I. TITLE : Review and Evaluation of Degradation and Bioremediation of Elevated Levels of Pesticides at Spill Sites - Agriculture 6.

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A. M.L. 91, Ch. 254, Art. 1, Sec. 14, Subd 6(b) Minnesota Resources Review Levels of Pesticides At Spill Sites — Appropriation : $300,000.00; Balance : $ 30,050.00

This appropriation is to the Commissioner of Agriculture for a literature search and publication of bioremediation technologies for pesticide spills; laboratory research on the fate of elevated levels of pesticides in soil; and evaluation of bioremediation techniques.

B. **Compatible Data :** During the biennium ending June 30, 1993, the data collected by projects funded under this section that have common value for natural resource planning and management must conform to information architecture as defined in guidelines and standards adopted by the Information Policy Office. In addition, the data must be provided to and integrated with the Minnesota Land Management Information Center's geographic databases with the integration costs borne by the activity receiving funding under this section.

C. **Match Requirement :** Not applicable.

II. NARRATIVE :

Each year incidents occur involving pesticide spills where soil must be excavated or managed. In the first nine months of 1990, approximately 120 incidents were reported to the Minnesota Department of Agriculture. In addition, many of the sites where pesticides have been mixed, loaded or handled in the past have contaminated soils due to incidental spillage. These elevated levels of pesticides in soils may result in ground water contamination. Studies indicate that pesticides detected in ground water near these spill sites often are at elevated concentrations and may exceed health advisory levels.

The purpose of this work program is to better understand pesticide transport and degradation processes and investigate bioremediation technologies for treatment of soil contamination. Effective soil treatment methods will aid in the prevention of ground water contamination from point source pesticide contamination. The project will include a review of the available information in conjunction with laboratory studies on fate and transport of pesticides and potential bioremediation technology.

III. OBJECTIVES :

A. **Review of Existing, Developing and Applicable Technology and Information.**

A1. Narrative : The purpose of Objective A of this proposal is to technically evaluate existing and developing information and technologies regarding bioremediation of contamination at pesticide spill sites. Specifically, Objective A of the proposal will: review the available literature; provide support for the research of Objectives B and C of this proposal; review the current ongoing research for applicability to spill sites; technically evaluate the feasibility and effectiveness of bioremediation for specific classes of pesticides; review state and federal regulations (MERLA, CERCLA, RCRA, TSCA, etc.) that may impact on the implementation of bioremediation technology; and produce a report summarizing the present state of the technology.

Petroleum produc tamination is the focus in much of the literature regarding b' iediation technology. Less information is available on the treatment of pesticide contami. on. Both literature areas will be reviewed for applicability. Past research has documented the effects of microorganism degradation and plant uptake on pesticide concentrations only at field application concentrations. Objective A will develop and evaluate existing information for practical application to current spill site situations with elevated levels of pesticides.

Bioremediation treatment technology offers a permanent solution for cleaning up soil and ground water contamination. Other common treatment methods, such as carbon filtration or air stripping of ground water contaminants, only transfer the contamination to other media. Bioremediation mineralizes the contaminants into water, carbon dioxide (aerobic degradation) and inorganic salts.

The effectiveness of bioremediation technology depends on the availability of microorganisms or plants that affect the concentrations of a particular class of contaminants. The rate at which the treatment is achieved is influenced by: the contaminant of concern; soil type; the number and type of microorganisms; environmental conditions such as oxygen availability, temperature, pH, and moisture; and the availability of macro- and micro-nutrients necessary to encourage the growth of the microbial population.

The term "bioremediation technology" encompasses many different treatment methods for ground water and for soil contamination. Bioremediation treatment technologies may take advantage of indigenous microorganisms or plants or may use "engineered microorganisms" (microorganisms developed to degrade a particular contaminant) or plants. The treatment method selected depends on site specific conditions, such as class of contaminant, hydrogeology, extent and magnitude of the contamination, amount of land available for treatment and cost. Examples of "in-situ" bioremediation technologies (contamination remains in place) include: closed loop circulation systems for the treatment of ground water; degradation of the contaminants in the vadose zone resulting from soil venting systems; and plant uptake of pesticides in the soil. Bioremediation technologies also can be applied to the contaminated media after removal by excavation or ground water pumpout systems. Examples of "on-site" or "off-site" treatment technologies include: treatment tanks for ground water; contained areas for contaminated soil; and land application or composting of contaminated soil.

Minnesota is the first state with a program and fund that provides reimbursement to eligible parties for the remediation of pesticide spill sites. Consequently, alternative treatment techniques are needed as spill sites are remediated. Land application of contaminated soil is the treatment method selected for most pesticide spill sites. Land application has limitations (e.g., legality if the pesticide is not currently registered, need for additional studies regarding pesticide persistence or the need for large tracts of suitable land if the contaminated soils contain high concentrations of pesticides) and usually requires removal of the contaminated soil from the spill location. This proposal will help provide for the development of alternative treatment technologies.

A2. Procedures : The literature search will be conducted by utilizing computerized, inter-library search techniques. Degradation processes and bioremediation of pesticides will be the focus. However, other contaminants also will be screened and reviewed to examine for adaptation potential. National and international sources will be evaluated. Current and developing technologies, published and unpublished will also be reviewed.

A3. Budget :

a. Amount Budgeted : $ 70,000.00
b. Balance : $ 30,050.00

A4. Timeline for Products/Tasks :

	July 91	Jan 92	June 92	Jan 93	June 93
Initiate Literature Review	**********				
Analyze Data and Information	**********	**********	**********		
Evaluate Strategies	**********	**********	**********	**********	
Final Report				**********	**********

A5. Status :

Introduction -

The contamination of soils and waters by pesticides at sites where agricultural chemicals are stored, handled, and distributed ("dealership sites") has received increased attention over the past five years. Increasingly, state programs are requiring the investigation and remediation of dealerships sites. However, remediation technologies are not broadly available for pesticide-contaminated media. Landspreading of pesticide contaminated soil is the treatment method most often used in Minnesota and in other states, but there are many cases where this is not feasible. Consequently, alternative, cost effective remediation technologies are needed.

Bioremediation, the use of microbial degradation in a controlled setting, has received attention as a promising technology for pesticide contaminated media. Bioremediation is attractive because it permanently removes or alters the contaminant rather than transferring it to another media (as do many of the common treatment technologies such as placement in landfills, carbon filtration, thermal desorption and incineration) and, at the same time, the media is not greatly altered. Bioremediation has been applied to contaminants from other waste categories (petroleum products, wood preservatives, explosive-related wastes). However, bioremediation has not been broadly applied on pesticides and thus its efficacy is unknown. A complicating feature is that pesticides come from a number of chemical families which feature a range of chemical and physical characteristics and which will consequently behave differently under the same treatment scenario.

This report reviews the existing and developing information on bioremediation technologies applied to contamination at pesticide spill sites and evaluates the likely efficacy of the different bioremediation technologies on pesticide contaminated media. Although the detailed report has applications beyond the stated target audience, the document is written for an audience of regulatory technical staff who must make decisions regarding the selection of remediation technologies at individual sites. Generally, these individuals have formal technical training and practical experience in geology, soils, and/or engineering but may not have a good understanding of the concepts of microbiological degradation of organic chemicals.

Microbial Degradation Processes -

Biodegradation refers to the microbial alteration or transformation of an organic compound; mineralization refers to the complete degradation of an organic compound to carbon dioxide, water and inorganic ions. Bioremediation relies upon biodegradation and mineralization of organic contaminants for the removal of those contaminants from soil and water. Microbes degrade contaminants using the enzymes and metabolic pathways central to standard cellular function. It is necessary to have an understanding of biodegradation processes in order to asses the likely success of their application in bioremediation.

At the simplest level, microbes "view" a contaminant or any organic molecule as a potential energy source and/or as a potential source of carbon (or sometimes nitrogen, phosphorus or sulfur) for the synthesis of cellular materials. The biodegradation of a contaminant is thus driven by the biological thrust for survival, which in the microbial world means increasing the size and mass of the resident population. Whether degradation occurs depends in part upon whether the resident microbial species, individually or in consort, produce the necessary enzymes to transform the contaminant.

The two primary mechanisms by which microbes degrade organic contaminants are growth-linked degradation, whereby the contaminant serves as a substrate for microbial growth, and cometabolism, in which the contaminant serves as neither an energy nor a carbon source, yet it is fortuitously transformed by enzymes produced during normal microbial cell function. Two additional microbially-induced transformation processes which can affect contaminant fate are: polymerization and conjugation, wherein the contaminant molecules are chemically linked with other molecules to form polymers, and non-enzymatic contaminant transformation caused by microbial activity, in which the microorganisms bring about some environmental change (ph, redox, etc.) which causes an alteration in the contaminant structure.

Environmental and Chemical Factors Affecting Biodegradation Processes -

A number of environmental factors influence either the extent or rate of biodegradation. These characteristics are extremely site-specific and should be determined prior to assessing the applicability of bioremediation systems for any give site. Factors which are discussed in detail in the full literature review include: oxygen availability, water content (for soils), pH, oxidation potential, nutrient supply, temperature, contaminant concentration, and formation of bound residues.

The nature of the pesticide contaminant itself also affects its biodegradation. The chemical characteristics of a contaminant indirectly affect the accessibility of a contaminant to microbial attack by determining how the contaminant behaves in the environment (how it partitions between the aqueous, gas and solid phases), thereby either increasing or decreasing ease of microbial access. In general, microorganisms are only able, or at least much more able, to transform molecules which are in solution rather than in a sorbed state. Thus, highly insoluble compounds and compounds which tend to bind tightly to soil solids are generally less amenable to biodegradation, although there are exceptions to this. For highly volatile compounds, volatilization may be the preferential loss mechanism rather than microbial degradation.

Agricultural Chemical Dealership Sites and Pesticide Contamination: Implications for Bioremediation Systems -

Agricultural chemical dealership sites feature certain physical characteristics which may influence the application of bioremediation technologies. In general, there are several areas at any given site which have become contaminated. Very often, high concentrations of pesticide residues are involved. High concentrations can inhibit microbial growth and degradation rates, although this response is very chemical specific. In most situations, several different pesticide contaminants will be present in any given contamination area. This also provides a challenge: the conditions conducive to the degradation of one compound may not support the degradation of another. Native soil is often replaced or covered with coarse fill material to support heavy traffic. Site soils are usually highly compacted and generally low in organic matter content.

Pesticides themselves present a potential challenge to bioremediation. Most organic pesticides are xenobiotics, or man-made chemicals. For many xenobiotics, there are few or no chemical structural analogues found in nature. This feature helps explain their common resistance to microbial degradation. In spite of this, the literature shows that most pesticides studied will be at least partially degraded if an appropriate microbial population is present and if environmental conditions are favorable. Many pesticides have been shown to be completely mineralized by microbes in laboratory experiments. The microbial degradation pathways of some pesticides

have been well documented while the degradation of other pesticide families have received little study. It is important to recognize, however, that the laboratory demonstration of the degradation of a pesticide species does not guarantee successful bioremediation.

Bioremediation Treatment Strategies -

There are several bioremediation treatment strategies or approaches which can be applied in the various technology formats discussed in the next section. These strategies can be thought of as bioremediation tools. The different strategies can be used alone or in combination. Many of these strategies have been applied to various pesticides in laboratory and bench-scale tests but not at the full field scale. The strategies which are discussed in the detailed report include:

1. Biostimulation: The enhancement of natural biodegradation rates by altering the chemical and physical environment (by addition of nutrient amendments or oxygen, adjusting soil moisture content or pH, or simply tilling) so that the growth of naturally occurring degraders is optimized. It is beneficial to know the needs of the desired degrading population so that their growth can be optimized.

2. Bioaugmentation: The inoculation of contaminated media with microbial strains which have a known ability to degrade the target contaminant(s). This can be successful but there are several barriers to successful inoculation at the field scale. The inoculant must successfully compete with the native microflora and must express the same degradative capabilities that were displayed in the laboratory. Inoculation in slurry systems, where the environment can be controlled, is most promising.

3. White Rot Fungus: These naturally occurring organisms have been shown to transform and mineralize a broad range of man-made chemicals, including several pesticides, in laboratory studies. The treatment strategy involves inoculation with white rot fungus cultures. Although this strategy has been broadly promoted, there appear to be some potential problems which would detract from its effectiveness on pesticides.

4. Anaerobic Treatment: Although this has not been broadly applied in the field, it appears to offer a viable treatment option for halogenated aromatic compounds (this includes many pesticides).

5. Sequential Anaerobic/Aerobic Treatment: This is based on the concept that anaerobic degradation often results in partially degraded metabolites which are more susceptible to aerobic attack than the parent compounds. This has been tested on certain pesticides and appears promising.

6. Chemical Pretreatment: This strategy uses ultraviolet irradiation and/or oxidizing agents such as ozone or hydrogen peroxide to perform the initial oxidative transformations of pesticide molecules. The degradation products are more amenable to microbial attack. This technology appears to have limited application to pesticide-contaminated soil but may be useful for waters.

7. Genetically Engineered Organisms: Several researchers promote the use of genetically engineered microorganisms on man-made chemicals. Microbes can be engineered to display pesticide degradative abilities and/or specific survival-enhancing characteristics. Inoculation of genetically engineered microbes is subject to the same potential constraints as inoculation of naturally occurring microbes. The use of genetically engineered microbes is not feasible at this time because the release of such microbes is restricted by law.

8. Cell-Free Enzymes: The enzymes responsible for pesticide degradation are extracted and used independent of the microbial cells. This offers certain advantages because enzymatic reaction is no longer dependent upon the microbial growth or survival.

Because enzymes can not reproduce themselves and because they are labile, it would be necessary to continuously supply the enzymes. This strategy has only been applied to a handful of pesticides in laboratory settings and has not been applied in the field.

Treatability studies are necessary for each project to test the effectiveness of the selected strategy(ies) on the pesticides of concern under site specific environmental conditions. Correctly designed treatability studies guide in the selection of the most appropriate strategy and are necessary to optimize the selected strategy for site specific conditions. It is necessary to know the expected degradation products for a given pesticide prior to implementing conducting a treatability study. If one of the common degradation products for a given pesticide is more toxic than the parent compound, the treatability study should show whether this compound is transient or tends to accumulate.

Existing Bioremediation Technologies -

Bioremediation technologies range from highly engineered processes requiring intensive management to relatively simple technologies requiring less oversight and management. All of the technologies rely upon the processes of biodegradation (discussed earlier) and most are flexible in their ability to utilize the different treatment "strategies" presented above. In general, bioremediation technologies can be divided into three broad categories: a) solid phase treatment, b) treatment in a reactors and c) in-situ treatment. Examples of each of these categories are discussed in the detailed report.

1. Solid Phase Technologies: In solid phase technologies, contaminated soil is excavated and treated "ex-situ." Landspreading and its variants, prepared-bed treatment and various compost formats are solid phase technologies. Biodegradation rates are enhanced by manipulating the physical and chemical characteristics of the soil matrix (biostimulation). Mixing or tilling is performed to ensure adequate mixing between contaminant molecules and microbes. In a prepared bed or compost format, bioaugmentation may be used.

2. Treatment in Reactors: This includes slurry phase reactors for soils and various reactor formats for waters. Several reactor designs exist. Because the waste is contained in a relatively controlled setting, the use of reactors potentially offers several advantages. In general, reaction rates are increased and acclimation times shortened. Reactors provide superior mixing which allows for more homogeneous chemical and physical matrix characteristics and for increased contact between contaminants and microbes. Inoculation with specific cultures has the best chance of success in bioreactors due to increased environmental control. Reactor technologies are generally more costly than solid phase technologies.

3. In-Situ Bioremediation: In-situ treatment means that soils or ground water and aquifer solids are treated in place. For soils, in-situ treatment may involve biostimulation or bioaugmentation. In general, in-situ soil treatment is only feasible in settings which are not vulnerable to ground water contamination. For ground water and aquifers, in-situ treatment usually involves injection of water amended with nutrients, electron acceptors, and/or microorganisms into the aquifer. Contaminated water is pumped and treated in bioreactors and then reintroduced to the aquifer with the active microbial biomass. This literature review found a single example of a field-scale application of in-situ treatment of pesticide contaminated ground waters.

Conclusions and Recommendations -

The use of bioremediation technologies on pesticide contaminated media appears promising but remains largely untested at the field scale. Bioremediation is an evolving technology which has been applied most commonly to specific wastes for which there is an established commercial market (petroleum-related, wood preservative and explosive-related wastes). The treatment

strategies and technologies which are currently available have been tested to varying extents on pesticides in laboratory studies and in bench-scale systems. Laboratory scale studies have widely demonstrated the biodegradability of many pesticide families under specific and well defined conditions. However, the demonstration of biodegradation under laboratory conditions does not guarantee successful bioremediation under actual field conditions at an agricultural chemical spill site.

Since very few of the existing technologies have been applied to pesticides, and since pesticide degradation is very compound specific, it is impossible to make conclusive statements regarding the use of the individual technologies on all pesticides. No single bioremediation approach will act as a "silver bullet" for all remediation needs. However, of the currently available treatment strategies and technologies, several stand out as especially promising. Sequential anaerobic/aerobic treatment appears to be applicable for a broad range of pesticides and makes particular sense for complex pesticide mixtures. Strict anaerobic treatment may be suitable for the older, heavily chlorinated pesticides, although sequenced treatment also appears appropriate. Aerobic or anaerobic composting appears promising for many of the compounds commonly found at sites in Minnesota; this is true not only from a technical viewpoint but also because the low-tech, low maintenance characteristics of composting are ideally suited to the small scale of most agricultural chemical dealership sites.

Each bioremediation project requires a correctly designed treatability study to test the application of a specific technology on site-specific contaminant and environmental conditions. Treatability studies guide in the selection of the most applicable biotreatment strategy and are necessary to optimize the selected strategy for the site specific conditions. Treatability study requirements for pesticide contaminated sites are currently not standardized for the industry. It would help the development of the technology if treatability study requirements were standardized. It should not be necessary to perform exhaustive pesticide fate studies as part of each treatability study. However, the expected degradative pathways of each contaminant must be identified from the existing literature so that potential toxic intermediates can be identified and tested during the treatability study.

Research should be promoted in two distinct directions. First, there is a great need for pilot- and field-scale demonstration projects of the existing strategies and technologies on pesticide contaminated media. Although many potential constraints may be addressed at the treatability study stage, some problems will only become evident in a "real world" situation. Secondly, basic research is also needed. As the general understanding of specific pesticide degradation mechanisms and the factors affecting them grows, bioremediation technology will become more available for practical application to pesticide contaminated soils and waters.

Publications/Presentations Resulting From This Project -

The literature review will be available for distribution to the public in the fall of 1993.

A6. Benefits : The review and evaluation of information regarding bioremediation techniques will enable contaminated soil clean-ups to be conducted more cost efficiently and effectively than currently possible. Broadening the understanding of the available technology base will provide more and better clean-up of soils, thereby protecting ground water.

B. Degradation and Leaching of Atrazine and Alachlor Under Simulated Spill Conditions.

B1. Narrative : The mineralization (degradation) and movement of atrazine and alachlor will be determined on one vulnerable and one non-vulnerable soil to determine the degradation and leaching potential at elevated herbicide concentrations common with point source contamination events in Minnesota. Methodologies to accurately document pesticide degradation (metabolites and parent compound) and movement will be determined building on existing knowledge and research on field-use rate levels. In-field leaching studies will be established at two sites which

have been under controlled conditions for 3-4 years and which have been used for previous field-use rate studies on parent compounds and associated metabolites. This will allow application of the degradation and transport knowledge obtained at field-use rates to the elevated spill level work. Some actual Ag dealership spill site soil cores for which there is existing analytical data may be investigated along with the controlled sites if funding allows.

^{14}C will be used to allow metabolite work. In addition to allowing more efficient mass flow characterization, this is the only reasonable approach to a truly comprehensive "balance sheet" study which includes metabolite characterization.

B2. Procedures : The experiment will utilize two field sites which have been used for ongoing research on atrazine and alachlor at field-use rates; one site is hydrogeologically vulnerable and the other not considered vulnerable to pesticide leaching. In-field leaching studies will be conducted in which elevated levels of atrazine and alachlor simulating spill conditions will be applied and monitored (these study areas receive regulatory oversight). Leachate from the columns will be analyzed for parent compound and associated degradation products. After leaching, the columns will be sub-sectioned into depth increments and the herbicide will be extracted from the soil and quantified. The movement of parent herbicide and metabolites will be determined. Pesticide mineralization and movement at these elevated levels will be compared with ongoing work at field-use rates at these sites.

B3. Budget :

a. Amount Budgeted : $130,000.00
b. Balance : $ - 0 -

B4. Timeline for Products/Tasks :

	July 91	Jan 92	June 92	Jan 93	June 93
Field Sample Collection/Methods Development	**********				
Sample Collection/ Extraction/Analysis		**********	**********	**********	
Data interpretation/ Extend Results Interpretation to Other Soils and Pesticide Problems					**********

B5. Status :

The degradation and transport of atrazine and alachlor at elevated levels common to spill and waste disposal sites were investigated in laboratory and field experiments. Two soils were used throughout the study, a Webster clay loam from Waseca, MN and an Estherville sandy loam soil from Westport, MN. Four studies were conducted:

1. The effect of atrazine concentration on persistence, and degradation was studied in laboratory experiments. Atrazine concentrations ranged from 5 to 5,000 parts-per-million (ppm).

2. The effect of alachlor concentration on persistence and degradation was studied in laboratory experiments. Alachlor concentrations ranged from 10 to 10,000 ppm.

3. F[illegible] studies were conducted to validate laboratory results and [illegible]valuate leaching b[illegible]ior in high concentration situations. Concentrations of each p[illegible]ide (atrazine and alachlor) approximated a range of 5 to 5,000 ppm.

4. Preliminary laboratory studies on the use of amendments to enhance atrazine degradation at in contaminated soils were conducted.

Study 1: Effect of Concentration on Persistence and Degradation of Atrazine in Soils - Laboratory Studies -

The persistence of atrazine under laboratory conditions was independent of concentration but differed between the two soil types studied here. Atrazine persisted longer in the sandy loam soil than in the clay loam soil, although in both soils the estimated 50% dissipation times were on the order of weeks for all initial atrazine concentrations.

The amount of atrazine degraded increased proportionally with increasing concentration in the clay loam soil and for low to moderate concentrations (below 500 ppm) in the sandy loam soil. The proportional amount of atrazine degraded decreased only slightly at high initial concentrations (500 and 5000 ppm) in the sandy loam soil. Degradation and mineralization (complete degradation to inorganic ions and CO_2) of atrazine were stimulated at higher concentrations in both soils, but more so in the clay loam soil. For both soils, the absolute amount of atrazine degraded increased with increasing initial concentration. Thus, there was no observed inhibitory effect of atrazine concentration on the microbial mineralization process for either soil. This finding was corroborated by observations that the introduction of high concentrations of atrazine into soil did not show any inhibitory effect on soil microbial activity.

Mineralization to CO_2 was the most important pathway for the dissipation of atrazine at all concentrations in the clay loam soil, and at concentrations of 500 ppm or less in the sandy loam soil. It was postulated that soil microorganisms were able to use the N and/or C from the *s*-triazine ring, and the addition of atrazine stimulated soil microbial growth and activity and thus stimulated the degradation of atrazine. This assumption was supported by an observed increase in soil respiration indicating increased microbial activity in the clay loam at atrazine concentrations of 500 and 5,000 ppm.

Study 2: Effect of Concentration on Persistence and Degradation of Alachlor in Soil - Laboratory Studies -

The effect of concentration on persistence and degradation of alachlor was similar in both soils. The persistence of alachlor increased with increasing concentration under laboratory conditions for both soils. At 10,000 ppm alachlor became virtually nondegradable. Estimated 50% dissipation times were on the order of years at the 10,000 ppm treatment, compared to a time scale of weeks for the 10 ppm treatment.

On an absolute basis, the amount of alachlor degraded was similar across concentrations. However, based on percent of applied alachlor, mineralization and formation of various degradation products and bound residues decreased substantially at higher concentrations. The results indicate that the capacity for alachlor degradation or mineralization was exceeded in both soils at high concentrations (1,000 ppm and greater). We feel that limited water solubility and the specific biodegradation mechanisms of alachlor may be the rate limiting factors for the degradation of alachlor at elevated levels. The overall soil microbial activity was slightly reduced at high concentrations, but it was assumed that the microorganisms involved in alachlor degradation were not affected based on other parameters measured.

"Bound" (or "unextractable") residues comprised a major proportion of the alachlor total soil residues at low concentrations, however, the formation of bound residues decreased significantly at initial concentrations above 100 ppm.

Study 3: C[illegible]rative Studies on the Persistence and Movement of [illegible]zine and Al[illegible]or in Soils at Normal and Elevated Levels Under Field [illegible]ditions -

To date, the field studies, which will be completed in October, 1993, have confirmed that alachlor, and to a lesser extent, atrazine, have increased potential to impact ground water when introduced into the soil at high concentrations. In both soils, increased concentration had a greater effect on alachlor as compared to atrazine in terms of the amount leached and the vertical extent of leaching.

For both soils, the 5 ppm concentration of alachlor (which approaches normal field use rates) showed little risk of leaching below the crop rooting zone. However, at 5000 ppm concentrations, alachlor moved to the lowest depth sampled (85 cm) and presumably below the rooting zone, posing a threat to ground water. Alachlor was detectable at lower depths in the soil profile, and often at concentrations an order of magnitude higher, than was observed for atrazine at similar high concentration applications. Preliminary field data and corroborative laboratory studies indicate that the persistence of alachlor will increase several orders of magnitude at high concentrations as well, compounding concern for potential environmental impacts.

For both soils, greater application rates of atrazine resulted in detections of higher concentrations at each sampling depth within the soil profile. However, the rate of travel, as determined by time required for atrazine to reach the 71 to 85 cm depth, differed little between the high and low atrazine application concentrations. The final round of field samples will be needed to make observations regarding the persistence of high concentrations of atrazine in the field.

The final round of field data for this study will be collected in late summer, 1993.

Study 4: Enhancement of Atrazine Biodegradation at Elevated Levels In Soil With Amendments -

Degradation of atrazine was affected by soil amendments in preliminary laboratory studies. All treatments used Webster clay loam soil and an initial atrazine concentration of 5,000 ppm. When soil was mixed with dairy manure, atrazine degradation rate was greatly increased. Degradation rates differed little in the soil amended with corn meal or ammonium phosphate fertilizer when compared to non-amended soils.

Mineralization was also influenced by the different types of amendments. Dairy manure stimulated mineralization in the 4 to 8 weeks following treatment. Mineralization was slower in the unamended soil but increased steadily with time. In both these treatments, adaption and stimulation of atrazine degradation by soil microorganisms was involved. Atrazine mineralization was inhibited in the cornmeal amendment treatment, indicating that organic carbon amendments such as cornmeal would not be effective in removing atrazine from the soil matrix. Ammonia phosphate amendment halted the mineralization of atrazine, illustrating that inorganic nitrogen may compete with the nitrogen in atrazine ring for use by the microorganisms. Although mineralization was inhibited in the cornmeal and ammonia phosphate treatments, atrazine was nevertheless partially degraded.

Atrazine and its nonpolar metabolites degraded rapidly in initial phases of degradation in manure amended soil, likely due to the abundant microorganisms already present in the manure. The possibility exists that non-selective microorganisms that degrade organic N-containing nutrients in manure cometabolized atrazine.

Conclusions and Recommendations -

This study illustrates that the effects of pesticide concentration on persistence, degradation and transport are highly compound-specific and vary with soil type. While the persistence of

atrazine is relatively unaffected by concentration, high concentrations of alachlor are considerably more persistent than low concentrations of alachlor. The difference between the effects of concentration on the environmental behavior of atrazine and alachlor have implications for the choice of remediation options for soils contaminated with these chemicals at high concentrations.

Laboratory results indicate that the degradation and persistence of atrazine at elevated levels in soil was dependent, to some extent, on soil type but independent of concentration. Although previous studies on atrazine and other pesticides have found that high concentrations of pesticides inhibit degradation, the laboratory studies conducted for this project indicate that the degradation of atrazine was not inhibited by high concentrations. Incubation studies conducted in this project showed that atrazine degradation at high concentrations was related to the stimulation of microbial activity, as indicated by the increase of CO_2 evolution. Remediation methods that accentuate increases in population and activity of relevant biodegraders would accelerate the dissipation of atrazine and thus could be effective in detoxifying contaminated sites.

This research suggests that it may be possible to treat soils containing high levels of atrazine in-situ, provided that other conditions (oxygen concentrations, pH, organic matter content, nutrient supplies) are optimized. This type of treatment would only be feasible at isolated sites with low potential for ground and surface water contamination. At sites which are vulnerable to ground or surface water contamination, this research would support treatment of soil contaminated with high levels of atrazine by stockpiling and treating ex-situ in an aerated compost format with appropriate safeguards to prevent leachate or runoff movement. For either of these treatments, appropriate amendments such as livestock manure may enhance degradation. These recommendations are based on atrazine degradation behavior observed in laboratory experiments. Behavior under field conditions may vary from that observed in the laboratory. For instance, there is conflicting evidence on the effect of "aged" residues (residues which have been contained in soils for extended periods ranging from months to years) on the extent and rate of degradation. It would be necessary to test either approach in a pilot scale study prior to implementation at an actual site.

Treatment of atrazine-contaminated soil by landspreading at or below labeled rates on labeled sites is a feasible remediation alternative if adequate land area is available. Land spread soil should be incorporated by tilling to ensure and sufficient mixing of the land spread soil with the clean soil and to prevent surface run-off.

High concentrations of alachlor pose a more serious threat than atrazine to ground and surface water resources. Degradation was severely retarded and leaching increased dramatically at elevated concentrations compared to low, field application rates of alachlor. Additionally, a low percentage of alachlor was in the form of bound residue when applied at high concentrations. This indicates that while most of the alachlor residues were still available for further biodegradation, they are also readily available for leaching. Based on this, and on the fact that alachlor degraded rapidly at concentrations at and below 100 ppm, landspreading should be used to treat alachlor contaminated sites and reduce leaching at the contaminated site. Exposure of diluted alachlor and its degradation products to abundant soil microorganisms after land-spreading would enhance the degradation process. Land spread soils should be tilled following application to insure adequate mixing with clean soils and to prevent surface run-off. Soils containing high concentrations of alachlor would not degrade sufficiently if left in place or if stockpiled without additional treatment. Thus, based upon this research, in-situ treatment of soils containing high concentrations of alachlor is not recommended.

The addition of dairy manure stimulated the degradation and mineralization of atrazine and merits further study to delineate the competition of inorganic and organic compounds with the biodegradation of atrazine in soil. Other amendment-related research should focus on the effect of amendment C/N ratio and the effect of readily available carbon sources, such as glucose, on atrazine degradation and mineralization. The effects of various amendments on alachlor degradation should also be explored.

