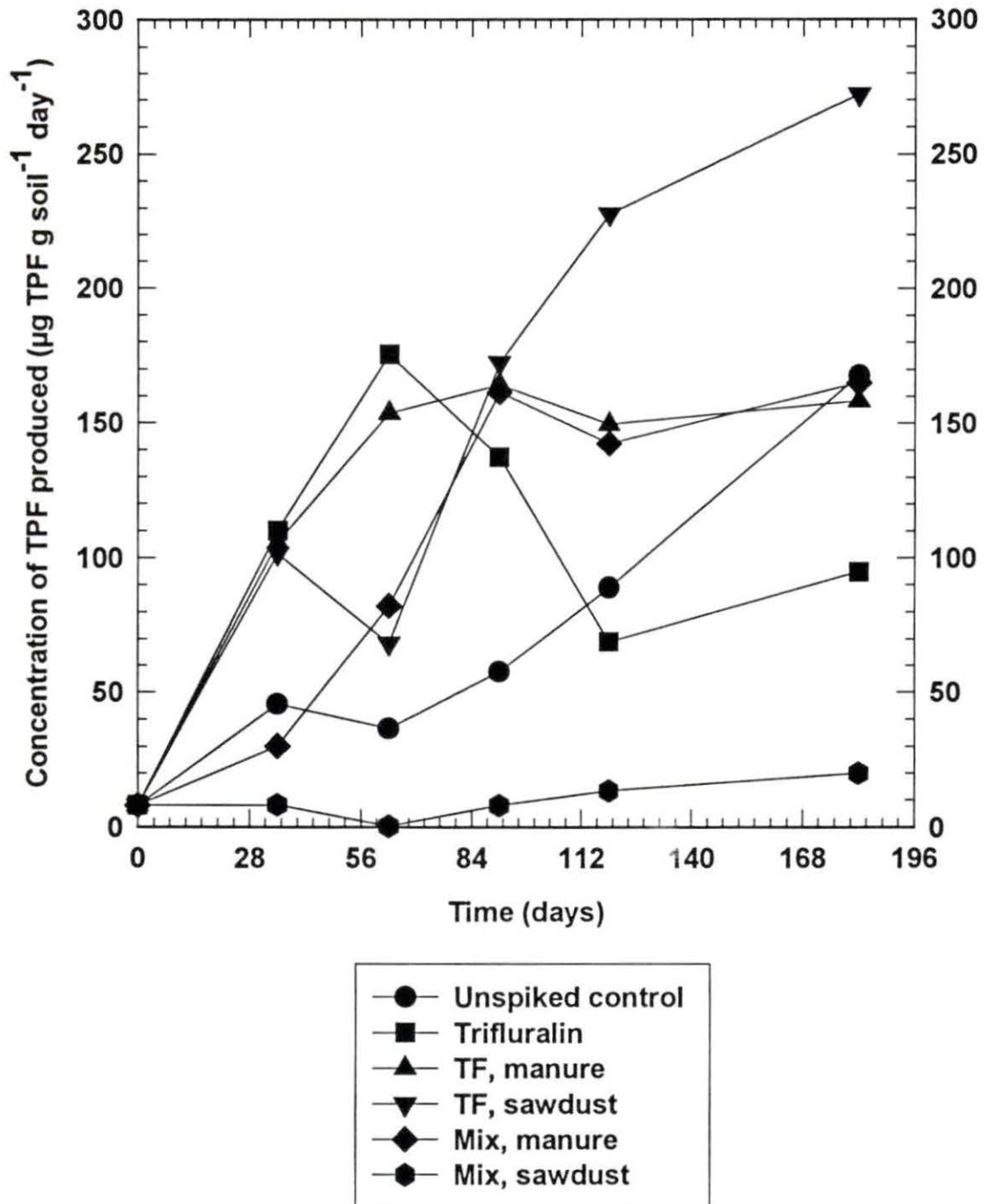


Figure 19. Biostimulation experiment II dehydrogenase results for the trifluralin treatments as compared to the unspiked control treatment.

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