Publications/Presentations Resulting From This Project -

Published or Presented:

1. Gan, J., W.C. Koskinen, R.L. Becker, D.D. Buhler, and L.J. Jarvis. 1992. Biodegradation of alachlor in soil as a function of concentration. Agron. Abstr. pp. 39-40.

2. Gan, J., R.L. Becker, W.C. Koskinen, D.D. Buhler, and L.J. Jarvis. 1992. Biodegradation of atrazine in soil as a function of concentration. Agron. Abstr. p. 40.

To be Published or Presented:

1. Gan, J., W.C. Koskinen, R.L. Becker, D.D. Buhler, L.J. Jarvis. Biodegradation of alachlor and atrazine in soils as a function of concentration. University of Minnesota and USDA-ARS, St. Paul, MN 55108. Proc. Fourth Nat. Pest. Conf., New Dimensions in Pesticide Research, Development, Management, and Policy. Richmond, Virginia. (accepted for presentation in November 1993).

2. Gan, J., R. L. Becker, W. C. Koskinen, D. D. Buhler, and L.J. Jarvis. (0000). Degradation of Atrazine in Soil as a Function of Concentration. To be submitted to J. Envir. Qual.

3. Gan, J., W. C. Koskinen, R. L. Becker, D. D. Buhler, and L.J. Jarvis. (0000). Effect of Concentration on Persistence and Degradation of Alachlor in Soil. To be submitted to Soil Sci.

B6. **Benefits :** The difficulties inherent in assessing the potential for pesticide movement at known or future pesticide spill sites, and the prohibitive costs for remedying such situations require a more thorough knowledge of the behavior of these compounds at elevated levels. Private and public resources will be conserved by utilizing more efficient and prioritized bioremediation methodologies. This project will provide the basis for making better management decisions regarding the clean-up or containment of existing and future point source pesticide contamination.

C. Investigate Innovative Bioremediation Techniques for Reduction of Elevated Pesticide Concentrations.

C1. **Narrative :** Atrazine is commonly detected in ground water and is commonly involved in pesticide spills. The focus of Objective C is to examine innovative approaches using plants and microbes to enhance biodegradation and removal of pesticides from spill sites. If enhanced degradation occurs in laboratory and greenhouse experiments, exploratory field studies may be undertaken as funding allows.

C2. **Procedures :** Atrazine is biotransformed by a number of reactions including oxidative dealkylation and conjugation with glutathione. It is proposed in this research to effect biotreatment of atrazine spills by enhancing the activities of microorganisms which catalyze these reactions. Two main approaches to the development of bioremediation methods will be attempted: investigation of enhanced degradation due to plant rhizosphere effects; and identification of microorganisms that metabolize atrazine. Plant species will be tested for tolerance of elevated levels of herbicide.

Previous work has shown that the conjugation product of atrazine with glutathione is tightly adsorbed to soil, mitigating against potential leaching of the pesticide into ground water (Clay and Koskinen, 1990, Weed Sci 38: 262-6). Typically, plants which display herbicide resistance manifest this resistance via oxidative and/or conjugative reactions to detoxify the pesticide. Some of these same types of biochemical reactions are known to be catalyzed by terrestrial and

aquatic m[illegible]organisms. We have extensive experience studying bacteria[illegible]xygenases which are crucia[illegible] the biodegradation of aromatic compounds and chlorinated [illegible]atic compounds (Wackett and Gibson, 1988, Appl. Environ. Microbiol., 54: 1703-8; Wackett, et al., 1989, Appl. Environ. Microbiol., 55: 2960-4: Fox, et al., 1990, Biochemistry 29: 6419-27).

Furthermore, we have previously investigated the major group of conjugating enzymes, known as the glutathione S-transferase supergene family, from bacteria (Scholtz, et al., 1988, J. Bacteriol. 170: 5698-704), fungi (Wackett and Gibson, 1982, Biochem. J. 205: 117-22), and mammals (Blocki and Wackett, unpublished data). We have an E. coli strain that produces up to 50 mM glutathione in its immediate environment via overexpression of cloned glutathione biosynthetic genes. The ability of these and other well characterized soil isolates to effect high-level atrazine biotransformation will be assessed. We also will examine other well-determined biodegradation regimes for activity with atrazine.

Similar analytical methods will be used to monitor pesticide metabolism in the soil and in pure culture experiments. Extraction methods and HPLC analysis of metabolites will follow procedures developed by Bill Koskinen. Previously described dealkylated and conjugated metabolites will be identified by comparison with authentic standard compounds. Heterocyclic ring side chain oxidation will be analyzed by trapping out ^{14}C - CO_2 using standard methods.

Selective enrichment cultures will be used to isolate bacterial populations (mixed or isolated) capable of growth on atrazine as a sole nitrogen source. All enrichment cultures will be subcultured into a homologous medium at two week intervals. Cultures will be successively subcultured under limiting conditions; subsequently, atrazine degradation will be quantified by HPLC analysis of the remaining atrazine in the medium. Positive enrichments will be harvested; attempts will be made to isolate pure cultures capable of degrading atrazine from the mixed cultures.

Atrazine mineralization assessment will be performed using uniformly ring-labeled [^{14}C]atrazine amended media inoculated with isolated cultures. Evolution of $^{14}CO_2$ will be measured; the atrazine medium will also be analyzed for residual radioactivity. Atrazine degradation pathways will be determined using ammonia 31 enzymatic analysis of ammonia in growth media and Thin Layer Chromatography analysis in addition to High Pressure Liquid Chromatography (HPLC) measurements of atrazine and its metabolites. Mass spectroscopy will also be used to further identify metabolites which coelute on the HPLC system.

Molecular probes to identify specific genes in microorganisms will be used to monitor potential enhancement of those microbial populations most active in pesticide detoxification. For example, gene probes are available for several bacterial oxygenases and they could be prepared from cloned genes that are known to be involved in conjugative reactions. We also have glutathione S-transferase gene family probes from bacterial, plant and mammalian sources. Higher levels of microbial populations containing these genes will be indicated by enhanced levels of atrazine detoxification. Since gene probe methodologies are relatively rapid, this will afford us a convenient tool for assessing the effectiveness of specific pesticide-detoxifying strains of bacteria.

Field testing will be exploratory and confined to one or two sites because of budget constraints. Based on previous experiments, the most likely candidates for field testing will be selected and inoculated in field plots at sites for Part B. These plots will be replicated and blocked. Soil samples at different depths in and below the root zone will be monitored for remaining parent compound and metabolites and compared to unplanted control plots.

C3. Budget :

a. Amount Budgeted : $100,000.00
b. Balance : $ - 0 -

C4. Timeline for Prod[illegible]ts/Tasks :

	July 91	Jan 92	June 92	Jan 93	June 93
Greenhouse and Growth Chamber Experiments	**********				
Cultures and Molecular Probe Analysis	**********	**********	**********		
Test Cultures on Aged Soil			**********		
Final Report					********

C5. Status :

This study used three major approaches in seeking innovative remediation techniques using microbes and plants to enhance biodegradation and removal of pesticides from spill sites: the use of enrichment cultures to isolate pesticide degrading microorganisms from soils exposed to repeated pesticide spills; conjugation of atrazine with glutathione-S-transferase; and enhancement of degradation by plant rhizospheres. The atrazine enrichment cultures yielded the most exciting results and were consequently pursued in greatest depth. The experiments which were performed are presented here in 4 parts:

Part 1: Isolation of atrazine degrading microorganisms from contaminated site soil using enrichment cultures. Preliminary determination of degradation rates.

Part 2: Determination of atrazine degradation pathway and rates in soil for the isolated mixed cultures.

Part 3: Application of isolated mixed culture to highly contaminated site soils.

Part 4: Preliminary attempts to: (a) enrich for alachlor degradation; (b) detoxify atrazine by glutathione conjugation; and, (c) enhance pesticide degradation by the use of plant rhizospheres. The success of these three approaches varied and all are worth reporting for the benefit of future research efforts.

Part 1: Isolation of Stable Mixed Bacterial Cultures Capable of Atrazine S-Triazine Ring Mineralization -

Mixed microbial cultures capable of degrading atrazine were isolated from soils from three agricultural chemical dealership sites in Minnesota using enrichment culture techniques. Multiple locations from all three sites yielded cultures capable of degrading atrazine, although cultures differed in their degradation abilities. In total, over 30 atrazine degrading bacterial cultures were isolated. Successful cultures were subcultured (small amount of culture was transferred into fresh enrichment medium) every two weeks to enhance degradation ability and to select for stable cultures. Selected stable mixed cultures were studied to determine degradation characteristics and to assess degradation rates in culture medium.

Although previous studies have shown atrazine to be biodegraded (but not mineralized) by whole soils, and mixed and pure microbial cultures, atrazine ring cleavage has been only rarely reported. Even side chain degradation (which results in partial degradation) demonstrated in previous studies has typically been slow. In contrast, the present study demonstrated rapid and extensive degradation of the atrazine ring to CO_2. The use of atrazine as the sole nitrogen source and/or the use of citrate and sucrose as mixed carbon sources may have contributed to the success of the enrichments in these studies.

These studies represent the most rapid rates of biological atrazine degradation ever reported in the literature as well as the first illustration of the degradation of elevated concentrations of atrazine. The half-time for degradation of 100 ppm atrazine was 0.5-2 days. Previous studies have shown half-lives on the time scale of weeks for microbial cultures and on the order of months for whole soils.

Degradation intermediates were identified to ensure that no toxic intermediates formed. The identification of hydroxyatrazine as an intermediate in the microbial degradation pathway was an unique finding and suggests a previously unidentified microbial degradation pathway. The formation of hydroxyatrazine is an environmentally positive reaction since hydroxyatrazine does not have pesticidal activity, is not toxic to animals, and is not a carcinogen.

Part 2: The Rapid Hydrolysis of Atrazine to Hydroxyatrazine in Soils by Mixed Bacterial Cultures -

The purpose of this study was to determine whether, and at what rates, a bacterial mixed culture, shown in Part 1 to mineralize atrazine in a liquid growth medium, could metabolize atrazine to hydroxyatrazine in soils. This is important because hydroxyatrazine formation has not been previously attributed to bacterial activity and it is widely reported that the formation of hydroxyatrazine in soil is due to a non-biological process.

The mixed bacterial culture (LFB6) successfully degraded atrazine in two soil types, a clay loam and a silica sand, from an initial concentration of 100 ppm. After 24 hours, more than 80% and 95% of the atrazine in the clay loam and silica sand, respectively, was degraded. Hydroxyatrazine was detected after the first hour and was rigorously identified as a transient intermediate. Observed degradation rates far exceeded those previously reported in the literature for native soils or bacterial cultures. Cell-free protein extracts of the culture were found to rapidly transform atrazine to hydroxyatrazine which was further degraded to a more polar metabolite after 24 hours.

Isotopically labeled water was used to investigate the mechanism of hydroxyatrazine formation. The results conclusively showed that dechlorination by the mixed culture occurred via hydrolytic dechlorination (the oxygen in the hydroxy group which displaces the chlorine comes from the surrounding water rather than from atmospheric oxygen). These findings force a reevaluation of the widely held belief that the formation of hydroxyatrazine in the environment is due to chemical mechanisms.

The elucidation of the hydrolytic pathway is important because it means that atmospheric oxygen is not required for the microbial breakdown of atrazine. This suggests that microbial dechlorination of atrazine should occur in oxygen limited environments such as ground water and subsoils if other conditions are optimized for the growth of the degrader population.

Part 3: Degradation of Atrazine in Contaminated Site Soils by Mixed Bacterial Cultures -

Treatment of contaminated site soils was attempted using two of the mixed bacterial cultures (LFB6 and LFA7) isolated in Part 1. The test soil was obtained from a contaminated site in Madison, MN where atrazine had been spilled in the early 1980's.

In the initial experiment, 10-g portions of contaminated soil were treated in oxygen-limited (water saturated) conditions in the laboratory. Soil was amended with the growth medium (containing sodium citrate and sucrose as additional carbon sources), inoculated with culture LFB6 or LFA7, and incubated in the dark for three weeks. For inoculated soils which were amended with an additional carbon source, 80% - 100% of the atrazine present was degraded. Control treatments indicated that the inoculated organisms could successfully compete with the indigenous microflora and that an additional carbon source was necessary for successful degradation to occur in the inoculated soils. It was also found that atrazine degrading organisms

were present in the site soil but required the presence of an additional carbon source for successful atrazine degradation to occur (the native degraders removed 40% of the atrazine present).

A greenhouse experiment was conducted with soil from Madison, MN to evaluate the degradation of aged atrazine residues in an upscaled system. The soil contained approximately 4,000 ppm aged atrazine. Treatments were conducted using 10L of soil in 5 gallon buckets. Soils were inoculated with the culture LFB6 and amended with sodium citrate as a carbon source. Soils were incubated for 28 days. Sixty percent atrazine degradation was observed for inoculated soils which had been amended with an additional carbon source; degradation did not occur without the carbon source amendment. Forty percent degradation was observed in non-inoculated soils amended with sodium citrate. These results support previous data that soils previously exposed to atrazine contain indigenous microflora capable of atrazine degradation in the presence of an additional carbon source (sodium citrate).

The mineralization of high concentrations (100 ppm) of atrazine in soils was studied by amending the soils with ring-labeled atrazine and measuring the production of $^{14}CO_2$. Two soils were used, a clay loam and a silica sand. Rapid initial mineralization occurred in both soils after inoculation with the mixed culture. However, following the phase of rapid mineralization, the mineralization rate decreased and leveled off at 20 to 25% total mineralization for the clay loam and silica sand, respectively. It is not clear at this point why the mineralization rate decreased. It was found that only 10% of the initial atrazine remained in the soil, indicating either that atrazine was partially degraded and the degradation products bound to soil or the method used to trap the CO_2 was inefficient.

Part 4: Preliminary Results of Alternative Remediation Approaches -

Enrichment cultures for the degradation of alachlor were initiated at the same time as those for atrazine degraders. A few fungal cultures and many bacterial cultures that degraded alachlor were obtained; two isolated bacterial cultures were particularly active. Because of the more dramatic degradation seen for atrazine and the availability of ^{14}C-labeled atrazine, we chose not to pursue further research on alachlor. However, it seems that isolation of bacterial cultures capable of alachlor degradation is feasible and warrants further work.

The use of a class of enzymes, glutathione S-transferases (GST), to detoxify atrazine was investigated. These enzymes catalyze the reaction of non-polar, hydrophobic compounds (such as atrazine and alachlor) with glutathione, thereby rendering hydrophobic compounds more water-soluble. Previous work has shown that the conjugation products of atrazine with glutathione are tightly adsorbed to soil, decreasing the potential of the pesticide to leach to ground water. To test the possibility of using this strategy as a remediation technique, studies on the conjugation of atrazine to glutathione in the presence of GST from rat liver crude extract were performed.

It was found that after 24 hours incubation of atrazine with the glutathione and rat liver GST, 50% to 60% of the atrazine was conjugated to glutathione. However, since conjugation of atrazine in soils was expected to be slower than conjugation under optimal experimental conditions, and since bacterial mixed cultures obtained from soils demonstrated higher atrazine degradation rates than these, further research concentrated on the degradation of atrazine by the mixed bacterial cultures.

Greenhouse experiments were conducted to study the survival of several plant species in elevated concentrations of atrazine and alachlor. It was found that only one of the tested plant species survived at herbicide levels higher than 25 parts per million (ppm). Sorghum plants were stunted, but did grow at levels of up to 50 ppm atrazine. Kochia, crimson clover, switchgrass and Indian grass seedlings survived when transplanted into 25 ppm alachlor. Soybeans grew at half the control rate in alachlor. Further testing of sorghum showed that there was no effect of the sorghum rhizosphere on atrazine degradation. Plant studies were thus

abandoned because it was difficult to find any plants that would grow at the elevated levels and because no rhizosphere effects were observed.

Conclusions and Recommendations -

This study successfully isolated mixed bacterial cultures capable of degrading atrazine at high concentrations in soils and water. The bacteria were obtained from spill sites in Minnesota, cultivated in the laboratory and used under defined conditions to break down atrazine into harmless endproducts. The mixed cultures were demonstrated to rapidly degrade atrazine to carbon dioxide and ammonium ion. The rates of atrazine degradation were very rapid and exceeded all rates previously reported in soils, waters, and mixed or pure bacterial cultures.

The bacteria were shown to degrade high concentrations of atrazine in water (100 ppm) to below detectable levels. Significant biodegradation of atrazine was observed when the bacterial cultures were added to heavily contaminated (4000 ppm atrazine) soils from a 10-year old spill site.

The metabolic degradation steps were investigated to rule out that any intermediates were toxic. The first metabolic step produced hydroxyatrazine. This intermediate is not toxic to plants or animals, nor is it a carcinogen. It is further broken down to be completely recycled into biological materials. It was shown that atmospheric oxygen is not required for the degradation of atrazine by the isolated microbes. This is advantageous because sites with oxygen-poor environments could be treated with the organisms obtained in this study.

The other biological methods investigated in this study showed various degrees of promise; these were not pursued so that resources could be focused on the more promising results of the atrazine degrading bacterial cultures. Mixed bacterial cultures with varying abilities to degrade alachlor were isolated. A class of enzymes, glutathione S-transferases, were investigated as a means of detoxifying atrazine, and although some decrease in atrazine was seen using this approach, it was generally less successful than the bacterial methods described above. Lastly, the effect of plants on the breakdown of atrazine was also investigated but was not found to be successful.

In general, these results highlight the potential to apply the bacterial cultures isolated in the study for the decontamination of atrazine contaminated soil and water. The conditions appropriate for the use of the microbes were investigated in great depth. The bacteria were obtained from natural sources and used without genetic manipulation. The efficacy and safety of a potential treatment system were studied. A large scale treatment of a field site would require further research on the preparation of the soil and the application of the bacteria to the soil in order to optimize treatment. The preliminary results on the isolation of alachlor degrading organisms also warrants additional research.

Publications/Presentations Resulting From This Project -

1. Allan, D.L. and R. Mandelbaum. 1991. Degradation and Bioremediation at Pesticide Spill Sites. Workshop on Pesticide-Soil Interaction Research. United States Department of Agriculture National Tilth Laboratory, Ames, IA. November, 1991.

2. Mandelbaum, R., L.P. Wackett and D.L. Allan. 1992. Hydrolytic Dechlorination is the First Step in the Degradation of Atrazine by Some Bacterial Consortia. American Society For Microbiology Conference on Anaerobic Dehalogenation. Athens, GA. September, 1992.

3. Mandelbaum, R., L.P. Wackett and D.L. Allan. 1993. Microbial Degradation of Atrazine. Environmental Science Workshop. The Gray Freshwater Institute. Navarre, MN. February, 1993.

4. Mandelbaum, R., L.P. Wackett, and D.L. Allan. 1993. Bacterial Degradation of Atrazine - New Mineralization Pathway. American Society for Microbiology Annual Meeting. May, 1993. Atlanta, GA.

5. Mandelbaum, R., L.P. Wackett, and D.L. Allan. 1993. Atrazine Mineralization by Microorganisms Isolated From Atrazine Spill Site. Conference on Bioremediation. Research Triangle, NC. April, 1993.

6. Mandelbaum, R., L.P. Wackett and L.D. Allan. 1993. Mineralization of High Concentrations of Atrazine by Stable Microbial Communities. Appl. Environ. Microbiol. 59: 1695-1701.

7. Mandelbaum, R., L.P. Wackett and L.D. Allan. 1993. Soil Bacteria Rapidly Hydrolyze Atrazine to Hydroxyatrazine. Environ. Sci. Technol. In Press.

C6. **Benefits :** This objective will evaluate the potential for treatment of contaminated soil at pesticide incident sites. Microbes isolated from spill sites will be evaluated for their ability to promote biodegradation or transformation of atrazine.

IV. EVALUATION :

For the FY92-93 biennium, this program can be evaluated by the development of new information regarding soils contaminated with elevated levels of pesticides. Each objective can be evaluated as follows:

(1) Objective A can be evaluated by the production of a report describing the existing and applicable technologies.

(2) Objective B can be evaluated by its ability to characterize the mineralization and movement of atrazine and alachlor at elevated levels representative of spill sites.

(3) Objective C can be evaluated by its ability to identify: (1) mixed and/or isolated microorganisms capable of rapid degradation of elevated levels of atrazine; (2) potential mechanisms of atrazine degradation; and (3) whether mixed cultures or isolated organisms can effectively reduce atrazine concentrations in contaminated soils.

The long term success of this project will be evaluated by the incorporation of the information generated into clean-ups and programs to remediate contaminated soils.

V. CONTEXT :

A. Recent studies have identified pesticide mixing, loading and handling sites as potential sources of ground water contamination. Efforts in Minnesota and the Midwest are beginning to attempt remediation of these sites. Very little is known of the fate and transport of elevated levels of pesticides in soil and the remediation of these soils. Virtually all efforts by pesticide registrants, EPA and University researchers to date has focused on fate and transport mechanisms for pesticides used at or near labeled rates in field situations. What little data exists nationwide indicates that elevated pesticide levels may overwhelm mechanisms responsible for degradation and retarding movement of chemicals, thereby increasing the potential for ground water contamination.

The only techniques available for clean-up of pesticide contaminated soils currently available are at out-of-state licensed hazardous material sites, landspreading techniques and on-site encapsulation.

B. Several reports suggest that the presence of live or decomposing plant roots have the potential to increase pesticide degradation or transformation. The enhancement of degradation has been

little studied, but confirmed for alachlor on alfalfa, and for atrazine on corn, where up to 25% or more of the atrazine was detoxified (mineralized or converted to metabolites) due solely to the presence of the plants. Most studies have simply looked for disappearance of the parent compound or evolution of CO_2. The work proposed here will elucidate mechanisms for enhanced degradation, determine application of this bioremediation measure for elevated levels of pesticides, and evaluate plant species appropriate for Minnesota conditions. This project will review existing information and develop new information in a field where little is known.

C. Past LCMR funded projects consisted of efforts to understand the fate and transport mechanisms under normal use. This project will build on existing and presently developing data from the University of Minnesota Center for Agricultural Impacts on Water Quality which has been partially funded through LCMR in the past. This project provides the unique opportunity to access the extensive database being generated at field-use levels to enhance this effort to characterize the mineralization and movement of elevated pesticide levels encountered with spills or mishandling. The equipment is in place to do the laborious and costly sample extraction and analysis.

Based on the results of this program, new projects may be proposed such as correlating laboratory studies to actual field conditions. Potential future studies may involve other bioremediation technologies and fate and transport studies on additional pesticides and under various conditions.

D. Not applicable.

E. Not applicable.

VI. QUALIFICATIONS :

1. Program Manager :

Deborah B. DeLuca
Hydrologist, Incident Response Program
Agronomy Services Division
Minnesota Department of Agriculture
M.S. Land Resources, University of Wisconsin, Madison, 1989.

Ms. DeLuca is a hydrologist in the Incident Response Program at the Minnesota Department of Agriculture. In this role, she provides the technical review for remedial investigations and corrective actions for agricultural chemical spills and incidents. Her areas of interest and expertise are environmental chemistry, site remediation, and regulatory policy on site remediation.

2. Major Cooperators :

A) Dr. Douglas D. Buhler
USDA/ARS and Associate Professor
Department of Agronomy and Plant Genetics
University of Minnesota

Ph.D. Agronomy (Weed Science), University of Nebraska, Lincoln, 1984.
M.S. Agronomy (Weed Science), University of Nebraska, Lincoln, 1982.

Dr. Buhler's expertise and interests are in herbicide mineralization and movement related to agricultural uses. He is the primary contact within the University of Minnesota Center for Agricultural Impacts on Water Quality specializing in field research aspects of the environmental fate of herbicides. Dr. Buhler's role will be to develop the field and laboratory components to derive soil and water samples for analysis.

B) Dr. William C. Koskinen
USDA/ARS and Associate Professor
Department of Soil Science
University of Minnesota

Ph.D. Soil Science (Chemistry), Washington State University, Pullman, 1980.
M.S. Chemistry (Physical Organic), San Diego State University, San Diego, 1974.

Dr. Koskinen's interests and expertise are in the sorption, degradation, and movement of pesticides in the environment. He is the primary contact within the University of Minnesota Center for Agricultural Impacts on Water Quality for pesticide analysis. His specialty is the development and use of analytical techniques to qualify and quantify herbicide sorption and degradation. Dr. Koskinen will have primary responsibility for developing methodologies for, and extraction and assaying of, samples.

C) Dr. Roger L. Becker
Assistant Professor
Department of Agronomy and Plant Genetics
University of Minnesota

Ph.D. Agronomy (Crop Physiology), Iowa State University, Ames, 1982.
M.S. Botany (Plant Physiology), Iowa State University, Ames, 1978.

Dr. Becker's interests and expertise are in weed control and environmental concerns associated with herbicide use. He is the primary contact within the University of Minnesota for Extension Service efforts addressing herbicide contamination of surface and ground water. His major role will be to coordinate Section B, to provide inputs on experimental design and focus, and to develop applied utilization of research results.

D) Dr. Beverly R. Durgan
Associate Professor
Department of Agronomy and Plant Genetics
University of Minnesota

Ph.D. Agronomy (Weed Science), North Dakota State University, Fargo, 1985.
M.S. Agronomy (Weed Science), North Dakota State University, Fargo, 1983.

Dr. Durgan's interest and expertise are in weed control and environmental impacts of herbicide use. She is the primary contact for extension efforts in weed control on Minnesota's agronomic crops. Dr. Durgan's role will be to provide inputs into the applied aspects of project design and implementation.

E) Dr. Deborah Allan
Assistant Professor
Soil Science Department
University of Minnesota

Ph.D. Soil Science, University of California, Riverside, 1987.
M.S. Agriculture, California Polytechnic State University, 1983.

Dr. Allan's primary interest is in root physiology and soil chemistry of the rhizosphere. She has expertise in greenhouse and growth chamber experiments and analysis of plant roots, their excreted compounds and the rhizosphere soil. Her primary role will be to coordinate and participate in the accomplishment of Objective C.

F) Dr. Lawrence Wackett
Assistant Professor
Biochemistry Department
University of Minnesota

Ph.D. Microbiology, University of Texas, Austin, 1984.
M.S. Microbiology, Louisiana State University, 1979.
Dr. Wackett's major research focus is the use of bacteria to biodegrade hazardous wastes. Expertise has been developed in understanding mechanisms of these processes and the use of that knowledge in bioremediation. He will primarily work on the microbiological aspects of Objective C.

VII. REPORTING REQUIREMENTS :

Semiannual status reports will be submitted not later than January 1, 1992, July 1, 1992, January 1, 1993 and a final status report by June 30, 1993.

1991 Research Project Abstract
FOR THE PERIOD ENDING JUNE 30, 1993
This project was supported by MN Future Resources Fund (MS 116P.13)

TITLE:	Review and Evaluation of Degradation and Bioremediation of Elevated Levels of Pesticides at Spill Sites
PROGRAM MANAGER:	Deborah DeLuca
ORGANIZATION:	Minnesota Department of Agriculture
LEGAL CITATION:	M.L. 91, Ch. 254, Art. 1, Sec. 14, Subd. 6(b)
APPROP. AMOUNT:	$300,000.

STATEMENT OF OBJECTIVES: To perform a literature review on the application of bioremediation technologies to pesticide-contaminated soils and water; to perform laboratory and field studies investigating the effects of high concentrations (characteristic of spill situations) on the persistence and transport of the pesticides atrazine and alachlor in soils; and to perform laboratory studies investigating the use of innovative bioremediation technologies using plants and microbes on pesticide-contaminated soils and waters.

OVERALL PROJECT RESULTS: The use of various bioremediation technologies on pesticide contaminated media appears promising but remains largely untested at the field scale. The literature indicates that many of the pesticide families can be biodegraded although the actual field conditions typical of agrichemical spill sites may present challenges to successful treatment. Laboratory and field studies conducted here indicate that at high concentrations typical of spill sites, alachlor degradation is severely retarded and leaching is dramatically increased relative to low, field application rates. The degradation and persistence of atrazine were shown to be independent of concentration but dependent on soil type. The effect of concentration on the environmental behavior of atrazine and alachlor has implications for the selection of appropriate bioremediation technologies for atrazine- and alachlor-contaminated soils. Lastly, microbes were isolated from contaminated soils, cultivated in the laboratory, and used under defined conditions to degrade high concentrations of atrazine in soils and water into harmless end products. The rates of atrazine degradation were very rapid. Conditions were defined for optimal use of the microbes. The bacterial cultures were added to heavily contaminated (4000 parts per million atrazine) soils from a site in MN; significant biodegradation of atrazine was observed.

PROJECT RESULTS USE AND DISSEMINATION:
Dr. Becker and Dr. Gan presented two posters at the Agronomy Society of America Annual Meeting (11/92, Minneapolis, MN). Dr. Allan and Dr. Mandelbaum made presentations at five professional conferences [Workshop on Pesticide-Soil Interaction Research (11/91, Ames, IA); American Society for Microbiology Conference on Anaerobic Dehalogenation (9/92, Athens GA); Environmental Science Workshop-The Gray Freshwater Institute (2/93, Navarre,MN); Conference on Bioremediation (4/93, Research Triangle Park, NC); and American Society For Microbiology Annual Meeting (5/93, Atlanta GA)]. Publications are being submitted to Journal of Environmental Quality and Soil Science by Drs. Gan, Becker, Koskinen, Buhler and Ms. L Jarvis. Articles have been published in Applied and Environmental Microbiology and Environmental Science and Technology by Drs. Mandelbaum, Wackett and Allan. The literature review will be distributed to the public in fall, 1993. Information from this project will be used in making remediation decisions for sites contaminated with agricultural chemicals in Minnesota.

I. TITLE : Review and Evaluation of Degradation and Bioremediation of Elevated Levels of Pesticides at Spill Sites - Agriculture 6.

Program Manager : Deborah B. DeLuca, Agronomy Services Division
Minnesota Department of Agriculture
90 West Plato Blvd.
St. Paul, Minnesota 55107
(612)297-7283 FAX: (612)297-2271

A. M.L. 91, Ch. 254, Art. 1, Sec. 14, Subd 6(b) Appropriation : $300,000.00
Minnesota Resources Review Levels of
Pesticides At Spill Sites Balance : $ 30,050.00

This appropriation is to the Commissioner of Agriculture for a literature search and publication of bioremediation technologies for pesticide spills; laboratory research on the fate of elevated levels of pesticides in soil; and evaluation of bioremediation techniques.

B. Compatible Data : During the biennium ending June 30, 1993, the data collected by projects funded under this section that have common value for natural resource planning and management must conform to information architecture as defined in guidelines and standards adopted by the Information Policy Office. In addition, the data must be provided to and integrated with the Minnesota Land Management Information Center's geographic databases with the integration costs borne by the activity receiving funding under this section.

C. Match Requirement : Not applicable.

II. NARRATIVE :

Each year incidents occur involving pesticide spills where soil must be excavated or managed. In the first nine months of 1990, approximately 120 incidents were reported to the Minnesota Department of Agriculture. In addition, many of the sites where pesticides have been mixed, loaded or handled in the past have contaminated soils due to incidental spillage. These elevated levels of pesticides in soils may result in ground water contamination. Studies indicate that pesticides detected in ground water near these spill sites often are at elevated concentrations and may exceed health advisory levels.

The purpo. this work program is to better understand pesticide t. rt and degradation processes and investigate bioremediation technologies for treatment of soil contamination. Effective soil treatment methods will aid in the prevention of ground water contamination from point source pesticide contamination. The project will include a review of the available information in conjunction with laboratory studies on fate and transport of pesticides and potential bioremediation technology.

III. OBJECTIVES :

A. Review of Existing, Developing and Applicable Technology and Information.

A1. Narrative: The purpose of Objective A of this proposal is to technically evaluate existing and developing information and technologies regarding bioremediation of contamination at pesticide spill sites. Specifically, Objective A of the proposal will: review the available literature; provide support for the research of Objectives B and C of this proposal; review the current ongoing research for applicability to spill sites; technically evaluate the feasibility and effectiveness of bioremediation for specific classes of pesticides; review state and federal regulations (MERLA, CERCLA, RCRA, TSCA, etc.) that may impact on the implementation of bioremediation technology; and produce a report summarizing the present state of the technology.

Petroleum product contamination is the focus in much of the literature regarding bioremediation technology. Less information is available on the treatment of pesticide contamination. Both literature areas will be reviewed for applicability. Past research has documented the effects of microorganism degradation and plant uptake on pesticide concentrations only at field application concentrations. Objective A will develop and evaluate existing information for practical application to current spill site situations with elevated levels of pesticides.

Bioremediation treatment technology offers a permanent solution for cleaning up soil and ground water contamination. Other common treatment methods, such as carbon filtration or air stripping of ground water contaminants, only transfer the contamination to other media. Bioremediation mineralizes the contaminants into water, carbon dioxide (aerobic degradation) and inorganic salts.

The effectiveness of bioremediation technology depends on the availability of microorganisms or plants that affect the concentrations of a particular class of contaminants. The rate at which the treatment is achieved is influenced by: the contaminant of concern; soil type; the number and type of microorganisms; environmental conditions such as oxygen availability,

temperature, pH, and moisture; and the availability of macro- and micro-nutrients necessary to encourage the growth of the microbial population.

The term "bioremediation technology" encompasses many different treatment methods for ground water and for soil contamination. Bioremediation treatment technologies may take advantage of indigenous microorganisms or plants or may use "engineered microorganisms" (microorganisms developed to degrade a particular contaminant) or plants. The treatment method selected depends on site specific conditions, such as class of contaminant, hydrogeology, extent and magnitude of the contamination, amount of land available for treatment and cost. Examples of "in-situ" bioremediation technologies (contamination remains in place) include: closed loop circulation systems for the treatment of ground water; degradation of the contaminants in the vadose zone resulting from soil venting systems; and plant uptake of pesticides in the soil. Bioremediation technologies also can be applied to the contaminated media after removal by excavation or ground water pumpout systems. Examples of "on-site" or "off-site" treatment technologies include: treatment tanks for ground water; contained areas for contaminated soil; and land application or composting of contaminated soil.

Minnesota is the first state with a program and fund that provides reimbursement to eligible parties for the remediation of pesticide spill sites. Consequently, alternative treatment techniques are needed as spill sites are remediated. Land application of contaminated soil is the treatment method selected for most pesticide spill sites. Land application has limitations (e.g., legality if the pesticide is not currently registered, need for additional studies regarding pesticide persistence or the need for large tracts of suitable land if the contaminated soils contain high concentrations of pesticides) and usually requires removal of the contaminated soil from the spill location. This proposal will help provide for the development of alternative treatment technologies.

A2. **Procedures:** The literature search will be conducted by utilizing computerized, inter-library search techniques. Degradation processes and bioremediation of pesticides will be the focus. However, other contaminants also will be screened and reviewed to examine for adaptation potential. National and international sources will be evaluated. Current and developing technologies, published and unpublished will also be reviewed.

A3. **Budget:**

a. Amount Budgeted: $ 70,000.00
b. Balance: $ 30,050.00

A4. **Timeline for Products/Tasks:**

	July 91	Jan 92	June 92	Jan 93	June 93
Initiate Literature Review	**********				
Analyze Data and Information	**********	**********	**********		
Evaluate Strategies		**********	**********	**********	
Final Report				**********	**********

A5. **Status:**

Chapter 1: Introduction and Background

1.1 Introduction

The contamination of soils and waters by pesticides at sites where agricultural chemicals are stored, handled, and distributed (*ag-chem facility sites*) has received increasing attention over the past five years (Habecker, 1989; Felsot et al, 1988; Myrick, 1990; Buzicky et al, 1992). Contamination at these sites may be the accumulated result of years of chronic, inadvertent, small spills and outdated management practices, or may be the result of catastrophic, accidental spills. In either case, the soil at these sites becomes contaminated with single or multiple residues at a range of concentrations. The elevated level of pesticides in soils at these sites may result in ground water contamination.

Increasingly, state programs are requiring the investigation and remediation of dealerships sites (AAPCO, 1992). However, remediation technologies are not broadly available for soils and waters contaminated with pesticides. Land application of pesticide contaminated soil is the treatment method most often used in Minnesota and in other states (AAPCO, 1992), but this is easily implemented only when the pesticides in the contaminated media are currently registered for use; and even then, when extremely high concentrations are involved, the required spreading acreage may be prohibitive . Consequently, alternative, cost effective remediation technologies are needed.

Selecting an appropriate remediation technology is always a difficult task. In the case of pesticide contaminated media, there is little precedent available to aid in this selection. The Minnesota Department of Agriculture (MDA) Incident Response Program is one of the first programs in the country developed specifically to address the investigation and clean-up of sites contaminated with agricultural chemicals. On a nationwide basis, few pesticide-contaminated sites have been investigated or remediated. Consequently, although there are several innovative technologies which have been broadly applied to other contaminants, most are unproven for pesticides.

Bioremediation, the use of microbial degradation in a controlled setting, has been promoted as a promising technology for pesticide contaminated media . Bioremediation is attractive because it permanently removes or alters the contaminant rather than transferring it to another media (as in placement in landfills, carbon filtration and thermal desorption) and, at the same time, the media is not greatly altered. Bioremediation has been applied to contaminants from other waste categories (petroleum products, wood preservatives, explosives). However, bioremediation has not been broadly applied to pesticides; therefore its efficacy is unknown. A complicating feature is that pesticides represent a number of chemical families which feature a range of chemical and physical characteristics thus resulting in different behavior under the same treatment scenario.

This literature review evaluates the existing information on bioremediation technologies and their applicability to contamination at ag-chem facility sites. Although this report should be useful to a broader audience, the document is written for a target audience of regulatory technical staff who must make decisions regarding the selection of remediation technologies at individual sites. Generally, these individuals have formal technical training and practical experience in geology, soils, and/or engineering but may not have a good understanding of the concepts associated with the microbiological degradation of organic chemicals.

This report is organized in the following manner: The introduction provides background information on agricultural chemical facilities, the nature of contamination at these facilities, and how such sites are investigated and addressed in Minnesota under the Incident Response Program. Chapter 2 is a primer on biodegradation processes, explaining the basic principles of the microbial degradation of contaminants. Chapter 3 explains how chemical and environmental factors affect the rate and extent of microbial degradation. Chapter 4 provides descriptions, along with supporting research, of broadly accepted and innovative bioremediation strategies, some of which appear promising for use on pesticide contaminated media. Chapter 5 provides descriptions of bioremediation technologies, such as land treatment, slurry treatment, and composting, which may employ the various strategies described in Chapter 4. Most of these technologies have not been applied to pesticide-contaminated soil and water. Whenever possible, examples of their use relative to pesticides are discussed. Chapter 6 is a discussion of treatability studies and treatability classes of pesticides. Chapter 7 includes the summary and conclusions.

1.2 Background - Agricultural Chemical Point Sources

Ground water contamination by agricultural chemicals from non-point (diffuse) sources came under scrutiny in the 1970's and early 1980's as localized studies in numerous states found evidence of ground water contamination associated with the regular use of agricultural chemicals in specific settings. This led to the development and implementation of ground water monitoring studies and programs by several states and the United States Environmental Protection Agency (USEPA). In some states, monitoring programs were accompanied by

programs aimed at the management of the non-point source pesticide problem. Although the primary goal of these programs has been the assessment and management of non-point sources, they brought attention to the possibility that point source contributions from mixing, loading and handling sites could have significant impacts on local water quality (Hallberg, 1986; Victor, 1986).

Federal law requires that pesticides be used, stored and handled in accordance with the guidance provided on the pesticide product label. Although label language prohibits off-target application of pesticide products, chronic small spills during the routine handling of bulk pesticides, as well as catastrophic accidental spills, have commonly occurred at commercial agricultural chemical dealership sites. This has resulted in contaminated site soils and, in situations where the hydrogeologic setting and spill volumes are conducive, contaminated ground water. Spill sources associated with routine handling operations include: incidental spills during mixing and loading of pesticide tank mixes for application; rinsing and washing of application equipment and spray tanks without collection of the rinsate; impregnation of fertilizers with pesticides; illegal dumping of left-over tank mix solutions; and improper disposal of containers (Habecker, 1989; Buzicky et al, 1992). Some of these spill sources involve pesticide spray mixes, which are comprised of pure formulations diluted with water for the purposes of applications. Pure (undiluted) pesticide product may also be involved in site spills. Catastrophic spills are often associated with storage and transfer of pure pesticide products from bulk tanks and thus often involve large volumes and high concentrations.

There is not a great deal of data available on the magnitude of the point source problem. A recent survey of state environmental and agricultural agencies was conducted by the State FIFRA Issues Research Evaluation Group (SFIREG) (AAPCO, 1992). The survey addressed issues associated with environmental contamination problems at agricultural chemical dealerships. Out of the 50 states surveyed, 28 responded to the questionnaire. Of these, 21 states had documented environmental degradation at commercial storage and mixing/handling facilities. Seven-hundred-seventy-four total commercial sites were reported in these states as having contaminated soil or ground water. This number probably severely underestimates the actual magnitude of the problem. Very few of these states have active programs designed to address the investigation and clean-up of agricultural chemical incidents. Consequently, it is very likely that many contaminated facilities go unreported and undocumented. To illustrate this point, of the 774 sites which were reported as having environmental contamination, 80% are located in five states (California, Illinois, Michigan, Minnesota and Wisconsin). Each of these states either has a unique program dedicated to sites contaminated with agricultural chemicals or maintains a focus on ag-chem facility sites within a more general clean-up program.

There has been very little site data collected and compiled for sites contaminated with agricultural chemicals. Habecker (1989) reported on contamination associated with 20 facility sites in Wisconsin. In total, 19 different pesticides were found in ground water and 17

pesticides were found in the soil. Contamination areas at each site were defined based upon site operations associated with each area. Mixing and loading areas, drainageways, equipment parking areas, acute spills, discarded container storage sites, burn piles, ponded depressions where run-off accumulates, impregnated fertilizer load-out and weigh scale pits were the areas with the highest concentrations. Alachlor, atrazine, cyanazine and metolachlor were the pesticides most frequently found in soil and ground water. Ground water data from ten farm chemical dealerships in Iowa have also been summarized. In total, 12 pesticides were detected ranging in concentration from less than 1 ppb to 17 ppb (Hallberg, 1986).

Agricultural chemical dealership sites feature certain unique physical characteristics which influence the nature of site contamination, and thus the use of bioremediation technologies. The following characteristics are supported by practical experience gained through the Minnesota Department of Agriculture's Incident Response Program as well as by a study of 20 Wisconsin sites (Habecker et al, 1989). Most site operations at ag-chem sites must be easily accessible to vehicles and these facilities receive a great deal of vehicular traffic. To support heavy traffic, the majority of site areas are covered with gravel fill. In most cases, the native topsoil has either been covered with fill, or the native topsoil has been removed during grading of the site and replaced with fill. The net result is that site soils are highly compacted and generally low in organic matter content.

Agricultural dealerships are often located in rural towns. Small towns in Minnesota usually rely upon ground water as a drinking water source; towns either have a public water supply and/or residents are supplied by private wells. It is common for residential properties to be located in close proximity to in-town facility sites. At several sites in Minnesota, private wells on adjacent properties have been significantly contaminated by agricultural chemical facility operations.

It is also common for dealerships to be located adjacent to railroad tracks, generally spurs off the main track, in order to facilitate transfer of product from train cars. In fact, it is not uncommon in Minnesota for dealerships to be located on land leased from a railroad company. Railroads are frequently sites of historic contamination by non-agricultural chemicals, although this has not been evident in the majority of Minnesota dealership sites that have been investigated.

Ag-chem facilities may have been in operation for several decades or a handful of years. The older sites may have handled a wide variety of pesticides over the course of their existence. In general, any single area of contamination at a site will contain not just one, but multiple, pesticide residues. Very old sites may exhibit a broader spectrum will generally represent a number of pesticide families. Additionally, concentrations of pesticides in soil profiles are frequently very high; although concentrations for a single pesticide may range from single parts-per-million (ppm) to thousands of ppm.

In summary, agricultural chemical contamination at dealership sites is common and usually involves high concentrations of multiple pesticide residues, which may or may not represent several pesticide families. Native soils at these sites are often removed and replaced by, or simply covered by, coarse fill material which tends to be low in organic matter content. The soils are further altered by compaction by heavy traffic.

1.3 Background -- The Minnesota Department of Agriculture Incident Response Program

In Minnesota, there are approximately 1,000 facilities which store, handle, and distribute agricultural chemicals as a major component of their business. There are approximately 350 permitted bulk pesticide storage facilities and about 700 facilities which deal solely with pesticides in non-bulk volumes. The Minnesota Department of Agriculture (MDA) maintains an "Agricultural Chemical Incident Response Program" to ensure that contamination incidents involving agricultural chemicals are reported, investigated and cleaned up so as to prevent unreasonable adverse environmental effects. This program was developed under the authority granted to the MDA by the Minnesota Comprehensive Ground Water Protection Act of 1989 to facilitate identified responsible parties to clean-up sites contaminated with agricultural chemicals and to implement programs to clean-up such sites. Under Minnesota statute, agricultural chemicals are defined as pesticides (including non-agricultural pesticides) and fertilizers. The Act also created the Agricultural Chemical Response and Reimbursement Account (ACRRA) and an ACRRA Board to oversee reimbursements from the account. The ACRRA account was established to reimburse responsible parties who clean-up sites identified by the MDA, provided that the site investigation and clean-up is performed with MDA oversight and approval. The ACRRA account is funded by annual surcharge fees on pesticide and fertilizer manufacturers, distributors, applicators and dealers. In addition to granting the MDA authority for the Incident Response program, the Legislature recognized that clean-ups may not proceed cooperatively and granted the MDA CERCLA (Superfund) authority in order to enable the MDA to order or compel responsible parties which are to clean-up sites.

Although any responsible party, including transporters, homeowners, and farmers, are eligible to apply to the fund, the emphasis of the Incident Response program to date has been investigation and clean-up of agricultural chemical facility sites. Manufacturers, distributors and applicators of sanitizers and disinfectants sought and received an exemption from contribution to the fund and, consequently, sites contaminated by sanitizers or disinfectants are not eligible for ACRRA funds.

The MDA Incident Response Program addresses both "Emergency Sites" and "Long Term Sites." Emergency sites are characterized by large, sudden releases associated with weather calamities, transportation accidents, and facility accidents. Emergency spills occur with greatest frequency in the spring and early summer. It is during this season that the majority of pesticide applications are made. Storage tank leaks resulting from hose breaks or valve malfunctions can release thousands of gallons of pesticide product while spray truck tip-overs

may ... hundreds of gallons of tank mix solutions. These sit... usually relatively simple to remediate if the response is immediate. The spilled product is excavated and analyzed, and since the spill almost always involves a single product, it is easily applied to a cropped field as originally intended. Over 200 emergency incidents are reported to MDA each year.

Long Term Project Sites are those where significant chemical contamination has adversely affected soil, ground water and/or drinking water. Long-Term sites often represent the net result of years or decades of chronic spills associated with routine mixing/loading operations. Emergency incidents which cause widespread or large scale environmental contamination are treated as Long-Term sites in the Minnesota Incident Response Program. The investigation and clean-up of these sites is more complex. Often, multiple areas at one facility are contaminated. More than one product is invariably involved in any single contaminated area, and often a large suite of chemicals may be present. Concentrations in soil can range from single digit part-per-million (ppm) to thousands of ppm for a single product in any single area. Minnesota has over 120 sites listed as current Long-Term sites. Fifty-one of these sites are proceeding through investigation and clean-up; approximately 27 sites have been closed or await closure. The remaining sites have been prioritized and await the availability of program staff time.

Twenty-four pesticides have been identified in ground water associated with 24 facility sites in Minnesota. Of these 24 pesticides, some have been detected frequently at numerous sites and have been detected at a large range of concentrations while other pesticides have only been identified at a single site and at low concentrations. For instance, alachlor has been identified at 19 of the 24 sites, at concentrations ranging from less than 1 ppb to 1800 ppb and atrazine has been identified at 18 sites with concentrations ranging up to 1400 ppb. At the other extreme, pendimethalin has only been detected once and at less than one ppb. The compounds most frequently found in ground water are atrazine, alachlor and metolachlor. At this time, Minnesota has not compiled the soil data for the sites that have been investigated. However, areas which are routinely contaminated at high concentrations include load areas associated with bulk storage and mixing areas (often water fill stations) associated with both bulk and small package storage. Other operation areas which are frequently contaminated include sprayer parking areas, areas where left-over spray solutions have been improperly dumped, areas where application equipment has been rinsed or washed without containment, impregnation towers, buried rinsate collection tanks, and burn piles. It is the norm for any given area to contain multiple pesticide residues. The pesticides most frequently found in soil include atrazine, alachlor, metolachlor, and trifluralin.

Under the Incident Response Program, sites are investigated in a flexible, phased approach. The first phase of the investigation is a site assessment during which an initial evaluation is made of the extent, magnitude, and potential impacts of contamination. During the second phase of the investigation, the horizontal and vertical extent of soil contamination in all contaminated areas is determined. The second phase of the investigation also includes, when

required, ... ydrogeologic investigation, which requires the installa... of monitoring wells. The hydrogeologic investigation describes the types and concentrations of contaminants in ground water and the extent and shape of the contaminated ground water plume. The hydrogeologic flow system is characterized to determine ground water flow direction and rate and to identify flow paths. An impact survey is conducted to identify potential receptors and impacts of contaminated ground water.

Following the site investigation, a Corrective Action Design for soils and/or ground water is developed and implemented, once MDA approval is granted. The Corrective Action Design describes the proposed remediation action and clean-up goals for the site. In general, soil is excavated according to the contamination extent identified by the site investigation; physical limitations of the excavation equipment must be considered. Land application is the most common, and generally the most cost effective, clean-up option for contaminated soils at agricultural chemical sites in Minnesota. Average total excavated soil volumes for a single site are in the hundreds of cubic yards range and generally do not exceed 1,500 yards. The total soil volume usually represents multiple soil piles from different contamination areas. Piles from different areas are handled separately since any one area will contain a unique suite of pesticide contaminants, and the pesticide content dictates the available land application options. In some cases, land application of pesticide contaminated media is not feasible (see chapter 5). Remediation options in these situations are limited. Remediation technologies used on soils contaminated with other types of contaminants have not been proven on pesticide contaminated media.

To date, ground water corrective actions have not been implemented under the MDA Incident Response Program, although some sites currently in the investigation phase will most likely require some type of corrective action. Ground water corrective actions are usually highly complex and expensive to install and operate. Very little data is available on the application of most accepted ground water corrective action technologies to pesticide contaminants.

Appendix 1 contains selected guidance documents developed by the MDA Incident Response Unit to guide consultants in the investigation of an ag-chem facility.

Chapter 2: Microbial Degradation Processes

2.1 Introduction

Biodegradation refers to the microbial alteration or transformation of an organic compound; the term encompasses both partial degradation (or transformation) and *mineralization* of organic molecules. The term *mineralization* refers to the complete degradation of an organic compound to CO_2, H_20, and NH_4. Bioremediation relies upon biotransformation and mineralization of organic contaminants for the removal of those contaminants from soil and water. Microorganisms degrade contaminants using the enzymes and metabolic pathways of

standard cellular function. It is necessary to have an understanding of biodegradation processes in order to assess the likely success of their application in bioremediation. The goal of this section is to describe the basic principles of biodegradation of contaminants.

At the simplest level, microbes "view" a contaminant or any organic molecule as a potential energy source and/or as a potential source of carbon (or sometimes nitrogen, phosphorus or sulfur) for the synthesis of cellular materials. Microbes in the subsurface exist in a relatively carbon-starved environment. At any given time, the majority (70-85%) of the soil population is dormant. When a potential substrate is present, those species capable of using the substrate as a nutrient source will show rapid population increase (Racke, 1990). These species will maintain a negligible base population in the absence of usable substrate. The biodegradation of a contaminant is thus driven by the biological thrust for survival, which in the microbial world means increasing the size and mass of the resident population. Whether or not degradation occurs depends in part upon whether the resident microbial species, individually or in consort, produce the necessary enzymes to transform the contaminant. Microbial species which are able to capitalize on the organic contaminant as an energy, carbon, nitrogen, phosphorus or sulfur source are provided with a selective advantage over the other species present (Karns, 1990).

2.2 Enzyme Systems

The biodegradation of all organic compounds is facilitated by enzymes which are secreted by or maintained within the microbial cell (Bouwer, 1992). Enzymes are proteins which control the rate of biochemical reactions. Enzymes tend to be highly specific in regards to the substrate on which they are capable of acting. They accelerate the rate of reaction and are not consumed in the process but are renewed for future use. Enzymes give living organisms control over when and where biochemical reactions occur. Like all proteins, an enzyme's structure is encoded for within the genetic material of the microorganism. Enzymes are either produced only in response to the presence of a recognizable substrate (*inducible* enzymes) or they are produced continuously regardless of the presence of substrate (*constitutive* enzymes).

A microbe's degradative capability is dictated by its genetic complement. In general, the genetic complement of a population evolves over time in response to its environment. Pesticides are considered to be *xenobiotic* compounds, which means that they are man-made compounds foreign to the natural environment. On an evolutionary scale, they have been present only for an instant (USEPA, 1989b). For the most part, microbes have not developed the enzymes necessary for the complete degradation of xenobiotics. Yet, microorganisms are capable of partial or complete degradation of most pesticides. The reasons why this is so can be lumped into two broad categories.

First, xenobiotics may be transformed because the substrate-binding specificity of enzymes is not exact (USEPA, 1989b). Enzymes display varying degrees of substrate specificity. A

portion of the xenobiotic molecule may be structurally similar to an enzyme's natural substrate. Consequently, the enzyme may bind the xenobiotic as an analog for the natural substrate. Very often, the structure of the xenobiotic degradation product varies sufficiently from the corresponding natural degradation product that it will not be altered by the subsequent enzymes in the degradation pathway. The xenobiotic degradation product may not be susceptible to further microbial attack and may therefore accumulate. Alternatively, the product may be sequentially degraded by several enzymes produced by different organisms within the microbial community.

Secondly, microbes have a unique ability to adapt to available substrate by "microevolution", or the relatively rapid acquisition of the necessary genetic material which encodes for the metabolic or structural systems needed to degrade the introduced substrate (Racke, 1990; Karns, 1990). The acquisition of the necessary genetic material can involve mutation of specific genes on the microbial chromosome or it can involve the transfer of mobile DNA in extra-chromosomal elements such as plasmids and transposons (Karns, 1990). This capability may, in part, explain why commonly used soil-applied pesticides often display reduced effectiveness following two or more years of consecutive treatment, a commonly observed phenomenon termed *enhanced* or *accelerated degradation* (Moorman, 1990; Karns, 1990; Racke, 1990). Several studies on a wide range of pesticide compounds (2,4-D, parathion, bromoxynil, EPTC, Butylate) have indicated that the microbial populations present in the soil developed the ability to degrade these pesticides when they were continuously applied to the soil (Karns, 1990).

If the newly acquired or mutated genes provide a selective advantage, the microbe possessing those genes will be successful in competing for the available substrate and the population of *degraders* will grow. The newly altered or acquired genes may code for enzymes with increased efficiency, enzymes with new degradative abilities or proteins involved in the transport of the contaminant molecule across the cell membrane (Racke, 1990). Alternatively, alteration of the gene may result in a change in the regulation of how often and when the gene is expressed. For instance, the gene for an inducible enzyme could be altered so that the gene was expressed constitutively (Racke, 1990). This could be beneficial in situations where the contaminant is present at low concentrations, because the gene would be expressed regardless of the concentration of the contaminant.

Plasmids may be of key importance in the transfer of unique genetic information within and between species (Racke, 1990). Plasmids are autonomous, circular DNA located outside the cellular chromosome. Plasmids can self replicate within the bacterial cell and can be passed (by "conjugation") to other bacteria. Plasmids may interact with the cellular chromosome; some plasmids can be inserted into the chromosome, and recombinant events are possible between the chromosome and the plasmid (Karns, 1990). Generally, plasmid genes encode for useful, but nonessential, functions. This allows for alteration of the unique genes without jeopardizing crucial genes on the cellular chromosome (Racke, 1990). Because plasmids can

self-[illegible]cate, several copies of a plasmid may be present in a [illegible] Since there are multiple copies of the gene present, there is increased opportunity for "translation" of the gene into the functional protein. This is known as gene amplification (Karns, 1990). Studies on several pesticides, most notably 2,4-D, have found that plasmids are associated with enhanced degradation (Karns, 1990). For instance, Skipper (1990) found that plasmids were associated with the enhanced degradation of carbamothioate herbicides, including EPTC and butylate. However, Moorman (1990) asserts that enhanced degradation has not been conclusively demonstrated to be due to the spread of plasmids between and within species as opposed to the growth of strains already containing the observed plasmid.

2.3 General Mechanisms of Biodegradation

There are two primary mechanisms by which microbes degrade organic contaminants (Bouwer, 1992; Bourquin, 1989; Norwood and Randolph, 1990; Alexander, 1981; USEPA, 1989b; Sims et al, 1989). The first is *catabolism*, or *growth-linked degradation*, whereby the contaminant serves as a substrate for microbial growth. The second is *cometabolism*, in which the contaminant serves as neither an energy or a carbon source, yet it is fortuitously transformed by enzymes produced during normal cell function of the microbe.

In most reviews on the subject of bioremediation, these are the sole microbial processes identified as contributing significantly to the degradation of a contaminant. However, Bollag and Liu (1990) identify three additional biotransformation processes of potential importance to pesticides. The first, *accumulation*, in which contaminants are sequestered or adsorbed by the microbe, has been used in the biotreatment of metal-related wastes but does not have direct relevance to the bioremediation of pesticides and thus will not be discussed further here. Although the two remaining processes may not serve to remove large quantities of pesticides from contaminated media, they are important to bioremediation because they may have significant effects on contaminant fate. *Synthesis reactions* are enzymatically regulated reactions in which the contaminant molecules are chemically linked with other contaminant molecules or naturally occurring molecules to form larger molecules or polymers. Lastly, *non-enzymatic transformation* occurs when microbial activity brings about some environmental change (such as pH, redox, etc.) which in turn causes an alteration in the contaminant structure.

The remainder of this chapter describes the processes of catabolism, cometabolism, synthesis reactions and non-enzymatic transformations.

2.3.1 Catabolism (Growth Linked Degradation)

In catabolism, the microbial population uses the contaminant as a growth substrate. The organic contaminant acts as an energy source and/or a source of elemental building blocks (usually carbon but also sometimes nitrogen, sulfur or phosphorus) for cellular materials. As

microorga[illegible]s convert an organic contaminant to inorganic produc[illegible]ey produce energy. Meanwhile the population makes use of some of the carbon (and potentially the nitrogen, phosphorus and sulfur) present in the contaminant by converting it to cellular constituents. The microbial biomass increases as energy is acquired for use in biosynthesis and as carbon is assimilated as cellular material.

Cellular growth is driven by oxidation/reduction reactions which are facilitated by cell enzymes. In these oxidation/reduction reactions, electrons are transferred in a step-wise fashion from an *electron donor* to a terminal *electron acceptor*. Organic contaminants are generally metabolized as electron donors and are oxidized to CO_2 in the process. In aerobic systems, the terminal electron acceptor is molecular oxygen. For chemotrophic microorganisms (those which derive their energy from chemical reactions rather than from sunlight) the transfer of electrons from the electron donor to the electron acceptor releases useful energy which is captured by the cell and used for growth, sustenance and motility. The electron donor, for chemotrophs, is thus considered an energy source.

Microorganisms must transform available organic substrate into forms which they can incorporate into cellular constituents (Bouwer, 1992). In general, cells use reduced forms of nutrients in biosynthesis reactions. However, the substrates available in the nutrient pool (including organic contaminants) usually contain elements in more highly oxidized forms. When a contaminant is used as a carbon source by an organism, the contaminant is first reduced to usable metabolic products which are then incorporated into the macromolecules of the cell (proteins, carbohydrates, etc.). The reduction of nutrients to usable, reduced forms requires energy and a source of electrons. As previously mentioned, many organic contaminants are metabolized as electron donors. However, organic contaminants also often supply carbon for synthesis of cellular materials. Thus, in growth linked degradation, a portion of an organic contaminant may serve as an energy source (and be converted to CO_2) while a portion of the contaminant is incorporated into biomass.

If a contaminant can be used in this fashion by a single microbial species or a group of microbes, it will be mineralized, or metabolized all the way to CO_2, H_2O and biomass. This is a desirable outcome for the purposes of bioremediation since the contaminant is completely destroyed and no questionable intermediates remain (Bollag and Liu, 1990).

In some situations, a contaminant is present at concentrations that are too low to support microbial growth, although the microbes present are capable of using the contaminant as a carbon and energy source. When this occurs, it is possible that the contaminant will not be degraded. However, the contaminant may serve as a secondary substrate while the microbial population uses another organic compound as the primary energy source. This is termed "secondary utilization" (Bouwer, 1992). Although the organic contaminant does support microbial growth, it contributes only negligibly to the energy and carbon required for growth, and a primary substrate must be supplied if degradation of the secondary substrate is to occur.

It is important to keep in mind that cellular reduction/oxidation reactions are catalyzed by enzymes. In order for a compound to be used as a substrate for growth, the microbial population must posses the enzymes necessary for the transformation of that compound. If the appropriate enzyme system is not present to degrade a contaminant, degradation will not occur. Many aspects of electron transfer and energy production are similar among organisms, but microbial species exhibit unique electron carrier intermediates and unique enzymes. This diversity allows for a wide range of abilities of microorganisms to degrade different types of contaminants (Rittmann et al, 1988).

2.3.2 Cometabolism

Cometabolism occurs when a contaminant is microbially transformed even though it serves as neither a nutrient nor energy source for the microbes. In this case, the microorganism relies upon a primary, or growth, substrate for both nutrient and energy source. The growth substrate must be supplied if the transformation of the contaminant is to occur. Essentially by chance, the enzymes produced by the microbe to degrade the growth substrate are also capable of transforming the contaminant; because of this, cometabolism is sometimes described as "fortuitous metabolism" (Bollag and Liu, 1990). Cometabolism is very important processes in the biodegradation of pesticides. While hydrocarbon degraders which are capable of using petroleum contaminants as a sole source of carbon and energy are widespread in the environment (Atlas, 1981), the same does not appear to be true of most pesticides.

In general, the enzymes responsible for cometabolic transformation of the contaminant are not substrate-specific and are often enzymes associated with the initial steps in degradation of the growth substrate (Bouwer, 1992). Cometabolism may result in only partial degradation of the contaminant since the resultant transformation products may not be vulnerable to further transformation by the enzymes catalyzing the subsequent break-down steps of the growth substrate. This means there is a possibility that one or more intermediates will accumulate if the microbial species present do not have the metabolic ability to further degrade the intermediates.

It is possible for the cometabolic product of one organism to be used as a growth substrate by another organism, or cometabolized by another microorganism present in the population (Bollag & Liu, 1990). If this is the case, a consortia or interacting community of microbial species, is needed to fully mineralize the contaminant since one species will supply the enzymes to degrade the cometabolic product of a different species. Thus, cometabolism may represent a single step in the complete mineralization process for a given contaminant.

In summary, the implications of cometabolism for bioremediation applications are that: 1) the contaminant does not support growth of the microbial population which can degrade it; 2) therefore, a primary growth substrate must be supplied if degradation of the contaminant is to occur; 3) usually a single microbe will not be capable of fully degrading the contaminant; 4)

consequently, intermediates might accumulate; and 5) the concept of consortia, or interacting communities of microbial species, is important since many different enzymes and microbes may be necessary to degrade the single product.

2.3.3 Synthesis Reactions

In synthesis reactions, including polymerization and conjugation, contaminant molecules are linked to each other or to naturally occurring compounds to form new molecules. Polymerization results in large, polymeric molecules in which the contaminant molecule, or its degradation product, is linked to other contaminant molecules or to naturally occurring complex molecules (such as humic substances). Microbially mediated conjugation results in methylated or acetylated versions of the contaminant or its degradation products (Bollag and Liu, 1990).

The significance of synthetic reactions to bioremediation is the potential role that polymerization plays in the incorporation of contaminant molecules into soil organic matter. The incorporation of contaminants into organic matter is one mechanism of *bound* (or *unextractable*) *residue* formation. Bound residues are viewed as semi-permanent removal of pesticides from the soil system although the permanence of the removal is equivocal. The formation of bound residues and their implication for bioremediation are further discussed in Chapter 3.

Polymerization of pesticides into soil organic matter occurs because of structural similarities between soil humic substances and pesticide molecules. Most pesticides contain aromatic rings and many pesticides or their transformation products contain phenol and aniline moieties. Humic substances, the base of soil organic matter, are comprised of phenolic and aromatic aniline moieties. During normal humification reactions, pesticide molecules essentially act as analogues for humic materials and as a result can become incorporated into soil organic matter (Bollag, 1991). Although polymerization can occur due to abiotic reactions, microbially mediated oxidation reactions involving phenoloxidase, laccase and peroxidase enzymes are the primary mechanism of humification (Bollag and Liu, 1990).

In the humified state, pesticides are less available for further microbial attack. However, they are also less available for plant uptake and leaching. Studies have shown that, once bound to organic matter, pesticide residues may persist in soil for long periods of time. For instance, one study showed that nine years after ^{14}C-labeled atrazine application, a sandy loam soil contained 50% of the initial radioactivity in bound form. These residues were distributed among the various humic fractions: 13% in humic acid, 33% in fulvic acid and 44% in humin (Capriel et al, 1985). The bound species included the parent compound as well its various hydroxy and dealkylated degradation products.

2.3.4 Non ymatic Transformation Caused by Microbial Activity

As discussed previously, the energy required for microbial growth is obtained from the oxidation of organic compounds which requires the reduction of a terminal electron acceptor. These coupled oxidation/reduction reactions can cause chemical changes in the near-cell environment which may be conducive to non-enzymatic, or secondary, transformation of organic contaminants (Bouwer, 1992). In these situations, the microbe does not directly alter the contaminant molecule, but microbial activity is responsible for a micro-environmental change which does encourage transformation of the contaminant molecule. Examples of environmental alterations which may occur as a result of microbial activities and which are conducive to transformation of organic contaminants include the creation of reactive intermediates, changes in pH and changes in redox potential (Bollag and Liu, 1990).

Reactive intermediates produced during normal metabolic activity can react with and alter contaminant molecules. As an example, hydrogen sulfide is produced by sulfate-reducing bacteria during respiration. Hydrogen sulfide is a nucleophile and a strong reductant. Hydrogen sulfide may thus be added to a contaminant molecule, or it may act to reduce organic contaminants present in the soil environment (Bouwer, 1992). Other reactive agents of microbial origin include amino acids, peptides, alkylating agents and organic acids (Matsumara, 1982).

The effects of redox state and pH on degradation are discussed further in the section on environmental effects on degradation. An example of biological influence on pH is biological nitrogen fixation, which results in a production of hydrogen ion, thereby decreasing pH (Paul and Clark, 1989). Changes in pH can result in the protonation or deprotonation of compounds and sorptive surfaces, affecting the structure, reactivity, solubility and adsorption properties of the affected contaminant. Additionally, pH can influence other microbiological reactions since environmental pH can select for or against a given species. As an example, nitrification, the microbially-mediated transformation of ammonia to nitrate, is highly sensitive to pH (Paul and Clark, 1989).

Microorganisms can bring about a change in the redox potential during normal cellular function (Bollag and Liu, 1990). Many reductive reactions occur non-enzymatically under anaerobic conditions (Matsumara, 1992). Redox state is important to the transformation of organic compounds (see Section 3.1.4). DDT, Methoxychlor, and heptachlor, which do not degrade significantly under aerobic conditions, have been shown to degrade under anaerobic conditions (Bollag and Liu, 1990). Smith and Willis (1977) observed toxaphene removal under anaerobic conditions but not under aerobic conditions. The reasons for the rapid degradation were not discernible, but the authors postulated that one possible explanation for the observed degradation was that microbes acted to produce a reducing environment which facilitated the reduction of a chemical species, which in turn caused the chemical degradation of toxaphene.

2.4 Summary

Microbial degradation of contaminants relies upon biochemical reactions which are mediated by enzymes. At the simplest level, a contaminant represents, to the microbial population, a potential energy source or a potential source of carbon for the synthesis of cellular materials. Whether biodegradation occurs depends in part on whether the indigenous microbial species can produce the necessary enzymes to transform the contaminant. Enzymes are proteins which display varying degrees of substrate specificity and which regulate the rate of biochemical reactions, thereby providing the living organisms control over when, where, and to what extent a given reaction may occur. The degradative capability of a microorganism is determined by its genetic make-up.

The two primary mechanisms by which microbes degrade organic contaminants are catabolism, wherein the contaminant serves as a substrate for microbial growth, and cometabolism, in which the contaminant serves as neither an energy nor a carbon source, yet it is fortuitously transformed by enzymes produced during normal microbial cell function. Two additional microbially-induced transformation processes which can affect contaminant fate are: synthesis reactions, wherein the contaminant molecules are chemically linked with other molecules to form larger molecules, and non-enzymatic contaminant transformation caused by microbial activity, in which the microorganisms bring about some environmental change (pH, redox, etc.) which causes an alteration in the contaminant structure.

Chapter 3: Environmental Factors and Chemical Characteristics Affecting Biodegradation

A number of environmental factors influence either the extent or rate of biodegradation. These characteristics are extremely site-specific and should be determined during the site investigation and prior to conducting treatability studies for bioremediation alternatives for any given site. Environmental factors which are discussed in this report include:

1. Oxygen Availability
2. Soil Moisture Content
3. Effects of pH
4. Redox Potential
5. Nutrient Supply
6. Temperature
7. Contaminant Concentration
8. Formation of Bound Residues
9. Miscellaneous Factors.

The nature of the pesticide contaminant itself also affects its biodegradation. A contaminant's chemical characteristics influence the accessibility of the contaminant to microbial attack, because how a contaminant behaves in the environment can either increase or decrease the

ease of microbial access. The chemical characteristics influencing contaminant behavior which are discussed in this report include the following:

1. Solubility
2. Sorptive Tendencies
3. Volatilization.
4. Molecular Structure

3.1 Environmental Factors Limiting Biodegradation

In order for bioremediation to succeed, the physical environment at the treatment site, or in a reactor, must allow the desired population of microbes capable of degrading the contaminant to colonize and proliferate (Johnston and Robinson, 1989). The physical environment can also influence contaminant behavior, thereby altering the accessibility of the contaminant to the degrader population. It is important to remember that the environmental factors discussed in this section do not function in isolation, but act in concert to influence the behavior of the contaminant in the environment and biodegradation rate and extent.

3.1.1 Oxygen Availability

The presence of oxygen will determine whether aerobic or anaerobic degradation processes are possible. Based solely on the thermodynamic considerations, oxygen is the preferred terminal electron acceptor because it offers the largest change in free energy per electron transfer in oxidation/reduction reactions. If oxygen is present, *aerobes*, which are microbes capable of aerobic respiration, will have a selective advantage over anaerobic strains. *Anaerobes* are microbes which can grow in the absence of oxygen and under reduced conditions. *Facultative anaerobes* are those which can grow in either aerobic or anaerobic environments. For *obligate anaerobes*, those organisms which can only grow in the absence of oxygen, molecular oxygen is toxic. Obligate anaerobes lack certain enzymes which ordinarily function to remove toxic intermediates formed during aerobic respiration (Gaudy and Gaudy, 1988).

To date, the vast majority of bioremediation applications have relied upon aerobic bacteria and technology (Stroo, 1992). In engineered slurry systems, oxygen content is controlled by aeration which is determined by process design and control. In soil systems, oxygen content is dependent primarily upon two factors: soil moisture content and consumption of oxygen by soil organisms (Sims et al, 1989). The soil moisture content is influenced by the soil pore volume. The total pore volume of a soil is shared by soil moisture and soil gases. As soil moisture content increases, the oxygenation of the soil decreases. Total pore volume in mineral soils ranges from approximately 50% to 60%. "Adequate aeration" is defined for agronomic purposes to be a minimum of 10% total soil volume. Soil moisture content at field capacity may range from 15-30% of total soil volume for a sandy loam to 40-45% for a clay soil. This same fine textured clay soil may have a total pore space of only 50%. Consequently, anaerobic

conditions can be established even under field capacity water content in similar fine textured soils (Paul and Clark, 1989).

Active aerobic organisms in the soil consume available oxygen and enrich the soil gases with CO_2. As the aerobes consume available oxygen and if oxygen diffusion to the soil pores is not adequate, an anaerobic environment is created. Because anaerobic organisms can thrive under these conditions, a succession of microbial species may become apparent (Sims et al, 1989). An oxygen concentration of 1% in soil gas is considered to represent the change from aerobic to anaerobic conditions (Paul and Clark, 1989). As a very rough guide, aerobic metabolism requires greater than 0.2 mg/l dissolved oxygen in soil solution (Sims et al, 1989).

The depletion of oxygen can be a very localized event, affecting single soil aggregates or soil crumbs. Studies have shown that the upper few centimeters of soil can contain up to ten times the concentration of anaerobic bacteria than at greater depths where anaerobic conditions would be expected (Paul and Clark, 1989). This would indicate that anaerobic *micro-sites* have developed in the plow zone which would normally be expected to be an aerobic environment. The existence of micro-sites is symptomatic of the heterogeneity of the soil environment and illustrates the importance of thorough mixing in soil systems to ensure contact between the desired microbes, oxygen and the contaminants.

3.1.2 Soil Moisture Content

For biodegradation in soil systems, the effects of moisture content are manifold. As was just described, soil moisture content influences the oxygen content of the soil, thus determining the dominance of aerobic or anaerobic organisms and degradative pathways. Water itself is necessary for microbial life, but microbes must work against the combined effects of matric and osmotic potentials to obtain water. The matric potential represents the attraction of water molecules to soil solid surfaces while the osmotic potential represents the concentration gradient created by solute concentrations in the soil water. Both of these potentials act to decrease the free energy of water, and are thus regarded as negative potentials. In unsaturated conditions, these two potentials have a significant effect upon the total water potential (Paul and Clark, 1989). The optimal water potential for microbial activity is 0.01 milliPascals (mPa). Microbial activity decreases as the potential approaches 0 mPa (soil becomes increasingly waterlogged) or as the potential becomes more negative (soil is increasingly dry). Individual species may function optimally at potentials above and below 0.01 mPa. For instance, fungi are more resistant to drier conditions than most microbial species (Gaudy and Gaudy, 1988).

Perhaps most importantly, soil water is the transport medium through which nutrients and organic contaminants move to reach the microbial cell (Sims et al, 1989). Soil moisture content affects diffusion, mass flow, and concentration of nutrients and energy sources. It is commonly believed that contaminants are most available to microbial degradation when

solu[illegible]ed in soil water (Pignatello, 1989). If moisture cont[illegible] extremely low, nutrient diffusion may be a growth limiting factor even if the nutrient concentration is sufficient (Paul and Clark, 1989).

3.1.3 Effect of pH

The pH, the negative log of the hydrogen ion concentration, is a primary factor in determining an environment's ability to support microbial populations and reactions. Every microbial species exhibits a pH optimum where growth is maximized. Although there are exceptions, some generalizations can be made regarding pH optimums of bacteria and fungi. In general, fungi are more tolerant than bacteria of acidic environments with pH values less than 5 while bacteria tend to prefer a pH range between 5 and 9 (Gaudy and Gaudy, 1988). Many bacteria will not grow at pH values below 5 (Johnston and Robinson, 1984). Microbes themselves can alter the pH of their local environment, thus creating zones of reduced or increased pH (Bollag and Liu, 1991).

Several factors affect the pH of a soil. Soil composition, vegetation, climate, management factors and regional geology will all play a part of determining prevailing soil pH (Johnston and Robinson, 1984). It may be necessary in an engineered setting to manage the pH of the system to maximize microbial activity (Sims et al, 1989). Soil pH can be made more acidic by the addition of sulfur or sulfur-containing compounds (Foth, 1984). Soil pH can be increased by the addition of calcium hydroxide, crushed limestone or other lime ($CaCO_3$) materials (Sims et al, 1986).

Winterlin et al. (1989) found that pH had a major effect on pesticide degradation half-life in studies on contaminated soil from an evaporation pit and on soil treated with six different pesticides. The effect of pH was different for anaerobic and aerobic soils. In general, high pH favored increased degradation rate for most pesticides studied under anaerobic conditions while the converse was true under aerobic conditions.

3.1.4 Redox Potential

The redox potential can be viewed as a measure of the electron density of an environment, or a measure of the tendency to donate electrons. There are two standard measures of redox potential, Pe, a unitless value, and Eh, expressed in millivolts. Pe is the negative log of the free electron concentration and is thus analogous to pH. In reducing environments, such as a flooded soil, the electron density is high, the tendency to donate electrons is strong and the Pe is low (Eh is more negative). In oxidizing environments, such as a well-aerated soil, the electron density is low, the tendency to accept electrons is strong and Pe is high (Eh is a smaller negative number).

The redox p[illegible]ntial determines which terminal electron acceptor will [illegible]referentially utilized in a given environment. Microorganisms will use the electron acceptor which, upon reduction, results in the optimal free energy release. The electron acceptors commonly used in microbial respiration (listed in order of maximum to minimum free energy released) are molecular oxygen (O_2), nitrate (NO_3-N), Mn(IV), Fe(III), sulfate (SO_4-), and CO_2. Although sulfate and nitrate are known to act as electron acceptors, their concentration in native soils is often too low to support large amounts of oxidation (Hanstveit et al, 1988). As oxygen is consumed during the oxidation of organic molecules, and if it is not replenished, anoxic conditions result and the electron density is increased (the Pe decreases). The alternative electron acceptors will be preferentially used in the sequence listed above, provided the microbial species present have the enzyme systems necessary. This can result in a succession of predominant microorganisms which are capable of using each of these species as electron acceptors (Bouwer, 1992). In bioremediation systems, it may necessary to manage the supply of the desired electron acceptor to maintain a critical biomass of the desired microbial species.

Redox potential is important to transformation of organic contaminants. For some pesticides, microbial transformation has only been observed under anaerobic conditions while others require aerobic conditions for biodegradation to occur. For example, organophosphates, carbamates and pyrethroids are preferentially metabolized under aerobic (oxidizing) conditions while DDT and toxaphene appear to more susceptible to degradation under anaerobic (reducing) conditions (Craigmill and Winterlin, 1985). Several studies have shown that heavily chlorinated contaminants which are resistant to degradation under aerobic conditions (including pesticides such as DDT and toxaphene) are degraded by reductive dehalogenation under reducing conditions (Kuhn and Suflita, 1989). Anaerobic treatment of pesticides is discussed further in Chapter 4.

3.1.5 Nutrient Supply

Although microbial growth requires that essential several nutrients are supplied, the bulk of these nutrients are rarely limiting (Gaudy and Gaudy, 1988). The chemical composition of the microbial cell drives its nutritional needs. Most microbes have similar elemental composition and therefore require approximately similar ratios of the required elements. Four elements, carbon, oxygen, nitrogen and hydrogen, comprise over 90% of the dry weight of the cell (Gaudy and Gaudy, 1988). These four elements plus phosphorus, potassium, sulfur, calcium and magnesium, are considered the major nutrients. Minor or trace nutrients include iron, manganese, boron, molybdenum, copper, zinc, chlorine, sodium, copper, vanadium and silicon (Dupont et al, 1988). Most of these nutrients are generally, but not always, present in adequate supply in soils.

Nitrogen (N) is the element which is of greatest concern in determining nutritional needs while phosphorus (P) may on occasion be the major factor limiting microbial growth (Sims et al, 1989). In situations when the contaminant at the site is very rich in carbon, such as in

hydrocarbon spills, the native amounts of N and P present may be insufficient to support continued growth of the degrader population. In such situations, N and P are limiting factors in the degradation process. In order for degradation of a contaminant to proceed satisfactorily, the concentration of the contaminant, rather than the concentration of nitrogen or phosphorus, should limit the degradation rate. A carbon to nitrogen ratio (C:N) of approximately 20/1 to 25/1 should ensure that nitrogen is not limiting (Gaudy and Gaudy, 1988). Similarly, a carbon to phosphorus ratio of 100/1 to 120/1 should ensure that phosphorus is not limiting (Dupont et al, 1988; Gaudy and Gaudy, 1988). Nitrogen and phosphorus may be added in the form of mineral fertilizers to amend the native supply of these elements in a controlled bioremediation system. The required nutrients must be supplied in a form which is usable by the degrader population; common forms of these nutrients are ammonium salts and orthophosphate or polyphosphate salts (Wilson and Brown, 1989).

3.1.6 Temperature

Temperature affects degradation rate by altering cellular growth and metabolic activity and by altering environmental physical properties. Each microbial species has a specific temperature range where its growth rate is maximized. The majority of microbes are classified as *mesophiles* which means they grow most rapidly between 20 degrees C and 45 degrees C. *Thermophiles*, which are important in composting systems, experience maximum growth rates at temperatures above 45 degrees C while *psychrophiles* grow most rapidly at temperatures below 20 degrees C (Gaudy and Gaudy, 1988). As temperatures increase above the optimum range, reaction rates slow and eventually halt due to the denaturation of enzymes and other cellular proteins. In general, as temperatures drop below a microorganism's optimal range, metabolic reaction rates and growth slow. Essentially all microbial growth is halted at temperatures below 0 degrees C (Paul and Clark, 1989). This has profound implications for Northern states such as Minnesota where the surface (top 5 cm) soil temperatures may range from a maximum of approximately 15.6 to 36.7 degrees C in the summer to a minimum of approximately -9.4 to -1.1 degrees C in the winter (NOAA, 1991).

Surface soils are subject to seasonal and diurnal (daily) temperature fluctuations. An in-situ treatment or a land farming system would be affected by these fluctuations. Below approximately 3 meters, both diurnal and seasonal fluctuations are leveled out (Foth, 1984). Factors which affect soil temperature and temperature fluctuations include degree of slope, direction of slope (aspect), shading, surface cover, and soil moisture (Paul and Clark, 1989).

Soil temperature can also affect adsorption of organics to soil surfaces. Increased temperature generally results in a decrease in sorption, thus increasing the amount of organic contaminant available to the microbial population (Sims et al, 1989).

3.1.7 Contanimant Concentration

Of all the factors which can influence the degradation of pesticides, the most consistent and prominent factor is pesticide concentration. Higher concentrations are more difficult to degrade (Schoen and Winterlin, 1987). Although high concentrations of pesticides may inhibit biological degradation, the effect appears to be chemical-specific. There also appears to be a chemical-specific threshold level, above which degradation is inhibited. In general, increasing concentration appears to slow the degradation rate although some researchers have observed instances where increased concentration stimulates microbial growth.

Young (1984) studied persistence and microbial response to high concentrations of 2,4-D and 2,4,5-T at spill sites, which tend to exhibit high pesticide concentrations, and in field plots, which exhibited relatively lower concentrations. Spill site residues (higher concentrations) showed greater persistence than aerially applied or soil incorporated residues. However, the high concentrations of pesticide present at the spill sites (29,000 ppm - 75,000 ppm) did not sterilize the soil. In fact, it was found that certain microbial species proliferated at the spill sites.

Junk et al. (1984) studied the dissipation of atrazine, alachlor, trifluralin, 2,4-D (ester and acid), parathion and carbaryl in "soil water systems" which consisted of buried 110 L garbage cans containing 60 L water and 15 kg soil. The containers were capped to minimize volatilization losses. The systems were tested with and without aeration, with and without added nutrients, at high and low concentrations (approximately 4,000 ppm and 200 ppm, respectively), as singular contaminants, and in a mixture of all six compounds all at equal concentrations (again, 4,000 ppm and 200 ppm). The authors found concentration had differing effects on the various pesticides. Atrazine did not degrade appreciably in any of the treatments. Alachlor was degraded marginally at lower concentrations but not at high concentrations. 2,4-D ester was degraded easily at both concentrations. Trifluralin was degraded at low concentrations but not at high concentrations. The presence of a mixture of compounds had a great impact. In all cases, degradation was inhibited relative to the singular contaminant treatment. The authors speculated that this was due to a prolonged latent period attributed to the extremely high total pesticide concentration rather than to toxic action on the microorganisms. None of the compounds was appreciably degraded in the high concentration mixture. Alachlor, trifluralin and atrazine were not degraded in the low concentration mixtures. It should be noted that the plastic containers did not provide satisfactory containment for the soil water system since several of them ruptured during the winter freeze thaw cycle (the experiments were conducted in Iowa).

Honeycutt et al. (1984) studied the degradation of diazinon at varying concentrations in soil by the purified enzyme parathion hydrolase. Degradation occurred very slowly (half life of 9 days) in the absence of the enzyme. In the presence of the enzyme, the degradation half-life increased with increasing diazinon concentration. The half life increased from 1 hour at 500

ppm .6 hours at 2,000 ppm. Above 2,000 ppm, the enzyme v... ...ess effective. The authors postulated that the higher concentrations inhibited the enzyme.

Gan et al. (1992a and 1992b), in work supported by the same Legislative Commission on Minnesota Resources (LCMR) grant which supports this report, investigated the relationship between concentration and biodegradation extent for alachlor and atrazine in two Minnesota soils, a sandy loam and a clay loam. In the case of alachlor, degradation extent was dependent upon concentration for both soils. Soils treatments ranged from 10 ppm to 10,000 ppm and degradation was followed for 280 days. Soil microbial activity and alachlor mineralization decreased at high concentrations. The microbial response to increasing atrazine concentrations was soil-specific. Soil treatments ranged from 5 to 5,000 ppm and degradation was followed for 280 days. In the clay loam soil, degradation and microbial activity were comparable for all concentrations. Microbial activity was stimulated at the 5,000 ppm concentration. In the sandy loam, degradation and microbial activity were depressed at high concentrations. These results indicate that atrazine degraders may be enhanced and enriched under the right conditions to degrade high (> 100 ppm) concentrations.

In laboratory experiments, Felsot et al. (1990) found that alachlor was not degraded in soil at concentrations of 1,000 ppm or 10,000 ppm while at lower concentrations (10 ppm and 100 ppm) alachlor was degraded to water soluble metabolites. Microbial populations and dehydrogenase activity were reduced in the simulated spill concentration range (1,000 to 10,000 ppm) relative to the lower concentrations. Technical grade alachlor behaved similarly to formulated concentrations, indicating that toxicity was due to high concentrations and not formulation effects. Amendment of soil with ground corn or soybean stubble enhanced alachlor degradation at 100 ppm but not 1,000 ppm. The authors hypothesized that binding mechanisms or adsorption potential may be very different at higher concentrations.

3.1.8 Formation of Bound Residues

Bound residues are pesticide residues, parent compound or phytotoxic degradation products, which cannot be extracted by the conventional methods used in pesticide residue analysis in soils (Khan, 1991b). Since bound residues are generally not detected by common analytical methods, they are not identified during routine analysis of contamination-site soils. Bound residues are problematic for the bioremediation of pesticide-contaminated soils because the nature of the bound residues is not known. The bioavailability, environmental fate, and permanence of bound residues are equivocal. The presence of bound residues could mean that the total pesticide-residue burden of site soils is underestimated by conventional analysis (Khan, 1991b). Or, the bound residues could remain essentially unavailable, thereby presenting a possible mechanism of permanent removal from the contaminated soil (Bollag, 1991). These situations affect the determination of treatment (end-point) standards for any given bioremediation project.

The existe... ...of bound residues became evident through the use of ra... ...otope-labeled (^{14}C) compounds in fate and transport studies throughout the 1970's and 1980's. These studies consistently showed that a fraction of the labeled compounds remained in the soil following extraction with non-polar and polar organic solvents. One problem with the majority of the studies is that they could not distinguish the molecular structure of the radiolabeled and bound compound. In most of these studies, the amount of bound residue formation was determined by combustion of the soil, following extraction, to produce $^{14}CO_2$. The $^{14}CO_2$ was quantitated by scintillation counting (Khan, 1991b). Whether the ^{14}C label was still part of an intact pesticide molecule, a degradation product, or a fragment could not be determined. More recent studies have used other techniques, such as pyrolysis in the absence of oxygen, high temperature distillation (HTD) and supercritical solvent extraction, which do allow for the identification of the labeled compounds (Khan, 1991b).

For the purposes of pesticide registration, the USEPA has defined a significant quantity of bound residue as being any amount of unextractable residue greater than 10% of the initially applied amount of parent compound still remaining one year after a single treatment (Helling and Krivonak, 1978). For registration purposes, bound residues are problematic because the pesticide may become available for plant uptake in future planting seasons, thereby creating an extra pesticide load and potentially killing a susceptible crop. Although this definition applies to a field use setting, it provides a reference point to assess the magnitude of bound residue contribution to the total pesticide concentration. Khan (1991b) summarized a number of recent laboratory and field studies on bound residues in different soils for a variety of herbicides. The amount of bound residues ranged from 7 to 90% of initially applied pesticide. This broad range illustrates that bound residues can constitute a large percentage of total pesticide load. The extent of bound residue formation is dependent on a myriad of factors, including soil type, organic matter content, redox potential, time elapsed since application, initial application rate and chemical structure of the pesticide; therefore, it is difficult to form conclusions regarding the tendency for bound residue formation of any single pesticide.

There are two proposed mechanisms for the formation of bound residues. The first mechanism is simply the formation of chemical bonds between pesticide residues or their degradation products and soil humic substances (humic acid, fulvic acid and humin, the three classically defined fractions of soil organic matter). Under the direct chemical binding model, the pesticide molecule is either bound to reactive sites on colloidal organic surfaces, or the pesticide molecule is incorporated into the structure of humic substances during the process of humification (Bollag, 1991). Binding may be induced by both biotic and abiotic agents. The nature of the chemical bonds may range from weak sorptive forces to irreversible covalent bonds that are more resistant to degradation (Bollag, 1991). The chemical binding mechanism is supported by several studies and is widely accepted (Khan, 1991b, Bollag, 1991). The second mechanism of bound residue formation is explained by the molecular sieve model in which a polymeric sieve-like structure is formed by hydrogen bonded moieties in humic materials. The sieve-like structure has voids and holes which essentially trap and hold intact

pesticide molecules or their degradation products (Khan, 1991b). This model is relatively recent and helps explain several experimental results which are do not fit the model of chemical bonding.

The difference between these two mechanisms is important for bioremediation. If pesticides are bound primarily by the first mechanism, it would be unlikely that the pesticides or their degradation products would be released in an unaltered form (Khan, 1991a). Additionally, it has been proposed that the first mechanism favors the binding of degradation products over parent compounds (Helling and Krivonak, 1977), which would mean that most bound residues are already partially degraded, even before release from their bound state. Under the molecular sieve model, the trapped molecule would be released intact upon disintegration of the humic polymer. Under the first mechanism, the formation of bound residues can be viewed as a means of permanent removal of the pesticide. Under the second mechanism, bound residues would provide a potential source pesticides for future release; in this case the operative question becomes at what rate are the bound residues released?

Helling and Krivonak (1977) investigated bound residue formation of six dinitroaniline compounds using thermoanalytic techniques and ^{14}C-labeled compounds. Their results indicated that stable chemical reactions between surface reactive sites on soil organic matter and aromatic amine groups of pesticide degradation products were responsible for bound residue formation. Essentially no pesticide residues were associated with the organic matter nuclei, clay interlayers or carbonate. Bollag (1991) studied bound residue formation using multi-chlorinated phenols and aromatic amines. His results indicated that chemical binding between the pesticide and humic substances is the dominant mechanism of bound residue formation, but that the pesticide residues are incorporated into the humic substance core during the humification process rather than being bound to surface sites. It is possible that different chemical structures favor binding to the surface or incorporation during humification. Either way, the results of both studies are consistent with the first mechanism of bound residue formation.

Khan (1991a) used thermoanalytic techniques to study the formation of bound prometryn residues in soil. Approximately 70% of bound prometryn residues were associated with the phenolic-OH groups and carboxyl groups of soil organic matter while approximately 20% were associated with the stable nuclei of organic matter. Both prometryn and its degradation products, hydroxypropazine and deisopropylprometryn, were present. The presence of these relatively unaltered pesticide products appeared to be inconsistent with a chemical binding mechanism (either binding to colloidal surfaces or, especially, incorporation during the humification process). Based upon these observations, Khan suggested the molecular sieve model to explain the release of unaltered parent compound upon destruction of organic matter.

The mechanism of bound residue release is not well understood, although it is believed to be primarily mediated by microbial activity (Bollag, 1991). There is strong evidence that bound

residues are persistent (Bollag, 1991). One study showed 50% of the originally applied atrazine was present in a mineral soil in the form of bound residues 9 years after application (Capriel et al, 1985). Studies on the release of ^{14}C-labeled 2,4-dichlorophenol (a derivative of 2,4-D) from humic complexes incubated for 12 weeks in the presence of various microorganisms showed release into the media of a maximum of 2.2% of the initially bound residue. The form of the released product was not characterized. At the same time, 4.8% of the initial ^{14}C was released as $^{14}CO_2$. The pattern of $^{14}CO_2$ release indicated that the source of CO_2 was a degradation product of 2,4-dichlorophenol and not the parent compound. The author felt this was evidence that bound residue formation may enhance mineralization (Bollag, 1991).

Studies with ^{14}C-labeled prometryn have shown that release of bound residues occurred in the presence, but not the absence, of microbial inoculum and that different physiological groups of microbes did not greatly affect the extent of ^{14}C release. The studies also showed that, following release, the prometryn was further degraded (Khan, 1991b).

Studies with ^{14}C-labeled atrazine investigated the release of bound atrazine by two Pseudomonas species (Khan, 1991a). Soil which had been treated with 25 ppm atrazine and incubated for one year contained 54% (13.5 ppm) of the initially applied atrazine in bound form. Atrazine accounted for 3.5 %, deethylatrazine for 2.1 %, hydroxyatrazine for 1.5 % and deisopropylatrazine for 1.1%. This soil was incubated with two Pseudomonas species known to metabolize atrazine. After 84 days, 30 - 35 % of the initially bound ^{14}C was released. The atrazine degradation products increased in concentration in the media over the length of the 84 days. The free atrazine concentration increased until day 35, then decreased. Released products were subject to further degradation. The distribution of the degradation products indicated that at least a portion of the degradation products were released in an altered form and not just further degraded upon release .

In summary, it is known that bound pesticide residues are formed in the soil environment. Two models of bound residue formation have been proposed. The first involves the formation of chemical bonds between the pesticide molecule and soil organic matter substances, induced by biotic or abiotic agents. The second involves entrapment of the pesticide molecule within a sieve structure formed by hydrogen bonds between reactive surface sites on humic substance molecules. Bound residues are not detected by common analytical techniques and pose a problem to bioremediation in determining treatment standards. Bound residues may effectively remove the pesticide molecule from the soil system and contribute to its mineralization, therefore representing a means of permanent removal. Or bound residues may represent an extra pesticide load which will act as an ongoing source of pesticide residues which is slowly released to the environment. If this latter perspective is correct, the rate at which bound residues are released to the environment is important in determining their contribution to the total pesticide load. These issues need to be addressed by both scientists the regulatory community in order to assess how treatment standards will be established.

3.1.9 Oth nvironmental Factors

The presence of compounds or conditions toxic to microbes, such as heavy-metal ions or very high salt concentrations, may act to inhibit the desired degradation. Non-target organic compounds present in the soil may act to stimulate or inhibit biodegradation. Stimulation may occur if the organic compound serves as a primary or growth substrate for cometabolism or secondary utilization. Inhibition may result from preferential degradation of the non-target compound by the degrading population or by competition for essential nutrients (Hanstveit et al, 1988).

3.2 Contaminant Characteristics Affecting Biodegradation

Chemical characteristics are important determinants of the biodegradative potential of a given contaminant. Certain chemical structural features can enhance resistance to microbial attack. Chemical properties also indirectly affect the accessibility of a contaminant to microbial attack by determining how the contaminant behaves in the environment, thereby either increasing or decreasing ease of microbial access. Lastly, the contaminant concentration can play an important role in determining degradation rate.

Soil is a complex media which is comprised of four broadly defined phases: 1) soil water; 2) soil gas; 3) organic matter; and 4) inorganic solids. The first two phases constitute the pore space of the soil; they comprise approximately 50% of the soil by volume. A contaminant may partition into any one of these phases, thereby influencing its behavior in the three remaining phases. The distribution of a contaminant among these phases will influence the potential degree of biodegradation and the tendency of abiotic loss or removal mechanisms such as volatilization. The following fate parameters define how a compound will partition between the phases, thus influencing the amount of compound available for biodegradation.

3.2.1 Solubility

A compound's aqueous solubility affects its accessibility to microbial attack. Microorganisms are only able, or at least much more able, to transform molecules which are in solution rather than in an adsorbed state (Pignatello, 1988). In general, hydrophilic molecular functional groups contain nitrogen, sulfur, oxygen or phosphorus. Hydrophobic molecular structures contain carbon, hydrogen, bromine, chlorine and iodine. The net solubility is dependent upon the summed effect and spatial orientation of the various functional groups (Sims et al, 1989).

3.2.2 Sorptive Tendencies

Adsorption or partitioning of contaminants onto mineral surfaces or organic matter complexes effectively renders a contaminant less available, or unavailable, to microbial attack. The tendency to adsorb is determined by the contaminant's chemical structure and soil chemical

and physical properties. The molecular structure of a chemical can be broken down into functional groups or chemical fragments. The chemical properties of the total molecule are, in effect, the sum of the chemical properties of the fragments, although a given fragment may be dominant in determining chemical behavior of the molecule (Dragun et al, 1988).

Dragun (1988) lists six factors which affect adsorption tendency:

1. Molecular Size
2. Hydrophobicity
3. Molecular charge
4. Hydrogen bonding of component chemical fragments
5. Three dimensional arrangement of molecular fragments
6. Coordinate bonding of molecular fragments

As molecular size increases, the tendency toward sorption increases. In general, compounds with molecular weights greater than 400 - 500 have a very strong tendency to exist in the sorbed state as Van-der Waal's forces become the dominant sorption mechanism. Hydrophobic compounds will tend to sorb or partition onto organic matter and clay/organic complexes. In general, carbon, hydrogen, and all halogen (such as chlorine, bromine, iodine) fragments will add to the hydrophobicity of a compound while nitrogen, sulfur, oxygen and phosphorus will tend to render a compound more hydrophillic (Dragun, 1988).

Functional groups which have a permanent charge or which can become charged under varying pH conditions generally tend to adsorb onto charged organic matter or mineral surfaces. Amine groups may become positively charged in low pH conditions while hydroxy and carboxyl groups may become negatively charged in high pH conditions (Dragun, 1988). Atrazine has two alkylamino side chains which take on a positive charge under low pH conditions. Consequently, atrazine tends to sorb more tightly in such a setting.

Functional groups with elements which contain lone electron pairs, such as oxygen, nitrogen and sulfur, or fragments with electron-rich pi-fractions, such as aromatics or alkenes, tend to form coordinate bonds in which the electronegative atom shares its electrons with adsorbed cations, forming weak coordinate bonds. Likewise, hydrogen bonds can form when two electronegative atoms, usually oxygen or nitrogen, are joined by the sharing of a hydrogen atom. The hydrogen atom is covalently bound to one of the electronegative atoms while it is electrostatically bound to the other. Thus, compounds with hydroxy groups, carbonyl groups, carboxyl groups or amine groups will tend to form hydrogen bonds with functional groups on mineral or organic matter surfaces (Dragun, 1988). Finally, the three-dimensional arrangement of a chemical, determined in part by electrostatic bonds between functional groups, can render a compound more or less susceptible to sorption. In general, planar molecules have a greater tendency to adsorb (Dragun, 1988).

The tendency to adsorb onto soil solid surfaces is described by partitioning coefficients such as the soil distribution coefficient (K_d) the organic carbon distribution coefficient (K_{oc}), the organic matter distribution coefficient (K_{om}) and the octanol water partition coefficient (K_{ow}). K_d describes the ratio between the concentration in the solid phase and the concentration in the soil water:

$K_d = C_s/C_w$
where C_s = pesticide concentration in soil
and C_w = pesticide concentration in water.

K_d is specific to a given contaminant and a particular soil type while Koc and Kom are normalized for soil organic carbon content and organic matter content, respectively.

$K_{oc} = K_d/oc$
and $K_{om} = K_d/om$
where oc = Soil organic carbon content (g organic carbon/g soil)
where om = Soil organic matter content (g organic matter/g soil)

K_{oc} and K_{om} are relatively constant for a given contaminant across a spectrum of soil types. Kow is the octanol-water partition coefficient and can be used as a relative indicator of the tendency to adsorb to hydrophobic surfaces when K_d, Koc and Kom are not available. For all of these coefficients, the larger the value, the stronger is the adsorbing tendency. Table 3.1 provides a guide to the practical implications of partition coefficient values; increasing mobility favors bioavailability to microbes.

Table 3.1

Relationships Between Partitioning Coefficients and Mobility (Increasing mobility favors increased bioavailability)

K_d	K_{om}	K_{oc}	Mobility Class
>10	>200	>2000	Immobile
2 - 10	60 - 200	500 - 2000	Low Mobility
0.5 - 2	20 - 60	150 - 500	Moderate Mobility
0.1 - 0.5	5 - 20	50 - 150	Mobile
< 0.1	< 5	< 50	Very Mobile

adapted from Dragun (1988)

3.2.3 Volatility

Volatilization may be a significant dissipation mechanism for certain chemicals in the soil environment. Volatile chemicals are those which, in pure state, tend to exist in gaseous form rather than in solid or liquid form. Three parameters can be used to estimate the tendency for volatilization, vapor pressure (Vp), boiling point (BP) and Henry's Law Constant (H_c). Both the boiling point (BP) and the vapor pressure (Vp) of a compound measure the strength of attraction between compound molecules. A compound which exhibits strong intermolecular attractive forces will tend to exist as a solid or liquid and will have a low vapor pressure and a high boiling point. In general, chemicals with vapor pressures greater than 10^{-2} mm Hg will tend to exist primarily in the soil air while chemicals with vapor pressures less than 10^{-7} mm Hg will be present in the soil air in only minor quantities. Chemicals with vapor pressures between these values will be present in both phases (Dragun, 1988).

The Henry's Law constant (K_H) describes the tendency of a chemical to partition between water and air and can be a useful as a gross estimate of the tendency for a chemical to exist in the vapor phase in soil. Henry's law states:

$Vp = K_H C$
where:
Vp is the vapor pressure of the chemical and
C is the aqueous concentration of the chemical.

In general, chemicals with K_H values less than 5×10^{-6} $(atm)(m^{-3})/mole$ will exist predominantly in the soil water while chemicals with K_H greater than 5×10^{-3} $(atm)(m^{-3})/mole$ will tend to exist predominantly in soil gas. A K_H between these two values indicates that a chemical is resistant to diffusion through either the aqueous or soil gas phases (Dragun, 1988).

Vapor pressure values are available for most pesticides. Henry's Law constants are generally available for compounds with high vapor pressures and large aqueous solubilities. For other chemicals, the K_H values are hard to derive in the laboratory (Dragun, 1988). When comparing literature Henry's Law Constant values, it is important to note that the units for Henry's Law Constant can take many forms since there are many options for vapor pressure and concentration units.

Several soil factors affect volatilization from soils. As temperature increases, volatilization tendency increases. Increasing aqueous solubility and increasing sorption tendency both cause a decrease in volatilization tendency (Sims et al, 1986). In general, high soil moisture content is accompanied by an increase in volatile losses (Dragun, 1988).

3.2.4 Mo ar Structure

Molecular structure is an important determinant of the biodegradability of an organic compound. Although there is no accurate mean of estimating degradation rates based upon chemical structure (Dragun, 1988), there is a fair amount of literature available which assesses the degradation of various structural families of compounds (USEPA, 1991a). Some classes of compounds have been clearly identified as biodegradable. For example, petroleum hydrocarbons, solvents such as alcohols, ketones and acetone, and simple aromatic structures such as benzene, toluene, phenol and xylene are all amenable to biodegradation. This type of information must be used with caution since compounds which had been previously identified as recalcitrant to degradation have been found, using improved experimental methods, to be biodegradable provided the necessary environmental conditions or microbial population are present (USEPA, 1991a).

Certain generalizations can be made about the effect of various molecular fragments or structural orientations on biodegradation rate. The degradability of an organic molecule is influenced by the behavior of its component fragments. The following list of generalizations can be used to assess the relative biodegradability of a series of similar organic compounds (adapted from Dragun, 1988):

1. Water soluble chemicals are generally more rapidly degraded than water insoluble chemicals.

2. Branched alkyl chains are generally more slowly degraded than straight alkyl chains. This may be the reason that the ethyl side chain of atrazine appears to be removed in preference to the isopropyl side chain (Montgomery, 1993).

3. The presence of hydroxyl, aldehyde, carboxyl, ester, and amide groups generally increases biodegradation rates (biodegradation is faster).

4. Benzene rings substituted with hydroxyl or carboxyl groups generally display faster biodegradation rates while benzene rings substituted with halogen, nitro and sulfonate groups tend to display slower biodegradation rates.

5. As the degree of halogenation increases, the rate of biodegradation decreases (becomes slower).

6. Multiple-ring aromatic structures having four or more rings generally display slow biodegradation rates.

7. N-alkanes, n-alkylaromatics, and aromatics which contain between 10 and 22 carbons are generally easily biodegradable.

8. N-a s, n-alkylaromatics, and aromatics containing betwee and 9 carbons are usually biodegradable. However, volatilization may compete as the predominant dissipation process for these compounds.

9. N-alkanes, n-alkylaromatics, and aromatics containing more than 22 carbons are generally rather insoluble in water and, resultantly, have slow microbial degradation rates.

10. Unsaturated aliphatic compounds degrade more quickly than similar saturated aliphatic compounds.

11. For straight chain aliphatic hydrocarbons, rate of degradation is dependent upon chain length, with longer chains degraded more quickly than short chains.

For pesticides, generalizations 10 and 11 rarely apply since few pesticides are aliphatic compounds. Generalizations 7 through 9 can be used as a rough guide to the effect of molecular size on biodegradation although the effects of other constituents may alter behavior. This type of information is best used to compare the relative biodegradabilities of a group of compounds having similar structures (Dragun, 1988). For example, one would generalization number 5 (increasing halogenation relates to decreased degradation rate) to compare a series of single ring aromatic compounds but would not be used to compare an aliphatic compound to an aromatic compound.

3.3 Summary of Pesticide Chemical Characteristics:

A summary of chemical characteristics for the pesticides most commonly seen at dealership sites in Minnesota is provided in Appendix 2. Appendix 1 contains selected guidance documents developed by the Incident Response Unit of the Minnesota Department of Agriculture; one of these guidance documents ("Analytical Lists For Pesticide Incident Investigations") lists the pesticides to be analyzed at sites investigated under the Minnesota Incident Response program.

Chapter 4: Bioremediation Strategies

4.1 Introduction

This chapter reviews several bioremediation strategies which can be applied in any of the technology formats discussed in the next chapter. These strategies can be thought of as bioremediation tools which can be tested and applied to suit the particular challenges presented by a given contaminated media. For example, land-treatment units or slurry reactors may be managed under anaerobic conditions to treat certain chlorinated pesticides. The strategies discussed in this report can be used in combination. As a hypothetical example, white rot

fungi may be added as an inoculant in a composting system which is the second phase of an anaerobic/aerobic sequenced treatment. When possible, examples of the use of these strategies on pesticide contaminated media are discussed.

Some of these treatment strategies are well established methods that have been applied in the field. Others are considered innovative and have not been applied in pilot or field scale projects. Because pesticides exhibit widely varying susceptibilities to biodegradation and because individual pesticides will require different environmental conditions for optimal treatment, treatability studies are crucial for both established and innovative practices. Treatability studies are discussed in Chapter 6.

4.2 Biostimulation

Biostimulation is the enhancement of natural biodegradation rates through alteration of the chemical and physical environment so that the growth of indigenous (naturally occurring) degraders is optimized. In general, biostimulation takes the form of adding nutrient amendments or electron acceptors (primarily oxygen), or changing physical environmental characteristics such as moisture content and pH. Biostimulation relies upon the native populations for degradative capacity rather than upon the addition of an adapted or selected microbial strain. Successful biostimulation requires that an indigenous microbial strain or consortia has evolved (over millennium) the necessary degradative pathways via genetic recombination or mutation (Hanstveit et al, 1988). Ideally, these degradative abilities should impart a selective advantage to the single strain or consortia, allowing the desired population to outcompete other indigenous populations until the contaminant is depleted.

Several laboratory studies have investigated the use of biostimulation on pesticide contaminated soils. In one study, soils collected from a California evaporation pit and fortified with six pesticides (atrazine, trifluralin, malathion, diazinon, chlorpyrifos, and parathion), each at a concentration of 1,000 ppm, were treated with various organic, mineral and nutrient amendments in aerobic and anaerobic environments (Winterlin et al, 1989). Degradation was generally enhanced by the addition of amendments, but certain combinations of conditions and amendments appeared to inhibit degradation of selected chemicals. Based on the results of this work, the authors recommended that, for soils contaminated with high concentrations of a mixture of pesticide residues, the most promising treatment appeared to be additions of high concentrations of lime, nutrients and an organic matter source, such as peat or manure, in an anaerobic environment. The study also illustrates that individual pesticides will require different nutritional and environmental conditions to optimize degradation.

Lab studies were conducted to determine the optimum conditions for degradation of various pesticides in soils taken from another California evaporation pit (Craigmill et al, 1990). Degradation was stimulated by additions of lime, blood meal, manure and alfalfa meal or wild rice hulls. Indigenous bacteria, when stimulated by carbon and nutrient amendments, were as

or more effective than bacteria inoculum in the form of sewage sludge or a commercial microbial mixture (Craigmill et al, 1990).

Mirsatari et al (1987) found that degradation of toxaphene in soil and sediment (at 10 and 500 ppm) was stimulated by addition of an organic amendment in flooded (anaerobic) conditions. Little or no degradation occurred in unamended soils under anaerobic conditions, and no degradation occurred in anaerobic, sterile soils. Degradation was not observed under aerobic conditions with or without amendment.

Felsot et al. (1990) tested the effect of amendment additions and inoculation on degradation in soils containing 100 and 1,000 ppm alachlor. Corn and soybean stubble were selected as amendments based upon their accessibility in Illinois, where the study was conducted. A Fusarium species which had demonstrated the ability to cometabolically degrade alachlor was used as an inoculant. Soil amendment was found to be the most important factor in enhancing the degradation of alachlor. Corn stubble, which had the lowest C:N ratio of the amendments, was the most effective for soil containing 100 ppm alachlor, followed by combined soybean stubble and ammonium nitrate. Soybean stubble alone was somewhat less effective. Soil microbial activity, measured as dehydrogenase and esterase activity, was greatly increased in corn stubble amended soils relative to unamended soils. Enhanced degradation was not observed in soils containing 1,000 ppm alachlor regardless of treatment. Based upon these results and the results of ^{14}C label fate studies which showed that alachlor was cometabolically degraded, the authors postulated that the amendments caused a proliferation of indigenous microbial species which were capable of degrading alachlor as a secondary substrate.

Later studies by Felsot and Dzantor (1991) found that soils fortified with alachlor and aged in the laboratory for 15 months did not behave the same as soils taken from a contaminated site. Lab-aged soils showed enhanced degradation of alachlor when amended with corn stubble while soil from the waste site containing similar concentrations of alachlor did not show enhanced degradation. Although the data were not presented, the authors reported that when the waste site soil was cut 10-fold with clean soil and amended, degradation was enhanced. This last treatment mimics land spreading (discussed in Chapter 5) wherein contaminated soil is applied to agricultural fields at very low application rates and incorporated into the field. This effectively dilutes the waste soil, although the dilution effect is greater than the 1:10 dilution used in this study.

4.3 Bioaugmentation

Bioaugmentation is the inoculation of contaminated media with microbial strain(s) which have a demonstrated ability to degrade the target contaminant(s) with the intent of enhancing degradation. Ideally, the selected microorganisms are capable of using the contaminant as an energy or carbon source. If inoculation is successful, the degradation rate is accelerated in comparison to the degradation rate in the non-inoculated medium. The majority of

bioa...entation studies covered in this literature review involve... laboratory scale studies and used involved relatively short incubation times. Unfortunately, there appears to be few studies which include side-by-side comparisons of inoculated and non-inoculated applications maintained over significant time frames or at the pilot- or field-scale.

Several types of inoculant sources have been proposed for the degradation of pesticides, including white rot fungus, acclimated strains, genetically engineered microorganisms, and sewage sludge. With the exception of sewage sludge, these strategies have the following in common: the inoculant has demonstrated the ability to degrade the contaminant of interest, and the inoculum represents a pure culture or consortia which has been maintained and grown in synthetic medium removed from the biotic and abiotic stresses which it may encounter in the treatment environment.

The most common source of inoculum is the isolation of microbes capable of degrading the specific contaminant from the contaminated environment using enrichment culture techniques. Once a degrader has been isolated, it can be grown in a fermenter or in the laboratory and added back to the contaminated media (Finn, 1983).

There have been successful laboratory experiments demonstrating enhanced pesticide degradation when soil was inoculated with selected cultures. Examples of full-scale projects in the literature are not as common. A pure *Pseudomonas* culture known to degrade 2,4,5-T as a sole carbon and energy source in a synthetic media was able to degrade 2,4,5-T in soil (from flower beds) at concentrations as high as 1,000 ppm in four days (Chatterjee et al, 1982). Degradation did not occur in the absence of inoculum. Soil temperature and moisture content were important parameters in determining rate of degradation. The degradation of 2,4,5-T was not affected by the presence of other carbon sources or by indigenous bacteria, although the incubation time may have been inadequate for acclimation of indigenous degraders to occur. Following depletion of the 2,4,5-T, the inoculated microbial population decreased exponentially.

The ability of a pure culture to degrade a contaminant in a synthetic media does not guarantee that inoculation of the culture into a natural soil or water will result in biodegradation of the contaminant (Goldstein et al, 1985). Microorganisms grown in pure culture are not exposed to the same ecological stresses present in the natural environment. For example, parasitism, predation and competition may contribute to the failure of an introduced organism to perform as expected. Additionally, abiotic stresses such as unfavorable pH, soil moisture and temperature may greatly affect an organism's ability to adapt to a natural environment (Goldstein et al, 1985).

It is important to recognize reasons why inoculation with a known degrader might fail in order that these constraints may be overcome. Goldstein et al. (1985) studied the inoculation of sterile and non-sterile soil and water containing varying concentrations of either 2,4-

dichlorophenol (DCP), a degradation product of 2,4-D, or p-nitrophenol (PNP). The inocula were Pseudomonas species with demonstrated abilities to degrade either DCP or PNP. The results were inconsistent. The known degrader could degrade DCP in sterile soil but not in non-sterile soil. In sterile lake water, additional nutrients and a simple carbon source were necessary for DCP degradation to occur. PNP was not degraded in sterile or non-sterile lake water but was degraded in sterile and non-sterile sewage. For both DCP and PNP, inoculation of surface soil was not effective unless the inoculum was extremely well mixed with the soil. Based upon these observations, the authors suggested a number of reasons why inoculation might fail in any given application:

1. The contaminant concentration is too low to support growth of the inoculated species.

2. The contaminated media may contain a substance which is toxic to, or inhibits growth of, the inoculated species.

3. The rate of predation by natural grazers (protozoa, for instance) may be greater than the growth rate for the inoculated species, causing decreased biomass.

4. The inoculated species may preferentially use other organics present in the environment.

5. There may be insufficient contact between the inoculum and the contaminant; for instance, there may be insufficient mixing of the inoculum with the contaminated media.

   Although the authors did not include the following point, it could act as the key constraint to successful inoculation:

6. Indigenous species may be more successful at surviving in the site environment and may outcompete the inoculant for use of the contaminant.

Inoculation may succeed in environments which do not feature these constraints. For instance, a contained reactor provides superior mixing of the contaminated media with the microbes and nutrients so that constraint # 5 is less relevant. For relatively sterile environments, constraints # 3 and #6 may not be an issue. Treatability studies using site soils are necessary to test (and possibly eliminate) the remaining constraints. Finally, as a part of the treatability studies for a project in which inoculation is being considered as a possible design element, it is necessary to compare degradation in non-inoculated media with the inoculated media. Incubation times must be long enough to allow for possible acclimation of indigenous strains. It may be that the conditions necessary to support growth of the inoculated species would be sufficient to support growth of native degraders which would be just as successful in degrading the target contaminant.

4.4 Genetically Engineered Organisms

Although the use of genetically engineered microorganisms (GEMs) has been promoted as a possible answer to the degradation of recalcitrant molecules (Karns, 1992), GEMs suffer from the same restrictions that other inocula do. Their demonstrated ability to degrade a contaminant in a laboratory setting does not guarantee their successful use in a field setting where alternative substrates may be present and where they will have to compete with indigenous strains.

Currently, there are considerable regulatory constraints to the use of GEMs in the field. Due to concerns over the environmental consequences of the release of GEMs, regulations dealing with the testing, notification and release of GEMs have been strongly debated over the past decade. The situation is explained succinctly in the following quotation (Stroo, 1992):

> "...regulations regarding the use of GEMs are in a state of flux, and have not yet been defined after almost a decade of controversy and discussion. At this point, even the use of naturally occurring organisms may require expensive and time-consuming notification and testing. Organisms used in contained systems may be subject to the same requirements as those deliberately released into the environment. The details of the notification process are not yet known, and not likely to be established in the near future. Finally, there is considerable uncertainty regarding the proprietary rights to naturally occurring organisms that are selected or modified for desirable traits. Until clear answers to such questions are available, no major commercial effort to develop and test such organisms is likely to occur."

The most likely use for GEMs would be in contained reactors where their potential for release could be carefully monitored. Due to this limited application and due to the regulatory questions surrounding their use, inoculation with GEMs is not a viable strategy for use on pesticide contaminated media in the near future.

4.5 White Rot Fungus

White rot fungi are naturally occurring organisms capable of degrading lignin. In laboratory studies, white rot fungi have been shown to transform and mineralize a broad range of xenobiotics and other recalcitrant molecules, including several pesticides. Unfortunately, it appears that very little work has been done in the field. Most work to date has been done with *Phanerochaete chrysosporium* although other white rot fungi species have similar capabilities (Bumpus and Aust, 1987).

The ability of white rot fungi to fully mineralize lignin is dependent upon a unique, non-specific lignin-degrading enzyme system. It is this enzyme system which has been shown to degrade DDT, 2,4,5-T, lindane, chlordane, and other pesticides as well as polychlorinated biphenyls (PCBs) and polynuclear aromatic hydrocarbons (PAHs). Lignin itself is a large, complex, insoluble, polymeric molecule comprised of aromatic complexes linked by ester bonds and containing many free hydroxyl and carboxyl groups. The degrading enzymes are non-specific peroxidase enzymes, commonly called ligninases, which are secreted by the fungus along with hydrogen peroxide. Ligninases and hydrogen peroxide produce oxidative free radicals which cleave bonds in the lignin polymer. The enzyme system has several unique characteristics which make it appear to be ideal for use in bioremediation of normally recalcitrant molecules. First, the ligninase system is nonspecific, it can, and does, work on a broad range of substrates. Secondly, the free radical mechanism may be able to oxidize compounds far removed from the active site of the enzyme. Since the enzymes are extracellular, large molecules can be attacked, and, as evidenced by the lignin itself, this may indicate that the contaminant does not have to be solubilized for effective degradation to take place.

Perhaps the most unique feature of the ligninase system is that it is activated by a nutrient-starved environment. Ligninase enzymes are produced in response to low concentrations of nitrogen, sulfur and/or carbohydrate. From the standpoint of bioremediation, the practical implications of this last feature are significant. Since the white rot fungus will produce the enzymes needed for degradation of the target chemical regardless of the presence of that contaminant, the microorganism does not need to be adapted to the contaminant. This means that a prolonged lag phase would not be necessary prior to initiation of degradation. Also, since the target chemical is not being used as a growth substrate, degradation of the contaminant should proceed even at low contaminant concentrations. The fungi do require the presence of sufficient carbonaceous substrate in the form of a woody plant product with a low C:N ratio such as sawdust, corncobs, or straw.

The requirement for nutrient starvation presents two potential problems with the use of white rot fungi on soils from an ag-chem facility sites. First, soil piles from these sites very often contain high concentrations of nitrogen due to historic incidental or catastrophic fertilizer spills. Since the fungus responds to nitrogen starved environment, this may mean that they will not flourish until nitrogen is consumed by other microbes. It may be possible to pre-treat such soils by flooding them and allowing denitrification to occur. Second, if treatment goals can not be reached, there will be a larger volume of contaminated media to dispose of by other means, because the fungi require the addition of considerable volumes of wood chips or other bulking agents.

Total dechlorination and mineralization of both DDT and pentachlorophenol by white rot fungi have been demonstrated in laboratory studies (Aust, 1990). Other agricultural chemicals which have been shown to be degraded extensively by P. chrysosporium include 2,4,5-T,

chlo[illegible]e and lindane. In a study by Kennedy et al. (1990) in[illegible]igating the degradation of alkyl halide pesticides with ^{14}C-labeled compounds, only chlordane and lindane were shown, by $^{14}CO_2$ release, to be mineralized to a significant extent. However, the remaining pesticides, including the normally recalcitrant aldrin and dieldrin, were transformed quite extensively as shown by disappearance of the parent compound. It seems possible that the transformation products of these compounds may be more amenable to further degradation than the parent compounds.

Table 4.1

Degradation of Alkylhalide Pesticides by White Rot Fungi, from Kennedy et al. (1990)

Compound	% Released as CO_2		% Disappearance	
	Soil	Water	Soil	Water
Mirex	4.0	2.0	15	19.8
Lindane	22.8	23.4	34.7	53.5
Aldrin	0.8	0.6	85.7	36.5
Dieldrin	0.2	0.5	28.0	15.5
Heptachlor	2.2	0.5	19.5	26.1
Chlordane	14.9	9.4	28	36.8

It appears that chlorination inhibits but does not prevent degradation. If chlorine is present, the molecule needs an additional substituent on the molecule in addition to the chlorine for degradation to occur. Thus, while hexachlorobenzene is not degraded by white rot fungus, pentachlorophenol, which is hexachlorobenzene with one chlorine replaced by a hydroxyl group, is readily degraded.

Unfortunately, little work has been performed with white rot fungus on the compounds most commonly found in Minnesota ag-chem facility sites. Aust and Bumpus (1987) report that atrazine was resistant to mineralization in ^{14}C-label studies, although the supporting study is not cited. Additionally, field applications of white rot fungus in full scale bioremediation systems have been rare. In fact, the use of white rot fungus appears to be essentially unproven at the field or pilot scale. Further investigation and refinement of this technique are needed before it is applied on a field scale.

4.6 Anaerobic Treatment

Although anaerobic treatment has not been broadly applied in the field (Suflita and Sewell, 1991; Stroo, 1992), it appears to offer a viable treatment option for halogenated aromatic and aliphatic compounds (Sims et al, 1990). These same compounds, especially the halogenated aromatics, tend toward recalcitrance in aerobic environments (Kuhn and Suflita, 1989). A

number o[illegible]aerobic processes remove halogens from aromatic an[illegible]liphatic compounds, producing dehalogenated compounds which tend to be less toxic and more susceptible to further microbial attack (Sims et al, 1990).

A majority of pesticides, both currently labeled and canceled or suspended products, contain halogens, most often chlorine or bromine. Aromatic pesticides may be halogenated on an aromatic moiety (atrazine, for example) or an aliphatic moiety (as in the case of alachlor). Of the standard analytical list of twenty compounds which the Minnesota Department of Agriculture employs in the majority of site investigations (Appendix 1, "Analytical Lists for Pesticide Incident Investigations), thirteen contain halogens. Many of the older chlorinated products, such as DDT, 2,4,5-T, and Lindane, were suspended or canceled by the USEPA precisely because their high degree of chlorination results in water insolubility which is conducive to biomagnification in food webs (Kuhn and Suflita, 1989). Often, the first step in the degradation of halogenated compounds involves the removal of the halogen(s) which is often the rate determining step in degradation of the pesticide (Kuhn and Suflita, 1989).

Anaerobic treatment potentially offers some practical benefits when compared to aerobic treatment. Soils and aquifers contaminated with complex, recalcitrant halogenated compounds often become anaerobic when oxygen is depleted by the aerobic metabolism of more easily degraded organic matter (Sims et al, 1990). One of the major costs of aerobic bioremediation is associated with the introduction and transport of oxygen, air, hydrogen peroxide or ozone into the subsurface to replace the depleted oxygen which is the predominant electron acceptor in aerobic settings (Suflita and Sewell, 1991). These costs may be lessened with anaerobic treatment because the alternative electron acceptors are generally more soluble and easier to introduce. If an exogenous source of electron acceptors is not needed, which may be possible in certain anaerobic settings, these costs would be eliminated.

Kuhn and Suflita (1989) recently published an excellent and highly detailed review of anaerobic dehalogenation of pesticides in soils and ground water. Anaerobic dehalogenation reactions were shown to transform a broad spectrum of pesticides in soil and ground water. Other good recent reviews include those by Sims et al. (1990, 1991) and Vogel et al. (1987). Table 4.2 lists and defines the anaerobic dehalogenation mechanism described in those reviews.

Table 4.2

Anaerobic Dehalogenation Mechanisms from Sims et al, 1990; Sims et al, 1991; and Vogel et al, 1987

Anaerobic Dehalogenation Mechanism	Description
Reductive Dehalogenation:	Replacement of a halogen by a hydrogen atom.
Hydrolytic Dehalogenation:	Replacement of a halogen by a hydroxyl group.
Vicinal Reduction:	(Aliphatic only). Reductive elimination of two halogens on adjacent carbons to form an alkene.
Dehydrohalogenation:	(Aliphatic only). Elimination of a hydrogen and a halogen on adjacent carbons to form an alkene.

Reductive Dehalogenation (aromatic and aliphatic compounds)

Reductive dehalogenation appears to be the primary pathway for dehalogenation of homocyclic aromatic compounds under anaerobic conditions (homocyclic compounds are those in which the aromatic ring contains only carbon). The reaction is rare in well aerated environments (Kuhn and Suflita, 1989; Sims et al, 1990). Most organic compounds are metabolized as electron donors; they are oxidized because oxidation is energetically favorable. However, due to the electronegative nature of halogen substituents, halogenated compounds are already relatively oxidized and may act as electron acceptors. Whether a halogenated compound is reduced or oxidized depends upon its structure and the prevailing environmental redox potential. The presence of halogens renders a compound more highly oxidized and makes it increasingly susceptible to reduction rather than to oxidation. The degree of oxidation, and the tendency toward reduction, increases as the number of halogens increases (Vogel et al, 1987). In general, highly halogenated compounds tend to be less persistent in anaerobic conditions than less-halogenated chemicals (Fathepure and Vogel, 1991).

The organisms which are capable of reductive dehalogenation of aromatic compounds generally require highly reduced (methanogenic) environments and long incubation times relative to those usually associated with aerobic metabolism (Stroo, 1992). Additionally, the reactions tend to be slow (Kuhn and Suflita, 1989). The need for long incubation times may be due to the induction of the necessary enzymes by the population responsible for dehalogenation (Sims et al, 1990). The need for increased incubation times should be reflected in the treatability studies for halogenated aromatic compounds. Reductive dehalogenation of aliphatic species tends to be faster than for aromatic species and the prolonged acclimation

period required for aromatic compounds does not appear to be necessary (Kuhn and Suflita, 1989). Although the reduction of haloaliphatic compounds is favored under anoxic environments, highly reducing environments are not required (Kuhn and Suflita, 1989).

The enzymes catalyzing the reductive dehalogenation of haloaromatics are generally highly specific within a chemical class while the reductive dehalogenation of haloaliphatics does not exhibit the same degree of specificity (Kuhn and Suflita, 1989). The position of ring substituents influence the ease of dehalogenation of aromatics. Haloaromatics substituted with phenols, ethers and nitrogen moieties (nitro or ammonia groups) are dehalogenated faster at ortho and para positions while carboxy-substituted compounds are dehalogenated preferentially at the meta position. Dehalogenation proceeds more quickly when ring destabilizing groups such as carboxy, hydroxy, cyano, and ether linked functional groups or nitrogen linked functional groups are present (Kuhn and Suflita, 1989).

Hydrolytic Dehalogenation

Hydrolytic dehalogenation is a substitution reaction in which the halogen is replaced by a hydroxy group. There is no transfer of electrons in hydrolytic dehalogenation. This relatively recently discovered microbial reaction appears to be the preferred mechanism for dehalogenation of heterocyclic aromatic compounds under anaerobic conditions (Kuhn and Suflita, 1989). Heterocyclic aromatics are those in which the aromatic ring contains elements other than carbon, such as the s-triazines wherein the aromatic ring is comprised of three carbon atoms and three nitrogen atoms. Kuhn and Suflita cite two separate studies which identified microorganisms capable of dechlorinating s-triazines. In both cases, aerobic microorganisms were involved, but molecular oxygen was not required and the reaction could proceed in anaerobic conditions.

Applications of Anaerobic Degradation of Pesticides

Several studies have investigated the anaerobic treatment of pesticide contaminated media from actual sites. In a series of laboratory and field studies on the biotreatment of soils from a California evaporation pit, it was found that, in general, anaerobic conditions were more effective than aerobic conditions for the degradation of endosulfan and other organochlorine pesticides (Craigmill et. al, 1990). In a study involving soil from another California evaporation pit, Winterlin et al. (1989) found that anaerobic degradation rates for six pesticides in a high concentration, mixed residue setting were increased under high pH conditions while low pH was conducive to shorter half-lives for most pesticides under aerobic conditions.

Mirsatari et al. (1987) studied the anaerobic degradation of toxaphene in soil. While degradation did not take place under aerobic conditions, regardless of amendment with organic carbon sources, toxaphene degradation was extensive in soil amended with alfalfa meal under anaerobic conditions. Anaerobic degradation was greatly enhanced by organic carbon

amendment, although the amendment type was important in determining the rate of degradation. When the toxaphene concentration was increased from 10 ppm to 500 ppm, the rate of degradation was slowed. No degradation took place under aerobic conditions at high concentrations.

DDT was found to degrade to a greater extent under anaerobic conditions as compared to aerobic conditions in a treatability study conducted on soils from a former pesticide manufacturing site (Thorn et al, 1991). The treatability study used a longer incubation period for aerobic treatment (26 weeks) than for anaerobic treatment (18 weeks). In spite of this, DDT was reduced by 48% under anaerobic conditions and 15% under aerobic conditions from an initial concentration of 94 ppm.

Two reviews cited anaerobic treatment under denitrifying conditions as a promising innovative bioremediation practice (Stroo, 1992; Suflita and Sewell, 1991). In this case, nitrate acts as the electron acceptor and is converted into N_2 or N_2O. Nitrate offers a practical advantage because it is very soluble and easy to introduce in an in-situ soil or aquifer setting. However, the remaining literature reviewed for this report did not provide evidence of the usefulness of this practice for the remediation of pesticide contaminated media. In fact, one study investigating the degradation of methoxychlor (1000 ppm) under aerobic, denitrifying and anaerobic conditions (undefined redox potential) showed that degradation was more extensive (73% as compared to 40% removal) under anaerobic when compared to denitrifying conditions. Methoxychlor was not degraded in aerobic conditions (Fogel et al, 1982).

4.7 Sequential Anaerobic/Aerobic Treatment

Anaerobic degradation pathways for halogenated compounds most often result in completely or partially dehalogenated and/or partially degraded metabolites; complete degradation is not as common. Usually, these degradation products are more susceptible to aerobic microbial attack than the corresponding parent compound (Fathepure and Vogel, 1991;). Consequently, sequential anaerobic/aerobic treatment may result in a more extensive degradation of chlorinated compounds which are recalcitrant to aerobic degradation. This approach makes sense from a practical standpoint for soils and ground water contaminated with a mixture of pesticides. In these instances, chlorinated pesticides which are resistant to aerobic attack but can be partially degraded under anaerobic conditions are often present in mixtures with pesticides which can be partially transformed or fully mineralized under aerobic conditions. Such mixtures would seen to be likely candidates for sequential anaerobic/aerobic treatment because the different pesticides would be preferentially treated under the different redox conditions.

Fogel et al. (1982) studied the degradation of methoxychlor under sequenced anaerobic/aerobic treatment and compared this strategy to isolated aerobic and anaerobic treatments. Radiolabeled methoxychlor was used in the study. Little or no evolution of $^{14}CO_2$ ocurred

under aerobic or anaerobic conditions over a three month period. Analysis of the soil extracts indicated that primary degradation of methoxychlor ocurred under anaerobic conditions but not under aerobic conditions. When soils which had been incubated anaerobically were exposed to aerobic conditions, $^{14}CO_2$ evolution was as much as 70-fold greater than for methoxychlor exposed solely to aerobic conditions. The authors projected that anaerobic/aerobic sequenced conditions could result in more rapid degradation of persistent chemicals than would be expected under a single environmental condition.

Winterlin et al. (1989) studied pesticide degradation rates in contaminated soils taken from a California evaporation pit and in clean soils treated with six pesticides (trifluralin, atrazine, chlorpyrifos, malathion, parathion, and diazinon) at concentrations of 1000 ppm each. These soils were placed in trays and subjected to various treatment conditions and amendment additions to determine the optimum conditions for degrading high concentration pesticide mixtures. Although the effect of various amendments and treatments were pesticide specific, the authors concluded that the most effective overall treatment strategy was alternation between moist (aerobic) and flooded (anaerobic) conditions (sequence not specified) with an added organic source, such as manure, and treatment with lime.

Thorn et al. (1991) performed a treatability study on toxaphene and DDT in soils from a former pesticide manufacturing site under three treatment scenarios: aerobic, anaerobic and sequenced anaerobic/aerobic. The authors postulated that the dechlorination of DDT and toxaphene via anaerobic treatment would increase pesticide susceptibility to ring cleavage under aerobic conditions, thus enhancing total degradation. The sequenced treatment consisted of eight weeks anaerobic incubation with a methanogenic culture followed by eighteen weeks of aerobic treatment which included inoculation with bacterial and white rot fungus cultures. Under anaerobic, aerobic and sequenced treatment, low DDT concentrations (19 ppm) were reduced 62%, 54%, and 82%, respectively. Moderate DDT concentrations (94 ppm) were reduced 48%, 15%, and 62%, respectively. The greatest losses in the sequenced treatment occurred during the initial anaerobic treatment. No significant reductions of toxaphene were observed in any of the treatments. Significant reductions in toxaphene leachability, 21 to 77 percent, were observed following sequenced treatment, based upon the Toxicity Characteristics Leaching Procedures (TCLP) test.

Based upon the treatability testing, the authors recommend a system consisting of a lined impoundment which could be flooded to create anaerobic conditions. As a first estimate, the authors recommend a 4 month anaerobic treatment period. Following removal of the flood water, which treatability tests indicate would be free of pesticide residues (data not provided), appropriate amendments and microbial cultures would be added to the soil. The soil would then be composted or simply tilled and monitored for the aerobic phase (Thorn et al, 1991).

4.8 UV-Irradiation and Ozone Pretreatment Followed by Microbial Treatment

Chemical pre-treatment using ultraviolet (UV) irradiation or oxidizing agents (ozone and/or hydrogen peroxide) has been proposed as a first phase in the biotreatment of pesticides in contaminated waters and soils (Kearney et. al, 1984; Kearney et al, 1990; Hapeman-Somich, 1992). The concept behind this strategy is that the products of photolysis or ozonation are more highly oxidized and more susceptible to further biological degradation than the parent pesticide compounds (Hapeman-Somich, 1991). The initial oxidative transformations, which, for many pesticides, represent the rate limiting step in the degradative pathway, are performed by the UV light or the oxidative agent. Because the photolysis and ozonation reactions are non-specific and the reaction rates are independent of contaminant concentration, chemical oxidative technologies would appear to offer certain advantages over biological treatment (Matsumara and Katayama, 1991).

Photolysis requires the use of a high energy lamp with a wavelength less than 254 nm. The emission spectrum of the lamp must include the absorption spectrum of the target pesticides (Hapeman-Somich, 1992). Ozonation relies upon a free radical mechanism for the transformation of organic chemicals. Oxidation is performed by the hydroxy radical generated during the decomposition of ozone; generation of the hydroxy radical can be enhanced by addition of hydrogen peroxide (Hapeman-Somich, 1992). In general, ozonation is effective with triazines, acetanilides, and phenoxyalkyl acids while organochlorines are resistant to oxidation by ozonation (Hapeman-Somich, 1992).

The development of this technology has focused primarily upon the disposal of pesticide rinsates generated from agricultural-chemical storage and handling facilities. A small scale reactor has been patented for this purpose. The reactor uses an ozonation pre-treatment followed by circulation of the rinsate through a soil column which has been augmented with an Pseudomonas species selected for atrazine degradation (Kearney et al, 1990). This reactor has been shown to degrade up to 90% of an initial rinsate concentration of 480 ppm atrazine in four days (Kearney et al, 1990). Although both irradiation and ozonation were considered in the development stages of the reactor, ozone was selected for the final design when it was determined that photolysis was ineffective on rinsates because their opaque nature does not allow for light penetration (Hapeman-Somich, 1992). The reactor design is still evolving based upon experience gained in testing it on field-generated rinsates (Hapeman-Somich, 1992).

Other studies investigated the use of both ozone and UV irradiation in similar reactor formats. It was found that UV irradiation produced different degradation products than ozonation for four pesticides tested (atrazine, alachlor, metolachlor and paraquat). In general, ozonation did not involve chlorine removal but did cleave double bonds and aromatic rings and removed or oxidized alkyl groups while photolysis tended to remove chlorine and alkyl groups but did not cleave the aromatic ring (Somich et al, 1988; Hapeman-Somich, 1991). Pesticides (atrazine, cyanazine and metolachlor) in a facility-generated rinsate were degraded by 45-90% from initial concentrations ranging from 17 to 82 ppm (Somich et al, 1988). Both irradiation and ozonation pre-treatment were shown to greatly enhance the rate and extent of degradation in laboratory and field studies (Somich et al, 1988; Somich et al, 1990, Hapeman-Somich, 1991).

The application of this technology to pesticide-contaminated soil appears to be limited. UV-irradiation is limited in effectiveness to those situations where light can penetrate the contaminated matrix; this automatically eliminates application to soils and to water containing sediment, humic substances or other organic matter. Ozone and other strong oxidants may be needlessly spent oxidizing non-target compounds such as pesticide inerts and soil organic matter. For these reasons, the usefulness of this strategy, in a practical sense, is limited to waters.

Chapter 5: Existing Bioremediation Technologies

5.1 Introduction

Bioremediation technologies range from highly engineered processes to relatively simple technologies. All of the technologies rely upon the processes of biodegradation discussed in Chapter 2, and most are flexible in their ability to utilize the different treatment strategies presented in Chapter 4. In general, bioremediation technologies can be divided into four broad categories (Bourquin, 1989): a) solid phase treatment, b) slurry phase treatment and/or treatment in reactors, c) in-situ treatment and d) the use of a, b, or c as one part of a treatment chain. The first three of these categories are described in this chapter. Where available, examples of their application on pesticide-contaminated media are provided.

Land application is treated in some detail in this review for three reasons. First, it is broadly used throughout the country. Second, as defined in this report, it is unique to pesticide contaminated media because contaminated soil is assumed to provide a benefit (pesticidal control) to the cropland at the application site. Also, due to its low cost per volume, it is the preferred technology when the contamination characteristics allow it to be utilized. The more highly engineered technologies can not compete with the cost of land application and, under the current laws in Minnesota, the more sophisticated technologies for soil treatment will probably be applied only in those situations where land application is not possible.

5.2 Solid Phase Technologies

In solid phase technologies, contaminated soil is excavated and treated "ex-situ" by land farming or one of its variants, in a prepared bed or in a compost-like setting (Bourquin, 1989). Biodegradation rates are enhanced by manipulating the physical and chemical characteristics of the soil matrix. This is done via the addition of nutrients, water, final electron acceptors (generally O_2), and bulking agents, by adjusting pH, and by providing regular tilling to ensure

adequate mixing and contact between the microbes and the contaminants. Most applications rely upon the stimulation of native microbial populations (*biostimulation*) although selected microbial strains may be used for inoculation (*bioaugmentation*) in some situations.

5.2.1 Land Treatment, Land Application, Land Farming and Landspreading

The terms *land treatment*, *landspreading*, *land farming*, and *land application* all refer to the application and/or incorporation of waste into the plow layer or upper portion of the soil profile with the intent that natural processes in the soil will cause degradation of the contaminants present. However these terms, as used in this review, denote different rates of application and varying degrees of engineering complexity. Under the Resource Conservation and Recovery Act (RCRA), *land treatment* is defined as "a hazardous waste treatment program that is designed to ensure that hazardous constituents placed in or on the treatment zone are degraded, transformed or immobilized within the treatment zone," (1988 40 CFR 264.271) while the treatment zone is defined as " a soil area of the unsaturated zone" (1988 40 CFR 260.10 Part B). *Land farming* (or *landspreading*), is an established treatment technology for the disposal of municipal waste water and sludges and has been defined as the "...spreading of waste contaminated-soil on agricultural or non-cropped land to stimulate degradation, transformation and/or immobilization of contaminants" (Felsot et al, 1992). In order to avoid confusion in this review, "land treatment" will be used as a broad category which includes land farming, land application, and prepared bed treatment. Landspreading and land farming are considered synonyms for the purposes of this review.

Land application, as the term is used in this review, is the application of pesticide contaminated soil or water to cropland in accordance with the pesticide product label with the intent of providing pesticidal control to the cropland. Land application of certain pesticides provides a potential beneficial use of the pesticides contained within the contaminated media. The label application rate is of key importance to land application. The application rate is expressed in pounds of pesticide active ingredient per acre and is specific for crop and soil type. The pesticide label is a legal document; compliance with the label application rate and all other application restrictions on the label is required by law.

Land application can be considered a variant of land farming unique to pesticide contaminated media. In land farming, the contaminated media is applied with the intent of waste disposal and degradation. Land application also places the contaminated media within the plow layer where it is expected that degradation and transformation of contaminants will be stimulated. The distinguishing feature of land application is that is that the applied soil or water provides, for certain pesticides, a potential pesticidal benefit to the application site. The contaminated media, soil or water, is considered the diluent for the pesticide product. Another distinguishing feature of land application of pesticide contaminated soil is that pesticides are intended to be applied to cropland and undergo an extensive registration process in order to ensure that their use, at label rates, does not cause unreasonable adverse effects to the

environment. In contrast, land farming introduces to the soil wastes which were not intended to be placed in the soil biosphere. The excavation of pesticide-contaminated soil from a facility or spill site with subsequent application of the soil to cropland is, in effect, the removal of the pesticide product from an unlabeled site and the transfer of the product to a labeled site.

Land application may not technically meet the definition of bioremediation. Relative to other bioremediation techniques, there is less control of operating parameters. It is included in this review, however, due to the ubiquity of its use and because its low cost makes it the most attractive remediation option in all cases where it can be applied.

A recent survey of state regulatory programs addressing the investigation and clean-up of sites contaminated with agricultural-chemicals included a question about what types of remedial technologies had been utilized for soil from such sites (AAPCO, 1992). Out of the 50 states surveyed, 28 responded to the questionnaire. Seventeen states responded that land application of pesticide contaminated media had been implemented in their state as a remedial action. Whether the label rate was considered in land application practices was not discussed in the report. Eleven states responded that bioremediation technologies had been used on pesticide contaminated media in their state. Nine of the bioremediation respondents were contacted by telephone by the Minnesota Department of Agriculture in an informal follow-up to the AAPCO survey. The only states where some type of engineered bioremediation technology had been implemented under the authority of the state were California and North Dakota. Of the seven remaining states, three practiced land application of pesticide contaminated soil on either a routine or occasional basis and considered this a bioremediation technology.

Land application is routinely practiced in Minnesota. Appendix 1 includes the guidance documents and submittal forms used by the Minnesota Department of Agriculture Incident Response Unit for land application proposals ("Proposal to Land Apply Soil" and "Instructions For Proposal to Land Apply Soil From Agricultural Chemical Incidents"). The MDA has recognized several situations where land application may not be feasible:

A. <u>The waste soil contains a mixture of contaminants which are not all labeled for the same crop (*incompatible products*)</u>.

   To apply this soil to the labeled crop for one pesticide would involve application of at least one pesticide which is not labeled for the crop. Crop damage may ensue if the incompatible pesticide is applied above a rate which is phytotoxic to the crop. Also of concern is the possibility of bioaccumulation of non-labeled residues in the food crop.

   In states like Minnesota, where one or two major crops are grown in a given region, contaminated facility soils frequently contain pesticides which are all compatible with a single crop. When there are incompatible products present, the off-label pesticides are often present at very low concentrations so that when excavated soil is spread according to

the concentration of the limiting pesticide (generally, but not always, the pesticide present at highest concentration), the incompatible product is applied at very low rates. In states where a large variety of crops are grown in the region serviced by a single agricultural chemical facility, contaminated soils may contain highly complex mixtures of pesticides which can not be applied to a single crop.

In Minnesota, two benchmarks are considered in determining whether a proposed application rate for an incompatible product is sufficiently low to be approved. The proposed rate must be less than 1/2 of the rate below which no phytotoxic effects will occur to the crop ("the phytotoxic rate") and/or less than 1/2 of the rate below which no residue accumulation will occur in the food crop (the "residue accumulation rate"). Minnesota has compiled a list of phytotoxic rates and residue accumulation rates from the registrants of the products most commonly seen at Minnesota sites. This information is treated as proprietary information and cannot be released. If there is no residue accumulation rate available for a selected pesticide and crop, which is often the case, then 1/10 of the phytotoxic rate, instead of 1/2 the phytotoxic rate, is used to determine whether a proposed application rate can be approved.

B. The products in the pile are all registered for the same product, but the concentrations are so high that it is not physically possible to apply the soil at a low enough rate.

This is best explained by example. An excavated soil pile from one site in Minnesota contained very high concentrations of alachlor, metolachlor and trifluralin (approximately 12,900 ppm, 115 ppm and 3,230 ppm, respectively) and had a volume of 250 cubic yards. If applied at a rate of 0.5 lbs/acre for trifluralin and 2 lbs/acre for alachlor (based upon the label for Cannon, the product which was spilled), the pile would require 3,840 acres of soybean land. Given the 250 cubic yard volume, this would be equivalent to less than 1/20 of a cubic yard per acre. The practical limitation of the spreading equipment is approximately 1 cubic yard per acre. There are other practical limitations for a soil containing very high pesticide concentrations. In the Minnesota case discussed here, even if the pile was mixed with a large volume of clean soil so that it could be applied to the required acreage, the amount of pesticide product present would still require 3840 acres. It would be extremely difficult or impossible for a facility to locate landowners with sufficient acreage willing to accept the soil.

C. The waste soil contains a pesticide product which is no longer registered for use or which was suspended or canceled by the USEPA for any previously labeled use.

To apply this soil would no longer be considered a legal application of the pesticide unless there are provisions for use of the product in the chain-of-trade in the USEPA cancellation notice.

D. The land application of the pesticide residue which is a listed hazardous waste is out of compliance with RCRA rules.

Several suspended and/or canceled pesticides are listed hazardous wastes under the Resource Conservation and Recovery Act (RCRA). Examples include 2,4,5-T, 2,4,5-TP, DDT, lindane, endrin, heptachlor, and chlordane. Some currently registered products are also listed hazardous wastes, including ethylene dibromide (EDB) and 2,4-D. However, under FIFRA, any currently labeled pesticide can be applied to a labeled crop, providing that the label directions are observed. One possible approach to dealing with the presence of trace levels of these contaminants is to define negligible or de-minimis concentrations. This would require the consent of the appropriate State RCRA authority.

5.2.1.1 Supporting Research for Land Application and Land Farming of Pesticide Contaminated Soil

Very little research has been conducted on land application or land farming in spite of its broad implementation. Several studies on landspreading of pesticide contaminated soils have been conducted in Illinois by Dr. Allan Felsot and various collaborators. These studies have investigated the effects of application rate on residue persistence, phytotoxicity to crops, weed control and shallow ground water quality. Some of the studies have looked at biostimulation effects of different organic nutrient amendments and the effects of inoculation with known degraders. This work is important both because of the paucity of research in the area and also because of the practical nature of Felsot's work.

The landspreading of pesticide contaminated soil from an Illinois facility was investigated (Felsot et al, 1988; Felsot and Dzantor, 1991; Felsot et al, 1990). Alachlor was the pesticide present in highest concentrations followed by atrazine, metolachlor and trifluralin (56.6 ppm, 31.1 ppm, 9.6 ppm and 1.5 ppm, respectively) (Felsot et al, 1988). This soil was applied at three different rates to corn and soybean plots and compared with plots which received freshly sprayed applications of the same pesticides at the same rates. The application rate was based upon alachlor due to its prevalence. Applications were made at 1x, 2.5x and 5x the label rate for alachlor which correspond to 3.0, 7.5 and 15 pounds of alachlor per acre, respectively. The contaminated soil was applied to the fields with a manure spreader. Soil applications were made prior to planting and the soil was disked twice in two directions to ensure incorporation. Table 5.1 indicates the effective application rates for the other three pesticides present. There were no freshly sprayed 2.5x plots. Residues were monitored for two growing seasons in soil, shallow ground water, and harvested grain. Phytotoxic effects to crops and weeds were also tracked.

Tab. 1

Effective Application Rates from Felsot et al. (1988)

TREATMENT	APPLICATION RATE (pounds per acre)			
	Alachlor	Atrazine	Metolachlor	Trifluralin
1x alachlor label rate	3	1.65	0.51	0.08
2.5x alachlor label rate	7.5	4.12	1.28	0.20
5x alachlor label rate	15	8.24	2.55	0.40

It is important to note that, by the definitions used in this review, only the 1x application would be considered land application. The 2.5x and 5x treatments would be considered land farming. The results should be interpreted in this context. During the course of the studies, there were practical difficulties in achieving the target application rates and in obtaining representative measurements of residue concentrations at the application sites. The application difficulties would be expected due to the heterogeneous nature of pesticide concentrations throughout a single soil pile and to the difficulties in achieving an even application rate with the spreading equipment.

Persistence

Alachlor and metolachlor residue levels in the 1x and 2.5x soil applied plots did not differ significantly from residue levels in the 1x freshly sprayed plots during the first or second growing seasons. The concentrations of alachlor and metolachlor in the 5x soil-applied plots were significantly greater than concentrations in the freshly sprayed 5x plots after two years. After two years, atrazine concentrations did not differ significantly for any of the treatments between soil-applied or freshly sprayed plots. Trifluralin residue levels were low after two years in all treatments, however concentrations in soil-applied plots were significantly greater than in freshly applied plots.

Pesticide concentrations in the undisturbed soil piles were also followed. Alachlor showed some degradation during the first year following excavation, but stabilized at about 60% of the initial concentration. Metolachlor and trifluralin showed essentially no dissipation. Atrazine did degrade significantly (from approximately 41 to 3 ppm).

Greenhouse and laboratory studies were conducted to determine the cause of the apparent recalcitrance of alachlor and metolachlor. It was found that high alachlor concentrations (1,000 and 10,000 ppm) depressed both microbial (bacterial and fungal) biomass and microbial activity as measured by enzymatic activity. Concentrations of 10 and 100 ppm did not appear to have a toxic effect as measured by enzyme activity and biomass. Pesticide persistence

seemed to be prolonged in simulated spill experiments which compared alachlor added alone to alachlor added in combination with the pesticides seen in the Illinois facility soil. High concentration mixtures (10,000 ppm of each species) were highly resistant to degradation while low concentration mixtures (10 ppm each species) degraded by 80% over the course of one year.

The effect of aged residues on degradation rate was explored as a second possible mechanism of pesticide persistence. Degradation rates of aged alachlor residues were compared to degradation rates of freshly applied alachlor. Both aged and fresh residues were present at an initial concentration of 100 ppm. The aged residues degraded faster than freshly sprayed residues.

Crop Phytotoxicity

Although the authors conclude that crop phytotoxicity is a potential problem (Felsot et al, 1990), only the field data for the higher application rates support this conclusion. Soybean crop injury was evidenced at the 2.5x and 5x rates for soil-applied treatments, but damage was much greater for the freshly sprayed 5x treatment. Soybean yield was not affected by any of the soil-applied treatments while the freshly sprayed 5x treatment resulted in a total loss of soybean yield in the first growing season following application. The crop injury and yield losses were suspected to be due to atrazine, which is not labeled for soybeans. There were no injury or yield effects in the second year for any of the soybean treatments. By the second year, atrazine concentrations had decreased in the 5x plots so that they are equivalent to the 1x plots. For corn, neither injury nor yield effects were evident in the soil-applied treatments. This is not surprising since trifluralin is the only pesticide present which is not labeled for corn in a pre-plant application and it was applied at rates which may have been below the rate which would be phytotoxic to corn.

Weed Control

The weed control was excellent in all treatments, soil-applied and freshly sprayed, during the first growing season. The 5x soil-applied treatment showed greater weed control than the 5x freshly sprayed treatment during the second year. This may be attributable to the apparent increased persistence of alachlor and metolachlor in the high rate soil-applied applications, as was demonstrated by the persistence data.

Ground Water Quality and Food Residue Results

There was no difference in shallow ground water quality between the freshly sprayed and soil applied plots. The low residue concentrations found in the harvested soybeans and corn were not significantly different between the land applied plots and the freshly sprayed plots and were all below federally set tolerances.

Stimulation and Augmentation

Since prolonged persistence had been identified as a possible problem for soil-applied alachlor and metolachlor, various routes of increasing degradation rates were explored. Inoculation with known degraders and amendment with corn stubble, soybean stubble, or soybean stubble plus nitrogen were investigated. The most significant effect was observed with amendment of the 100 ppm alachlor-containing soil with corn stubble. All of the amendments enhanced degradation, independent of C:N ratio. Inoculation with a Fusarium sp. that was able to degrade 75% of 100 ppm alachlor in a liquid medium did not enhance alachlor degradation.

Felsot et al, 1990 summarized their results by stating that the following factors may be problematic in land farming of pesticide contaminated soils:

1) the possible crop phytotoxicity and inability to generalize the feasibility of landspreading to specific sites;
2) lack of degradation of pesticide in waste soil when concentrations are very high; and
3) slower than expected degradation of pesticide in land applied contaminated soil.

These problems are only supported by the data for application rates greater than the label rate. When the soil was applied at the label rate for alachlor, there was no crop damage or yield loss. Concentrations of all pesticides but trifluralin remaining in the soil applied plots were not significantly different than concentrations remaining in the freshly sprayed plots; trifluralin levels were too low to be able to make solid conclusions. The lack of soybean crop damage is interesting because atrazine is toxic to soybeans in a pre-plant application, and atrazine was applied at a rate of 1.65 pounds per acre. Given that increased persistence may be problematic for alachlor, metolachlor and trifluralin at high application rates, it may be prudent to apply at levels beneath the label rate. In fact, Felsot suggests that persistence problems may be overcome by spreading the waste over a sufficiently large area.

Felsot's studies support the site-specific nature of determining appropriate landspreading rates. The pesticide concentrations in the soil piles must be known, and the choice of crop and application rate must be based upon the concentration of pesticides in the soil pile. Ideally, the soil should be applied to previously cropped land which will be in production during the subsequent growing season. By applying the soil to crop land previously treated with the pesticides in the waste pile, there is an increased possibility that the native microbial population will have enhanced ability to degrade the pesticides applied in the waste soil (Moorman, 1990).

In summary, landspreading and land application projects must be assessed on a case-by-case basis. Pesticide concentrations, label application rates and label restrictions must be considered. Soil should be applied so that the limiting pesticide is applied at a rate equivalent to or less than its label rate. Additionally, the application site must also be assessed for

environmental considerations which may restrict land application, such as steep slopes, proximity to wells, proximity to wetlands, proximity to surface water or sinkholes, and shallow depth to bedrock or ground water.

5.2.2. Prepared Bed Treatment

Prepared bed treatment is essentially land farming in a controlled setting. The excavated soil is placed in a treatment bed where it may be tilled, watered, amended with nutrients and additional carbon sources, and inoculated, if desired. The treatment bed is generally lined with a synthetic or clay liner to prohibit transport of contaminants out of the bed. The treatment bed may be prepared at the site of the excavation; the excavated soils are replaced following bed preparation. Alternatively, the bed may be located in an area far removed from the excavation (Sims et al, 1989). The bottom of the bed is generally sloped to allow for drainage. Leachate is collected via a system of perforated pipes set atop the liner. The leachate is discharged to a collection tank from which it may be recycled to the treatment bed, treated on site and then recycled to the treatment bed, or disposed. The bed can be left uncovered, or it may be entirely covered with a plastic film. If the system is enclosed or covered, volatile emissions can be controlled and/or treated. In general, an irrigation system is provided (Bourquin, 1989). Although inoculation may be attempted, a general guideline is that inoculation would is only likely to be successful in a setting where the inoculated population is known to have a large metabolic advantage compared to the indigenous species, or where the indigenous population is especially sparse (Golueke and Diaz, 1989).

Prepared bed technology was used at a North Dakota site to treat soil contaminated with 2,4-D, MCPA, alachlor and trifluralin and carbofuran (Bourquin, 1989). The contamination at the site was due to a facility fire which resulted in contamination of soil, ground water and an adjacent creek. The soil at the site was divided into two classes; the highly contaminated soil was treated in slurry reactors while less contaminated soil was treated in a prepared bed treatment unit. Initial treatability studies showed that solid phase treatment could be used to reduce concentrations of 2,4-D from 90 ppm to less than 10 ppm. Based on the treatability data, a prepared bed was constructed and used to treat 10,000 cubic yards of soil. The bed was five acres in size with an engineered clay liner 12 inches thick and a drainage system to collect leachate. Leachate was recycled and placed back on the treatment bed. The contaminated soil was placed to depth of 15 inches. The bed was operated for three months with daily tilling. Soil moisture was maintained in the range of 8-15%. During the three months of operation, the combined concentration of 2,4-D and MCPA dropped from 86 ppm on average to 5 ppm on average. This surpassed the clean-up criteria of 10 ppm combined 2,4-D and MCPA residue established by the State of North Dakota (Personal communication, Martin Schock, North Dakota Department of Health and Consolidated Laboratories, March 3, 1992).

For this treatment project, 2,4-D and MCPA were selected as marker compounds for following the extent of degradation. These compounds were selected because they were the contaminants

pres[illegible] the highest concentrations. Tests showed that alachl[illegible]d carbofuran were present at low initial concentrations (less than 3 ppm) and that trifluralin could be reduced from 81 ppm to 13 ppm over 20 days by solid phase treatment (Stroo et al, 1988).

Prepared bed treatment appears to be a promising technology for pesticide contaminated soils if the caveat is raised that treatability studies must be conducted prior to approval and implementation of a project. In Minnesota, a likely application of this technology would be on soils contaminated with incompatible products or very high concentrations of pesticides. In these situations, it would be useful to treat the pile to the point where it could be land applied. Another possible application would be for sites contaminated with canceled pesticides or pesticides listed as hazardous waste. Since many of these compounds are recalcitrant, treatability studies would be especially important to determine the biotreatability of the soil.

5.2.3 Compost Treatment

Composting is a solid phase treatment wherein contaminated soils (or other solid matrix) are placed in a pile into which nutrients and bulking agents are incorporated to provide drainage and air penetration. Composting can be thought of as a modified land treatment process. Compost piles are sometimes referred to as "aerated heap treatment" (Stroo, 1989), "biopiles," or "soil mounds" (Norwood and Randolph, 1990). Composting relies upon controlled biodegradation for removal or alteration of the organic components in the matrix (Dragun, 1988). Although the literature did not provide examples of pilot or field scale projects applied to pesticide contaminated soils, the technology has been applied to petroleum and polynuclear aromatic hydrocarbon (PAH) contaminated soil (USEPA, 1992). Compost treatment is sometimes used as a pre-treatment strategy for the treatment of highly contaminated soils (Norwood and Randolph, 1990).

There are several common compost formats which can be broadly classified as either *open* or *closed* systems (Dragun, 1988). Open systems include *windrows* and *static piles.* For windrows, the soil is piled in long, narrow, low mounds and aeration is accomplished by turning the material mechanically with a front-end loader or similar equipment. For static piles, which are also termed "the Beltsville system," the compost matrix is stacked in a rectangular-based, low-lying mound which is aerated via a series of perforated pipes installed under the mounds. The pipe is attached either to a pump or a fan and air is either drawn or forced through the pipe (Dragun, 1988; Hantsveit et al, 1988). Some studies have indicated that forced air gives the best results in terms of microbiological activity, chemical and physical quality of compost and progression of the project (De Bertoldi et al, 1982).

Closed systems generally involve some kind of enclosed digester. There are several potential advantages to a closed system. Greater control is afforded over temperature, moisture content, aeration, capture of volatilized gases, and, if desired, inoculation. Because of the more intensive material handling involved with the closed system, costs will be higher than for the

relatively [illegible]pensive open systems. There are several possible diges[illegible]mats for the closed system. One common format is the "Dano" system in which waste is placed in a horizontal drum which rotates on its longitudinal axis (Hantsveit et al, 1988). Moisture and aeration can be adjusted accurately, and as a result of the intensive mixing, composting is rapid. After the compost material is removed from the drum, it is mounded in order to complete the composting process. The Kneer reactor is a vertical cylindrical vessel; waste is fed into the top of the reactor and compost is removed from the bottom. Air and water are introduced at the base (Hantsveit et al, 1988).

Although composting is usually considered an aerobic process, compost systems can be conducted anaerobically (Dragun, 1988). Generally, "anaerobic composting" refers to a two-stage process consisting of a preliminary anaerobic slurry treatment using high solids content followed by an aerobic composting stage (Kayhanian, 1993). Sayles et al (1992) are currently conducting a study on the effectiveness of sequential anaerobic/aerobic composting and land farming techniques on soils and sediment contaminated with DDT, other pesticides and other chlorinated aromatics. Although this literature review found no applications using anaerobic composting applied to pesticide contaminated media, it would appear highly applicable, given the success of aerobic/anaerobic sequenced treatment on halogenated pesticides (see section 4.7). Compost systems can be mesotrophic, operating in the temperature range of 15 to 25 degrees Centigrade; or thermophillic, operating at 45 to 65 degrees Centigrade (Dragun, 1988).

The composting concept is fairly simple. The contaminated soil is prepared prior to placement in a pile or a digester by mixing it with bulking agents, fertilizers and additional carbon sources (Dragun, 1988). Bulking agents, such as wood chips, increase pore space to allow for better transfer of oxygen and to help maintain moisture levels. Fertilizer is added to maintain a C:N ratio of between 26 and 35 (Poincelot, 1974). Additional organic matter, including manure, may be added to help increase the general biomass. After the compost material is prepared and placed, the pH and moisture, oxygen and nutrient contents must be measured and adjusted routinely. The ideal moisture level range is about 40 - 60% by weight (Poincelot, 1974) and the pH optimum is approximately 5.5-8.0 (Fogarty and Tuovinen, 1991).

The pile initially contains an extensive, heterogeneous population of microorganisms. As the microorganisms grow, and consume available carbon, nitrogen and nutrients, the temperature of the pile is increased by the heat generated from biological oxidations. Due to the low surface-area-to-volume ratio achieved with the pile format, there is little opportunity for dissipation of the heat (Hogan et al, 1989). Additionally, the organic matter acts as an insulator (Poincelot, 1974); consequently the heat is retained and the temperature of the pile increases. The pile temperature profile for a representative compost pile can be divided into four phases. During the *mesophilic phase* (up to 37 degrees C) the composting process is initiated by mesophilic bacteria. At 40 degrees C, many of the mesophiles have been killed off or are in a dormant state and thermophiles which can withstand higher temperatures have

become predominant. The temperature range from 40 degrees C to approximately 65-70 degrees C is the *thermophilic phase*. Most of the microbial decomposition and biomass growth occur during this phase, and the rates of degradation are high (Fogarty and Tuovinen, 1991). At this stage in the process, one gram of compost material can contain one trillion microorganisms (Poincelot, 1974). Eventually, most of the readily available organic carbon has been consumed and microbial activity declines. Consequently, the temperature declines, resulting in the third, or cooling, phase. During this stage, fungal mesophiles predominate; at this point the more recalcitrant organics, such as lignins and waxes, are degraded (Fogarty and Tuovinen, 1991). The final stage is the *maturation stage* during which the compost ages and additional decomposition, degassing and stabilization occurs (Dragun, 1988).

Temperature, moisture, pH, aeration and C:N ratio are important controlling parameters in composting. Temperature is perhaps the most important. If excessive heat is generated during the thermophilic phase, the temperature may become prohibitive (at 65 degrees C and above), even to most thermophiles, and bioactivity will drop. Consequently, the pile may cool off prematurely and the composting process will not be complete. Additionally, during the thermophilic stage, a large temperature gradient can exist between the outside of the pile and the center (Poincelot, 1974). Turning the pile will help solve both these problems. In static piles, adequate air flow can control temperature. In a contained system, the temperature can be controlled so as to achieve a gradual temperature ascent, plateau and descent (Fogarty and Tuovinen, 1991).

Composting has been widely used in this country for municipal solid waste, sewage sludge and animal manures (Hantsveit et al, 1988). It has been applied successfully to treat soil contaminated with PAHs and petroleum (USEPA, 1992) . The literature does not contain examples of field applications of composting on pesticide-contaminated soils; although two references mention that unidentified pesticides have been successfully treated in compost systems (Dragun, 1988; Alpert and Epstein, 1981). Composting is used much more widely in Europe than in the United States for the treatment of toxic or hazardous wastes (Dragun, 1988; Hantsveit et al, 1988).
The degradation of thirteen pesticides representing several pesticides families was evaluated in an artificial compost media (The Snell Environmental Group, 1982). The media consisted of shredded newspaper, manure, waste water treatment plant sludge, sawdust, peat moss, soil, powdered milk and fertilizer. The pesticides were mixed individually with the compost matrix in separate cells which were specially designed to be inserted in a larger compost pile. The pesticides were mixed with the matrix to achieve a concentration of 500 ppm. Pesticides were analyzed in the compost mixture 7 days and 30 days following application. Compounds were tested individually, and no attempt was made to optimize treatment conditions for any given contaminant. Compost temperatures reached a maximum of 60 - 65 degrees C. The composting digester was provided with aeration intermittently.

The pesticides demonstrated varying susceptibilities toward degradation, ranging from trifluralin which was degraded by 83% to toxaphene which was degraded by 4%. The authors grouped the compounds by susceptibility to degradation. The pesticides tested, the percent degradation achieved, and the assigned susceptibility class are listed in table 5.2. The authors felt that compounds with "moderate" susceptibility or greater would be treated successfully in a large scale commercial system.

Table 5.2

Percent of Pesticide Degraded in a Compost System from The Snell Environmental Group (1982)

Pesticide	Percent Degraded	Susceptibility Class
Trifluralin	83	High (76 - 95%)
Methoxychlor	74	Moderately High (51 -75%)
Lindane	73	Moderately High
Atrazine	68	Moderately High
2,4,5-T	53	Moderately High
Endrin	52	Moderately High
Chlordane	44	Moderate (31 - 50%)
Silvex Acid	36	Moderate
Chlorpyrifos	31	Moderate
2,4-D	28	Moderately Low (16 - 30%)
Dieldrin	11	Low (0 - 15%)
DDT	7	Low
Toxaphene	4	Low

While most of the pesticides were maximally degraded in the first 7 days, dieldrin, DDT, chlordane, toxaphene, and chlorpyrifos did not degrade at all in the first 7 days and it is impossible to know when in the final 23 days that these compounds began to degrade. It is possible that an adaptation period was needed for these chemicals; this raises the possibility that they could be further degraded given more time and amendment of the pile with additional carbon sources. Proof of this supposition would require additional study. It should be cautioned that this study did not present evidence of biodegradation, but only disappearance of the parent compound.

The results of this study indicate that composting may be an effective means of degrading pesticides in highly contaminated soil piles to the point where they could easily be landspread. Unfortunately, because the study focused on lawn and garden pesticides, no representatives of the acetanilide class (such as alachlor) were included in the study.

The study was intended as a phase I investigation to determine whether composting could be used successfully for the biodegradation of organic compounds. Phase II, which was apparently never conducted, was intended to optimize the compost process for commercial application. Thus, several questions were left unanswered (The Snell Environmental Group, 1982). The concentration range at which treatment is effective was not determined. The effect of chemical mixtures was not determined nor was the ideal ratio of media to substrate. No attempt was made to perform a mass balance study, so that it could not be determined whether biodegradation was occuring, whether volatilization was a major disipation process or whether degradation intermediates were accumulating.

Muller and Korte (1975) studied the extent of degradation of monolinuron and dieldrin during composting. No information was provided as to the type of compost media used or the maintenance of the compost system. The material was composted for 3 weeks. ^{14}C-labeled compounds were used in the study. Only 2.7% of the dieldrin was degraded while 13.8% of the linuron was degraded. Given the lag phase present prior to dieldrin degradation in the Snell Environmental Group (1982) report, the three week incubation period may not have been adequate. Also, given the paucity of detail provided on the compost conditions, it is hard to assess what factors may have contributed to the poor degradation response.

A group of researchers from Virginia Polytechnic Institute (VPI) have studied the use of composting as part of a pesticide wastewater disposal system. Petruska et al. (1985) developed a bench-top model compost system to test compost feasibility on individual compounds and compost material. The model allows for all major routes of transformation to be traced. The study used radio-labeled diazinon and chlordane to test the efficacy of the model. Volatilization, CO_2 evolution and metabolite formation were investigated. The compost media consisted of cow manure and sawdust mixed in a 1:1 ratio. Chlordane and diazinon were added such that the concentration of each was 100 ppm. The system allowed for temperature and aeration control. A scrubbing system was used to trap CO_2 and volatile organics.

Overall, hydrolysis and volatilization were the major loss routes. Diazinon was successfully degraded while chlordane was persistent. After 3 weeks of aerobic incubation, 15% of the initial diazinon was volatilized as either the parent product or the hydrolysis product. No parent diazinon remained in the compost matrix. Essentially all of the chlordane remained unconverted. Approximately 50% of the chlordane was volatilized while 47.8% of it remained in the compost matrix. The authors felt that, because volatilization was an active loss mechanism for diazinon and chlordane, there is a possibility that these pesticides may volatilize and then recondense on the outer edges of a larger compost pile. The authors also felt that longer composting periods may have resulted in greater extent of degradation (Petruska et al, 1985).

This system appears to be a valid method to test the efficacy of composting on individual pesticides or pesticide mixtures. All dissipation routes can be followed. Unfortunately, it does

not appear that the bench top model was used to further explore the fate of other pesticides in compost.

Mullins et al. (1989) presents a series of field studies conducted by the VPI group designed to examine the efficacy of diazinon disposal using adsorption to peat moss which is subsequently treated in a compost environment. Two nutrient sources and a range of diazinon concentrations were tested. The nutrient amendments tested were cornmeal and dairy manure. Diazinon was applied to the peat moss by hand sprayer. The peat moss was placed in a plastic lined "disposal pit" which was constructed of pressure treated wood. Diazinon disappearance was rapid and nearly complete. After one week of incubation in the dairy manure/peat moss matrix, 6-14% of the initial diazinon concentrations remained from starting concentrations of 166, 1365 and 3719 ppm. After 3 weeks, less than 1% of the initial diazinon remained. With the cornmeal matrix, less than 1 to 61 ppm, respectively remained from initial concentrations ranging from 4,000 to 32,000 ppm after 8 weeks. After 18 weeks, 1 and 7 ppm remained. Volatile losses were not included in the mass balance, although ambient air diazinon concentrations were measured in the enclosed pits and were in the ng/Liter range.

Based in part on the previous work by VPI researchers, a pesticide wastewater disposal system which includes a composting phase was developed and field tested (Hetzel et al, 1989). The system uses organic material during an adsorption phase. The adsorbent is subsequently separated from the liquid and transferred to degradation pits for the second, or disposal, phase. The organic absorbents used were either peat moss, augmented with cornmeal and fertilizer, or processed wood products. The pits were maintained over several years. The disposal pits were the same as those described in Mullins et al. (1989). Four pesticides, diazinon, chlorpyrifos, chlordane and carbofuran, were tested. The rates of degradation for different pesticides varied. Diazinon was applied over a four year period and degraded rapidly. Diazinon concentrations dropped from a range of 39,000 to 42,000 ppm to a range of "Not Detected" (ND) to 3.4 ppm. Chlorpyrifos degradation was not as rapid. Lime was added to adjust pH to 6.6 in certain chlorpyrifos pits; degradation occurred at a more rapid rate in these pits. Carbofuran levels dropped from 7,070 ppm to 75 ppm while chlordane concentrations dropped from 3,850 ppm to 300 ppm over a three year period. The authors predict that continued exposure in a single pit would selectively enrich for more effective degraders. This study further illustrates the chemical specific nature of degradation mechanisms and supports the need for treatability studies for each proposed bioremediation system.

Mullins et al (1992) describes an improved wastewater disposal system. The system featured a third, or separation, phase for improved separation between the adsorbent and the liquid. In this version, the composting phase is conducted in Rubbermaid containers. The disposal phase of this system was assessed in preliminary studies using carbofuran and atrazine. The compost matrix consisted of peat moss, cornmeal, and crushed limestone (67:22:11), mixed with horse manure, agricultural soil and activated peat moss. The activated peat moss came from five year old disposal pits used for previous pesticide treatment. After 3 weeks, carbofuran was

degraded to undetectable levels from an initial concentration of 749 ppm. Atrazine was present at a higher initial concentration (7231 ppm). After 26 weeks, 14% of the initial atrazine remained and the hydroxyatrazine intermediate was shown to be transient.

As a wastewater disposal method, the VPI system is practical and inexpensive. The fate of additional pesticides during the compost process and means of optimizing the process need further evaluation. The bench top compost model developed by Petruska et al. (1985) would be a useful tool for accomplishing this. The authors feel that the composting portions of their research supports the use of composting on contaminated site soils (Mullins et al, 1992). Although an in-situ format is suggested, better control over aeration, nutrient addition and pH adjustment would be provided by a windrow or static pile format.

In summary, no pilot or field scale composting projects have been performed for pesticide-contaminated soil. This technology appears to show promise for many pesticide compounds. A likely application of composting technology would be in the situation where pesticide concentrations are too high for land application. The compost pile would be used to bring the concentrations down to a level which could be applied practically. Effective bench scale models have been developed to test compounds for their treatment potential in composting (Petruska et al, 1985; Hogan et al, 1989). These models could be used to optimize the selected system for a given body of pesticides. Composting is not a panacea. The studies conducted at VPI (Petruska, 1985; Mullins et al, 1989; Hetzel et al, 1989; Mullins et al, 1992) and by the Snell Environmental Group (1982) illustrated that certain chemicals are resistant to degradation in an aerobic composting environment.

5.3 Treatment in Reactors

Bioreactor treatment includes slurry phase reactors for soils and various reactor formats for waters. Because the waste is contained in a relatively controlled setting, the use of reactors offers several advantages. In general, reaction rates are increased and acclimation times shortened. Reactors provide greater process control and superior mixing which allows for greater overall control of, and more homogeneous, physical/chemical matrix characteristics and increased contact between contaminants and microbes. Inoculation with specific cultures has the best chance of success in bioreactors relative to other technology formats due to increased control of the physical/chemical environment. Reactor technologies are generally more costly than solid phase technologies.

There are differences in the design considerations for slurry and aqueous phase reactors. In general, due to the increased handling steps involved in slurry treatment, more equipment and associated operation costs are involved. Slurry treatment invariably involves a dewatering phase that requires extra handling and disposal processes for both solids and water. In spite of these differences, there are basics concepts of reactor design which can be discussed. There are many available reactor designs for both slurry and water treatment. The discussion presented

here is not meant as an exhaustive discussion of reactor design, but is meant to provide a basic understanding of the differences between the various reactor formats which are commercially available.

Most of the available reactors are variants of two primary process types, fludized processes and fixed bed processes (Gaudy and Gaudy, 1988). In *fluidized processes*, the growing biomass is held in suspension in the aqueous slurry or water being treated. Following treatment, the biomass must be separated from the treated media. This is usually done in a settling tank. The classic example of a fluidized process is *activated sludge* treatment. Fluidized processes are also called *activated sludge processes* or *sedimentation and recycle* processes. The latter term refers to the separation of the biomass from the treated water (or slurry) by sedimentation of the biomass in a settling tank. Part of the biomass is recycled from the settling tank back into the treatment tank to initiate the biomass for next batch of contaminated media. The recycled biomass is termed "activated sludge" because the microbes have become acclimated to the contaminants, resulting in a reduced lag phase and reduced total treatment time. Slurry treatment generally relies on some variant of the activated sludge process.

In *fixed bed processes*, the active biomass are fixed or immobilized on a neutral support and the contaminated media moves around and past the fixed biomass. Fixed bed processes are also termed *fixed film* or *biofilm* processes. The neutral support may be plastic, ceramic, glass, or stone. Ideally, the support provides a large surface area so that a large total biofilm surface can develop. Although the cells are retained by attachment to a neutral support, some biomass does come detached from the neutral support and must be separated from the treated media; to accomplish this, most fixed film tanks discharge to a sedimentation or settling tank. In general, fixed bed processes are not effective for slurry treatment.

In both processes, sedimentation is affected by the formation of *flocs*. Based on their size and density, individual microorganisms would be expected to remain in suspension in the liquid media. However, both in activated sludge and in the biomass washed from fixed beds, microorganisms tend to exist in aggregates, or flocs, consisting of millions of cells. The aggregates have sufficient size and density so that, in a quiescent environment, such as a settling tank, they settle out in a matter of hours (Gaudy and Gaudy, 1988).

In both fluidized and fixed bed systems, biomass must increase sufficiently so that the pollutant concentration in the substrate feed is decreased to achieve a pre-ordained treatment standard in the time that the biomass is in contact with the contaminated media (Rittman, 1992). The desired rate and extent of degradation is controlled through the parameters of substrate supply (including both contaminant and additional carbon sources), cell retention and cell/media contact time. Substrate supply is determined by type and concentration of contaminant and additional carbon sources in the influent. Cell retention assures that sufficient biomass is built up and maintained; ideal design allows for small reactor volume and high cell concentrations. Process loading assures that there is adequate time for contact between microbes and the

con[illegible]nts for sufficient degradation to occur to meet the tre[illegible]t criteria. Determination of proper loading rate requires an understanding of the kinetics of the degradation process. For activated sludge systems, the important loading parameter is cell retention time while for biofilm processes, the loading rate, or substrate flux, is the important loading parameter.

Reactors can be designed to operate in *batch mode* or *continuous flow* mode. In batch flow systems, the reaction occurs in a closed environment with no inflow or outflow, and product is removed from the reactor at the end of the treatment period. In the continuous flow mode, substrate enters continuously at a set flow rate while effluent continuously flows out of the reactor at the same flow rate. Continuous flow reactors feature different types of mixing regimes. The two extremes of mixing regimes are *plug flow* and *complete mixing* (Gaudy and Gaudy, 1989). In plug flow, all molecules entering the reactor, including substrate, nutrients and suspended cells, have the same residence time within the reactor. There is no forward or backward mixing within the reactor. On a conceptual level, plug flow is approached in long lengths of pipe where the cross section area is small in comparison with pipe length (Gaudy and Gaudy, 1989). In completely mixed reactors, the substrate and other influent material are instantaneously mixed with the contents of the reactor and there is a continuous flow out of the reactor. In completely mixed regimes, molecules experience a range of residence times within a reactor. In reality, most reactors feature a mixing regime somewhere between these two extremes.

5.3.1 Slurry Phase Treatment

In slurry phase treatment, contaminated soil is mixed with water to create an aqueous slurry (USEPA, 1990b). The slurry is treated in a reaction vessel which is generally a stirred tank reactor, although it may be as simple in design as a lined lagoon. Reactors are generally mobile and are transported to the contaminated site and operated on site. Slurry treatment is essentially an activated sludge process. Although batch mode and continuous flow are possible, batch mode is most common. Slurry treatment can be used to treat soluble organics in soils at concentrations of up to 250,000 ppm (USEPA, 1990b), although this number may high for most organic substances. Surfactants and dispersants can be added to increase solubilization of the contaminants.

Generally, the excavated soil is screened to remove debris and objects larger than 0.25 inches prior to forming the slurry. The soil is mixed with water to form a slurry and the slurry is fed into the treatment tank. Solids content ranges from 10-40% by weight; although the upper end of this range would probably require specialized mixing design. The water used for the slurry may be contaminated ground or surface water (Ross, 1990). The treatment tank is aerated by compressors and spargers or by floating or submerged aerators (USEPA, 1990b). Nutrients and inoculants may be added to optimize the degradation process (Ross, 1990). Mixing is necessary to keep the soils suspended and is performed via aeration alone or by mechanical mixing. Coarse soils may cause shearing of mechanical mixing equipment. The soil type,

mixing a[illegible]eration equipment, chemical and physical properti[illegible] the contaminant, contaminant concentration and desired removal rates will drive the retention time and other operating variables (Ross, 1990). The degree of solubilization of the organic contaminant and the size of microbial population which can be maintained are both rate limiting (Stroo, 1992).

When treatment is completed, the slurry must be dewatered. The dewatering step involves a intensive materials handling and specialized equipment. Part of the process water may be recycled for the next batch. The recycled water contains a large population of acclimated microbes so that recycling is desirable. The remaining waste water may need to be treated on site prior to discharge, which can be very costly, or may be discharged directly, depending upon ambient concentration and required permits (USEPA, 1990b). Dewatering may not be needed if the slurry is added to a solid phase treatment system such as a prepared bed treatment unit (Ross, 1990). In order this treatment scheme to be approved for a given project, it should be demonstrated that the slurry phase treatment is needed as pretreatment as opposed to direct application to the solid phase treatment bed. If air emissions are a concern, the unit may be designed to contain and treat volatile organic compounds (Ross, 1990).

Slurry phase treatment does provide certain advantages relative to solid phase treatment (Bourquin, 1989). The soils are treated in a well-mixed, enclosed environment. Because the unit is contained and the solids are slurried, there is greater control over operating parameters such as nutrient delivery and aeration. The system is homogeneous, ensuring increased contact between microorganisms and contaminants. Treatment times are generally shorter than for solid phase treatment. Slurry phase systems are especially useful in situations where available land is at a minimum and quick treatment times are desirable (Ross, 1990). Due to the greater degree of environmental and process control, inoculation would appear to be much more likely to be successful in a slurry treatment system than in a solid phase system. The major disadvantage of slurry phase treatment is that the costs are higher than solid phase treatment due to the increased materials handling and equipment needs (Bourquin, 1989). Disposal or additional treatment of the process waters may also pose a problem.

A mobile slurry reactor was used to treat 750 yards of soil containing up to 1,500 ppm 2,4-D and MCPA combined residue at the North Dakota site discussed in the section on prepared bed treatment (Bourquin, 1989). Treatability studies conducted for the project indicated that while soils with concentrations less than 200 ppm 2,4-D and MCPA could be treated in a solid phase system, highly contaminated soils would require treatment in a slurry system. The treatability studies indicated that the slurry system could be used to successfully treat moderately and highly contaminated site soils (400 ppm and 13,000 ppm 2,4-D, respectively). The treatability study explored slurry treatment with and without inoculation. The inoculant was added at four day intervals. The moderately contaminated soils were treated to 15 ppm in 16 days. The highly contaminated soils were treated to 2,600 ppm in nutrient-amended treatments and to 2,200 ppm in inoculated, nutrient-amended treatment. Inoculation did not affect rate of degradation. The treatability results indicated that soils containing 200 ppm 2,4-D could be

treated in the slurry unit to the cleanup criteria of 10 ppm within 2 weeks. The systems became nutrient limited after exhibiting initial growth and nutrient addition was needed to achieve the lowest final concentrations.

Three 2,600 gallon slurry reactors were used to treat the soil. Each reactor could treat 60 yards of soil in a batch. A trammel unit was used to screen and slurry the soil. Debris and stones greater than 0.25 inch in diameter were removed. The average concentration in the reactors dropped from 800 ppm (400 ppm in the slurry) to less than 10 ppm in 13 days. The observed 2,4-D half-life (2.1 days) was similar to that seen in the treatability study for moderately contaminated soils. The slurry was spread on the solid phase treatment unit upon removal from the reactor.

The literature review did not yield other examples of slurry treatment of soils contaminated with agricultural pesticides. However, slurry reactors have been commonly used to treat sites contaminated with pentachlorophenol (PCP) and polynuclear aromatic hydrocarbons (PAHs) (Ross, 1990). In one slurry phase treatability study for a site with pentachlorophenol contaminated soils, PCP was found to be successfully degraded when the slurry was inoculated with a PCP-degrading consortia (Ross, 1990). The study tested slurries with a range of solids contents (5-40%) and consequently, a range of PCP concentrations (approximately 40, 90, 160, and 275 ppm). For all but the 25% solids slurry (160 ppm), the PCP was degraded to less than 2 ppb within 10 days following inoculation. Determination of the concentration distribution among various size fractions of the soil indicated that the highest PCP concentration was associated with the coarse soil fractions (greater than 60 mesh size). Because the coarse soil fractions will not keep suspended in a slurry, soil washing was used to remove PCP from the solids larger than the 16-mesh size. The resulting liquid and the solids smaller than 60 mesh size were treated in a slurry reactor using 20% solids content. Two 25,000 gallon slurry reactors were operated in batch mode. PCP concentrations in the reactor were degraded from 370 ppm to less than 0.5 ppm in 14 days.

Slurry reactors were used to treat 3,700 yards of soil contaminated with PCP (USEPA, 1992). PCP concentration decreased from an average of 680 ppm to less than 0.5 ppm. Treatment was performed in the field and reactors were run in batch mode. Treatment resulted in 99% decrease in PCP concentration over a 24-day period. The full volume of soil was treated in 12 months. Contaminated ground water was used as make-up water in the slurry reactor.

In summary, slurry treatment provides greater control over treatment conditions and hence, over biodegradation processes, than solid phase treatment. Slurry systems tend to be more expensive than solid phase treatments but generally require less land area for operation. The few bench scale and full scale projects which have been conducted appear successful. Treatability studies will be necessary for any given site since individual pesticides, unique complex pesticide mixtures, and the site-specific physical and chemical environment will all impact the treatment effectiveness. Furthermore, individual vendors' systems may be unique

in their effectiveness for a given pesticide or blend of pesticides (USEPA, 1990b).

5.3.2 Bioreactors for Treatment of Contaminated Waters

Ground water can either be removed from the aquifer with pumping wells and treated in reactors, or can be treated in place using various in-situ methods. Removal of water with subsequent treatment above ground is commonly referred to as a *pump-and-treat* technology. Details on removal (pumping) methods and the potential problems associated with pump and treat technologies will not be discussed in this report. Good reviews on the subject are provided in Mackay and Cherry (1989) and USEPA (1990a).

Several bioreactor formats are available for the treatment of waters. Fixed bed, fluidized bed and immobilized cell reactors will be discussed here. In general, engineering design and control for aqueous phase bioreactors is fairly well established because reactor designs are similar to those used in conventional wastewater treatment, which has a long history in the use of biotreatment technologies (Stroo, 1992). However, there are few examples of the use of bioreactors on the treatment of pesticide contaminated ground water.

Fixed Bed Reactors

The basic concept behind fixed bed reactors was discussed in the beginning of this section (5.3). The active biomass is immobilized and forms a slime, or biofilm, on a neutral support. The neutral support, which may be made of glass, ceramic, plastic or gravel, offers greatly increased surface area for the attachment of microbial cells. The influent water moves around and past the biofilm. Due to the convoluted surface area offered by the neutral support, the water is never in free fall and there is ample contact time between the biofilm and contaminants for degradative reactions to occur. An example of one type of support configuration is provided in Kumaran and Shivaraman, 1988. This light, sturdy support structure is made of alternating flat and vacuum-formed PVC sheets bonded together. A variant on the fixed bed process uses rotating biological contactors (RBCs). The support configuration for RBCs is a honeycombed or corrugated disk; the biofilm attaches to the disk. Multiple disks are placed on a shaft, and the disks are rotated through a vessel containing the contaminated water (Tchoganoglous and Schroeder, 1985). With RBCs, the biofilm is moved through the contaminated water instead of the contaminated water being fed through the fixed bed by gravity flow.

Frick et al. (1988) developed and field tested a fixed film reactor for the treatment of water contaminated with pentachlorophenol (PCP). The reactor was modeled after a successful bench-scale reactor developed in previous studies. Ceramic saddles were identified as the most effective support matrix based upon tests of four possible support matrices. The active biofilm (a consortia capable of PCP degradation) was transferred to the ceramic saddles. A pilot scale reactor was designed and assembled; fresh saddles were mixed with the colonized saddles and

add[illegible]the reactor. The biofilm was developed in the reactor b[illegible]irculating PCP-containing medium through the reactor column. Lastly, the reactor was field-tested with PCP contaminated ground water. The reactor was run in recirculating batch mode during the development of the biofilm but was switched to single-pass operation for the pilot scale tests.

The reactor was moved to and installed at the treatment site (a pole treatment operation in Minnesota) where flow rate and operating parameters were optimized using process water from site operations. Next, the system was used successfully to treat contaminated ground water from the site which contained PCP in concentrations ranging from 5 ppm to 100 ppm. The reactor consumed more than 95% of the influent PCP, in addition to removing unidentified halogenated aromatics, and greater than 95% of the PAHs present in the water (Frick et al, 1988). This article offered excellent examples of the practical problems encountered in the development of an operable system and how such problems are resolved.

Fluidized Bed Reactors

Fluidized bed reactors are a sort of conceptual mixture between fixed bed and activated sludge systems. The support matrix in a fluidized bed reactor is mobile rather than fixed and consists of small particles of sand, coal, activated carbon, ion-exchange beads or metal oxides which form a bed of porous media (Rittman et al, 1988). The biofilm attaches to the particles, and the porous media is "fluidized" by the upward flow of water or by gas turbulence. During the fluidization process, the bed of porous media expands in volume by 10 to several hundred percent (Rittman, 1992).

Conceptually, fluidized bed reactors can be used with any biological reaction (Rittman, 1992). Liquid fluidization (when an upflow of liquid, usually water or effluent recycle, is used to expand the bed) can be used with anaerobic, denitrifying or aerobic reactions. Gas turbulence or air-lift fluidized beds (configurations in which gas or air are used to expand the bed) have primarily been used in aerobic applications but can be used for anaerobic processes with certain adaptations.

Fluidized bed processes technology offers several advantages. Fluidization allows for greatly increased surface area for the attachment of cells without clogging by the biofilm, as could occur in an immobilized support matrix with similar surface area. Fluidization also affords for improved mass transfer and mixing between nutrients, microbes and contaminants. Increased biofilm accumulation per reactor volume allows for more compact reactor size and shortened detention times (Rittman, 1992).

Mixed microbial cultures were used in a fluidized bed reactor to treat chloro-s-triazine wastewater from a manufacturing plant (Hogrefe et al, 1986). The mixed culture inoculant had previously demonstrated the ability to remove > 80% s-triazine (starting concentration not indicated) when tested in the laboratory. The biofilm was immobilized on quartz sand

particles. [illegible]removal efficiency of 75-80% was achieved and m[illegible]ined when certain operating conditions were met. The biofilm thickness had to be limited such that anaerobic zones were avoided. The continuous addition of an external carbon source (unspecified) was required so that a C:N ratio of 12 was achieved. The S-triazines were consumed as the sole nitrogen source in this setting. The concentration of atrazine in the influent water was not indicated.

Immobilized Cell Reactors

Immobilized cell reactors are a refinement of fixed bed techniques. In immobilized cell reactors, whole cells are embedded in, or entrapped into, a solid matrix of alginate, polyurethane foam, porous diatomaceous earth, chitin, cellulose, or hollow glass fibers (Stroo, 1992). Whole cell immobilization involves the entrapment of cells onto a matrix which contains a ligand to which the cell binds, making it accessible to the contaminated medium surrounding the matrix (Portier et al, 1988). Immobilization provides a certain degree of protection to the microbial cells. Immobilized cells are more resistant to washout than fixed cells and are also more resistant to high concentration spikes in the contaminant load and to the presence of toxic substrates (Stroo, 1992). The support material is placed in a packed bed format, much like a fixed bed reactor. Immobilization allows for the use of specialty organisms and is thus an ideal setting for inoculation. Immobilized cell technology is relatively recent, having been developed only in the past decade (Portier et al, 1990), and has not been used widely for treating contaminated ground water.

A Flavobacterium species was immobilized on Ca-alginate beads after a variety of support media were tested in several reactor designs (O'Reilly and Crawford, 1988). The immobilized bacteria were used to treat up to 200 ppm pentachlorophenol in water in batch reactors. An immobilized Pseudomonas species was shown to treat up to 250 ppm creosol in water. Reaction rate was dependent upon aeration method, with oxygen gas air-lift reactors being the most effective.

Dr. Portier's research group from Louisiana State University has studied various immobilized bed reactor formats for the treatment of pesticide-contaminated ground water. Adapted microbial strains were immobilized on a porous diatomaceous earth support (Portier et al, 1988). The treatment unit consisted of two bioreactor modules connected in series. The reactors were each self-contained units with pH/Eh, temperature, and flow controls. The reactors could be run under aerobic or anaerobic conditions. This format was tested on ground water containing toxaphene and other pesticides. The reactor was run in plug flow configuration under aerobic conditions. Influent concentrations of toxaphene ranged from 50 to 450 ppb. The reactor effectively removed toxaphene when concentrations were high. At low concentrations (< 50 ppb), removal efficiency was decreased. It appeared that there was an (undefined) substrate threshold concentration necessary for "enzymatic recognition." The average reduction of toxaphene was 90% for this reactor, although optimization of flow rates

apparently increased the removal rate in later studies. Diatomaceous earth was found to provide an acceptable support medium for immobilization. It is difficult to determine whether the observed removal rates in this experiment were due strictly to biodegradation or to other dissipation processes such as adsorption. This is because degradation was determined by comparing influent and effluent concentrations, which effectively measures disappearance. A mass balance approach would provide better evidence of biodegradation.

A similar reactor configuration was later used in a field pilot test to gain information for estimating scale-up needs and to determine whether ground water contaminated at dilute concentrations could be treated to meet state permit requirements (Portier et al, 1989). The reactor contained two biological towers connected in series. The immobilization support for both towers was diatomaceous earth; the first tower contained selected organochlorine degraders while the second tower contained a small bed of organochlorine degraders and a top layer containing selected organophosphate degraders. Toxaphene (an organochlorine) was the primary contaminant in the pilot study. Two ground water streams were used: a dilute stream containing 50 - 150 ppb toxaphene and a more concentrated stream containing greater than 200 ppb toxaphene. Threshold effects were evident. Removal efficiency was greater for the more concentrated waste stream than for the dilute stream. For the concentrated stream, the toxaphene removal rates were 65% for the first reactor and 54% for the second reactor. Total organophosphate removal rate was only 35%; the authors attributed this poor performance to lack of separation of microbial populations between reactors. An activated carbon unit was used as a polishing step for the unit. Carbon requirements without biological pre-treatment were 2.5-10.0 grams carbon/Liter ground water. With the biological system in place, carbon requirements dropped to 0.10 to 0.25 g carbon /L ground water. With this experiment, as in Portier et al. (1988), biodegradation was not definitively proven since degradation was measured by disappearance of the parent compound.

The authors found the diatomaceous earth support to be effective for the reactor function. Microbial populations were effectively insulated against spikes in pesticide concentrations in the influent. At this point in the development in the technology, it appeared that the reactor was most applicable as pre-treatment for activated carbon treatment.

Portier et al. (1990) field-tested two immobilized cell reactors on ground water from a formulation site. The ground water contained elevated concentrations of organophosphate insecticides (malathion, methyl parathion), organochlorine insecticides (aldrin, chlordane, toxaphene) and other non-pesticidal organics. A diatomaceous earth support was used in the first bioreactor; this reactor was operated in plug flow. The second reactor used plastic 2-inch diameter "pall rings" as the immobile support phase; this reactor was operated as a continuously stirred batch reactor. A granulated activated carbon pre-treatment unit was in-place for use when toxaphene levels exceeded 250 ppb. The microorganisms used for immobilization had been isolated previously and adapted for degradation of industrial effluents, including organochlorines.

Removal efficiencies varied with the compound. Toxaphene was the major contaminant; feed concentrations varied between 50 - 540 ppb toxaphene. The average toxaphene reduction was 80% for the first reactor and 61% for the second (at a flow rate of 80 gallons per day). Removal efficiency was increased at the higher concentration. Aldrin feed concentrations ranged from 15-35 ppb and removal ranged from 50 - 100% with better results at higher concentrations; the results for chlordane were similar. Malathion removal was essentially complete in the first reactor at all feed concentrations (15-60 ppb); methyl parathion removal was not as effective and may have been impaired by phosphorus levels in the influent. The authors felt that the reactors were generally very effective for removal of pesticides from water (Portier et al, 1990). In reviewing this study, the caveat must be raised that the study does not provide evidence of biodegradation of the target compounds. Disappearance of the target compound is documented, but a mass balance approach is needed to ascertain biodegradation.

At the North Dakota site discussed in the section on prepared bed technology, bioreactors of undefined format were used to treat more than 1 million gallons of water contaminated contaminated with 2,4-D and MCPA (Bourquin, 1989). Laboratory treatability studies had indicated that contaminated site water containing 100 ppm 2,4-D could be treated to less than 1 ppm in four days using a proprietary inoculum. Other pesticide contaminants, including alachlor, trifluralin and carbofuran, may have been present in the water but appear not to have been tracked in the treatability studies or in the final application.

These applications again indicate that bioremediation systems must be tested for individual pesticides and for site-specific pesticide mixtures. Treatability studies of careful experimental design are crucial for each individual project to determine biotreatability of the site-specific pesticide mixture and to establish optimal reactor format and operating parameters.

5.4 In-Situ Treatment

In in-situ bioremediation, contaminated soils and ground water are treated in-place. In-situ treatment of soils involves the enhancement of microbial degradation without excavation of the contaminated soil. For ground water, in-situ treatment involves the introduction of nutrients, oxygen (if the system is aerobic), and, possibly, microorganisms (although this is not currently practiced at field level) to the subsurface via injection wells or infiltration trenches to enhance degradation of the contaminant plume. In-situ treatment has been more widely applied to contaminants in the saturated zone than to unsaturated soils (Stroo et al, 1992). Neither practice has been widely demonstrated on pesticide contaminated media.

5.4.1 In-Situ Soil (Unsaturated Zone)Treatment

Excellent reviews of in-situ soil treatment (not specific to pesticide contaminated soils) are provided in Dupont et al. (1988); Sims et al. (1989) and Sims et al. (1986). The practice is simple in concept: the soil physical/chemical environment in the area of contamination is

alte[illegible]nd maintained to achieve a state where microbial deg[illegible]on processes, and ideally selected degradation processes, are enhanced. Treatability studies should be performed to determine whether the site soil contains the necessary indigenous degradative capabilities and to determine the physical/chemical conditions necessary to optimize degradation. Routine monitoring of the treatment area is essential to ascertain that the desired environmental conditions are being maintained and that degradation is occurring. Effective sampling for these purposes can be difficult due to the natural heterogeneity of soil properties.

Adjusting and maintaining the correct chemical/physical environment means manipulating the soil moisture content, soil pH, soil redox potential and soil nutrient concentrations at levels appropriate for the growth of an active biomass (Sims et al, 1986). Soil moisture content is maintained by irrigation and drainage. In-situ soil treatment is generally an aerobic process and oxygen may be delivered either by regular tilling or by drawing air through the soil by the use of vacuum pumps. This second system is termed *bioventing* and is usually employed for contaminants with high volatility. The passage of air through the unsaturated zone volatilizes these contaminants while providing oxygen for the enhancement of biodegradation (Stroo, 1992).

Reducing environments for anaerobic processes are generally maintained by flooding the soil and keeping it in a saturated state (Sims et al, 1986). This has the obvious disadvantage of promoting leaching of the contaminants unless a subsurface drainage system is installed, in which case a prepared bed approach is probably more effective. Soil pH is adjusted by the addition of crushed limestone or other lime products to raise pH or by the addition of acid-producing materials to lower the pH. Soil pH adjustments must take into account the buffering capacity of the soil. Soil nutrient levels are adjusted by the addition of commercial fertilizer to maintain a correct C:N:P ratio. Finally, the addition of non-specific organic carbon amendments may be used to improve soil tilth and the general microbial activity, population size and diversity. Possible organic carbon amendments include manure, plant material, corn meal, and sludge. The effect of different amendments on microbial activity, the formation of bound residues and degradation product formation should be addressed in treatability studies.

Inoculation has been suggested for situations where the native soil does not exhibit the necessary degradative capabilities (Thomas and Ward, 1989). However, the problems associated with inoculation (Goldstein et al, 1985) are probably most germane to in-situ treatment. Inoculated organisms must survive the possible presence of predators and toxic contaminants and they must be able to compete with the native microflora. The introduced species must be able to access the contaminant molecules, and the introduced species must use the contaminant as its preferred substrate (see section 4.3 on bioaugmentation). In addition, regulatory approval is required if genetically engineered strains are involved.

There are few documented cases of in-situ treatment of pesticide-contaminated soils in the literature. In-situ soil treatment of most ag-chem facility sites appears to be fairly impractical

due to the p[illegible]sical nature of most of these facilities. The most high[illegible]ntaminated areas at agricultural chemical sites tend to be mix/load areas, water fill stations and impregnation towers. These areas are heavily trafficked, and tend to be highly compacted. The surface soils usually consist of gravel fill. Even if the appropriate levels of organic amendments and nutrients were incorporated, tilling and irrigation would require that site operations be moved elsewhere. Due to the compacted nature of the soils, tilling and incorporation of nutrients, and oxygen and pH adjustment amendments would be difficult. In ponded areas and run-off areas, which are often physically removed from areas of heavy traffic, in-situ treatment could be attempted. However, treatability tests would be required to determine whether degradation, volatilization or bound residue formation will occur, and to establish necessary operating parameters. If in-situ treatment is selected as a remedial action, maintenance and monitoring schedules must be planned and carefully followed.

An attempted in-situ soil treatment project at a northern Minnesota wood preserving operation illustrates a poorly conceived and conducted in-situ soil treatment effort. The site was inherited by the Minnesota Department of Agriculture (MDA) after work had been initiated at the site. Both ground water and soil were contaminated with pentachlorophenol (PCP). The investigation at the time it came under MDA jurisdiction had focused on soil contamination. A small (50' x 50') in-situ soil treatment plot had been established at the site prior to the MDA involvement in the site. There were several problems with the in-situ treatment approach; however the two largest problems were the lack of an initial thorough site investigation, and failure to adequately maintain and monitor the treatment unit.

A thorough site investigation had not been conducted prior to establishing the treatment plot. Consequently, the extent of contamination was not well defined. The treatment plot was located in an area where pentachlorophenol had previously been stored in two 4,000 gallon tanks. Additional site investigation conducted later showed that soil contamination extended well beyond the tank storage area. In fact, the highest levels of soil contamination were approximately 100 feet south of the treatment area where a former process tank overfill had occurred in the 1970's.

A treatability study had been contracted out to a microbial technology consulting firm. The goal of the study was to characterize the degradative capability of the soils and to determine the chemical factors affecting microbial growth. Three soil samples from the site were used for the study. The total number of indigenous bacteria present and the number of PCP degrading bacteria present were determined. The effect of high PCP concentration on the growth rate of the PCP degraders was investigated by diluting bacterial suspensions of the site soils onto agar plates with PCP provided as the sole carbon source. Two PCP concentrations were used (35 ppm or "low" and 100 ppm or "high"). The incubation period was 10 days. Degradation was measured by disappearance of PCP and growth of PCP degrader populations.

The consultants justified the choice of 100 ppm as representing "high" concentrations because the incomplete site investigation conducted to that point had indicated that site PCP concentrations were less than 35 ppm. As it turns out, the sampling scheme for the site had missed the most contaminated portion of the treatment area. Samples collected as a part of the monitoring activity later showed that PCP concentrations in this portion of the treatment area ranged from 175 - 230 ppm (PCP concentrations in the tank overfill zone, which was discovered later, were in the thousands of ppm range).

The treatability study concluded that the indigenous population should be capable of degrading the PCP in the designated treatment area and that additions of ammonia and orthophosphate (to achieve a N:P ratio of 5:1 to 10:1) should enhance degradation. Based upon this study, the treatment unit was approved and treatment commenced. A maintenance and monitoring schedule was developed. Sampling was to occur on a monthly basis while thorough tilling was to occur on a weekly basis for three months.

The treatability study design was inadequate. No attempt was made to test degradation in the actual soils from the site. The presence of PCP degraders was not sufficient proof that the degraders were capable of the desired degradation in the treatment unit. For example, copper napthenate tank was located adjacent to the treatment unit; it is very possible that high copper concentrations could have been toxic to the degraders.

It appears that the microbial consultants had intended the treatability study as an initial investigation to gauge whether further work was warranted and that the engineering consultants decided to design and initiate the treatment unit based upon the initial study. The engineering consulting firm may have felt that the initial results were promising enough to attempt a pilot scale project and that, since the treatment area was relatively small, it could serve as the pilot project effort. The relatively low capital outlay required may have justified the attempt to them. In any event, the in-situ treatment was commenced in the fall of 1988 and it was anticipated that the project would be completed in the spring of 1989, although what this estimate was based on is unclear.

Although it appears that the maintenance schedule was followed during the first 6 - 12 of the project, the discouraging monitoring results caused the consulting firm to request an extension for the project. At this point, it appears that the maintenance schedule was abandonned. At the time that the MDA first visited the site in the fall of 1990, miscellaneous debris, including a parked truck, was placed in the treatment area.

The poor degradation results appeared to be due in part to the lack of initial site characterization and the design of the monitoring sampling scheme. For monitoring purposes, the unit was divided into 25' by 25' quadrants. A single discrete sample was collected from the middle of each quadrant. Unfortunately, this sampling scheme was not used prior to the start of the treatment period so that there was no baseline for comparison. Discrete samples were

apparently selected based on the desire for "reproducibility". In view of the ongoing tilling operations, composite samples probably would have produced more representative results; alternatively, more discrete samples from within each quadrant would have produced given some idea of the variability of PCP concentrations. Furthermore, the more highly contaminated soil from one quadrant of the treatment unit became mixed with the other quadrants as the area was tilled. Since the highly contaminated quadrant had not been represented in the baseline sampling, the high concentrations came as a complete surprise. The analytical results consistently indicated that the PCP concentrations in three of the quadrants (including the highly contaminated quadrant) were increasing. The PCP concentrations appeared to decline significantly in one quadrant, although the sampling scheme was inadequate to determine whether this was occurring.

This case study illustrates the possible pitfalls which can occur when regulatory and consulting staff who are inexperienced in the area of bioremediation and microbial degradation are involved in the planning and approval of a bioremediation project plan. The role of the regulatory authority has not been discussed here, but clearly, this project should not have been approved. This type of work does damage to the reputation of an emerging technology. As a result of this project, the engineering consultant may be wary of bioremediation efforts in the future. The responsible party may become a confirmed skeptic and spread his distrust to his peers through trade association channels. The regulatory personnel involved may view all future bioremediation proposals as suspect and too risky to try again. It is unfortunate that the technology was not given a fair trial in this misguided effort. This type of mistake should be avoided on agricultural chemical sites. The technology should be appropriately tested and responsibly applied. Only in this manner can definitive answers be derived regarding the success of full-scale systems on agricultural chemicals.

It probably should be mentioned that the story does have a somewhat happier ending. The site was eventually opened up to a more extensive site investigation. All potential source areas were investigated. Considerable ground water and soil contamination exist at the site. The site is approaching the corrective action stage and it is likely that bioremediation will be a possible option.

5.4.2 In-Situ Treatment of Ground Water

In-situ ground water treatment technologies were first developed in the 1970's and evolved in the 1980's (Thomas and Ward, 1989). The basic design elements used in the 1970's are still germane today (Lee and Ward, 1985): the growth of indigenous microbial populations is stimulated by the addition of nutrients and oxygen which are introduced into the contaminated aquifer by injection wells or infiltration galleries. The nutrients and oxygen are moved through the contaminated zone using a system of injection and withdrawal wells. Nutritional requirements, limits of degradation and other operating parameters are determined in laboratory studies, using subsurface materials, conducted prior to designing the system.

Var. ...s include inoculation with acclimated or specialized ... ...organisms and anaerobic systems. In-situ treatment offers several potential advantages to the alternative, pump-and-treat technologies. It can be faster and less expensive than pump-and-treat technologies, and can, in some cases, reach contaminants which are commonly inaccessible to pumping, such as those trapped in pore spaces or strongly sorbed to the aquifer matrix (Wilson and Brown, 1989; Lee et. al, 1988).

In-situ ground water treatment has been widely practiced on sites contaminated with petroleum hydrocarbons (Sims et al, 1992; Litchfield et al, 1989). In the correct setting, a well-designed in-situ treatment system can be used to successfully treat a hydrocarbon contaminated aquifer much more quickly and at a much lower cost than pump and treat technologies (Wilson and Brown, 1989). For example, Lee et al. (1988) cites one case in which an in-situ system at a hydrocarbon site was able to achieve clean-up standards in 18 months while it had been estimated that pump and treat systems would require 100 years to achieve the same levels. However, it must be stressed that pesticide contamination presents different challenges than hydrocarbon contamination for in-situ ground water treatment. In-situ treatment generally relies upon indigenous microbial populations which must have the capability to degrade the contaminant of concern. Hydrocarbon degraders are widely dispersed in the subsurface (Atlas, 1981). Biostimulation of indigenous populations is viable for petroleum contaminants and has been successfully demonstrated in many settings (Litchfield et al, 1989). In contrast, it is not clear that microbes capable of degrading specific pesticides are widely dispersed in the subsurface. Consequently, biostimulation may not be effective at all sites on all pesticides.

Hydrocarbon degraders are capable of using petroleum contaminants as a sole source of carbon and energy (growth-linked degradation) (Atlas, 1981) while many pesticides are degraded by cometabolic processes. In-situ treatment systems based on growth-linked degradation are well established, but few in-situ systems have been designed to exploit degradation of contaminants by cometabolism (McCarty, 1988). The effects of co-metabolic degradation on degradation rates and on the design and operation of in-situ systems are not well understood. This does not mean that such systems would not be successful, but this does once again illustrate the necessity of carefully designed treatability studies in the design of a bioremediation system.

The basic concepts behind in-situ ground-water treatment are fairly simple, although execution requires sophisticated knowledge and skills. The primary concepts and the progression of an in-situ project can be summarized as follows (Litchfield et al, 1989; Sims et. al, 1992):

1. The site is characterized through the site investigation. The extent of contamination and the fate and transport characteristics of the waste and the aquifer are determined. Contamination sources must be identified and removed and/or addressed in the in-situ treatment plan. If there is free product present at the water table, this must be removed by pumping (Thomas and Ward, 1989). If the free product is not removed prior to in-situ treatment, degradation of the free product will consume large proportions of the added

nutrie... ...d oxygen.

2. Indigenous microbes at the site must have the capability to degrade contaminants. Most likely, if they do have the capability, the degradation rate is unacceptably slow because one or more environmental or nutritional factor are limiting microbial growth.

3. Microbial degradation abilities and the factors limiting microbial growth are identified in laboratory studies. Treatability studies are conducted to determine whether biological treatment is likely to be effective and to identify the conditions needed to optimize the growth of the target populations. Compatibility of the nutrient amendments with the subsurface formation is also determined in laboratory studies (Thomas and Ward, 1989).

4. A system of injection wells, or infiltration trenches, and recovery wells is designed to deliver the required amendments to the subsurface and to circulate them through the contaminated zone.

5. The system is built and operated. A monitoring program is designed and maintained until the project has met regulatory clean-up standards.

Perhaps more than any other bioremediation technology, the success of an in-situ treatment system depends upon a thorough site investigation. Contaminant sources must be identified and removed or addressed in the treatment plan. Type and extent of contamination must be delineated. A complete understanding of the hydraulic properties of the subsurface is necessary since nutrients and oxygen must be introduced into the aquifer and delivered throughout the zone of contamination (Thomas and Ward, 1989). Rate and direction of flow are critical factors because they determine the rate at which oxygen and nutrients reach the microbes (Dragun, 1988). In general, in-situ treatment requires a hydraulic conductivity (K) of 10^{-4} cm/sec or greater; the subsurface must be permeable enough to enable perfusion of the required amendments (Thomas and Ward, 1989). Due to the scale of heterogeneity typical of most sites, K values should be determined for several locations. Other hydrogeologic parameters to be determined include depth to water table, depth to the zone of contamination, specific yield of the aquifer and rate and direction of ground water flow.

Treatability studies are needed to determine the degradation capabilities of the indigenous microbial population and the subsurface factors which will maximize rate and extent of degradation. Nutrient needs are highly site specific (Lee and Ward, 1985). For hydrocarbon contamination, nutrient additions are generally limited to nitrogen and phosphorus, although other trace elements may be necessary. Common forms of these nutrients are orthophosphate or polyphosphate salts and ammonium salts which are delivered in aqueous solution (Wilson and Brown, 1989). *Microcosm evaluations*, employing a soil/water slurry of site material, are often used for the treatability study. Nutrient additions are adjusted to achieve optimal degradation. It is important to use subsurface formation samples rather than ground water

samples to determine the degradation potential in treatability studies because wells often contain non-native microorganisms which are not characteristic of the subsurface (Thomas and Ward, 1989). Degradation rates and degradation pathways are evaluated. This information is used to estimate treatment time (Wilson and Brown, 1989). Once the necessary amendment concentrations are identified, the compatibility of the selected nutrient amendments with the subsurface formation must be determined (Thomas and Ward, 1989). For example, certain nutrient forms may precipitate or form complexes in the wrong chemical environment, and thereby become inaccessible to the microbes and plug the system.

Historically, most in-situ systems have been aerobic and, consequently, oxygen is the most common electron acceptor which is delivered to the subsurface. Aerobic pathways have been preferred because, for most of the contaminants which have been addressed by in-situ treatment, they are quicker and are more likely to degrade contaminants to CO_2, biomass, water and salts (Litchfield et al, 1989). Oxygen supply is usually a rate determining process for in-situ treatment, especially in low permeability settings (Lee et al, 1988). There are several possible modes for delivering oxygen. Atmospheric air or pure oxygen can be introduced by spargers, using diffusers placed down wells and attached to air compressors. Air sparging can achieve a maximum of 8 - 12 mg/L dissolved O_2 while sparging with pure oxygen can achieve a maximum of 40 - 80 mg/L dissolved O_2 (Lee et al, 1988). Hydrogen peroxide (H_2O_2) solutions have often been used as a means of introducing O_2 at hydrocarbon-contaminated sites (Lee et al, 1988). The H_2O_2 decomposes to produce O_2 and water. Hydrogen peroxide is infinitely soluble but can be toxic to microorganisms, even at low concentrations (Thomas and Ward, 1989). Hydrogen peroxide toxicity is very species dependent and can be tested during the treatability stage. Ozone has also been demonstrated as an oxygen source for in-situ treatment. To deliver oxygen using ozone, ground water is withdrawn, treated with ozone and reintroduced (Lee and Ward, 1985).

A system must be designed to deliver the nutrients and oxygen to the contaminated zone. Infiltration galleries may be used for shallow aquifers while the use of injection wells is more common for deeper aquifers (Litchfield et al, 1989). In either case, withdrawal wells are used to control migration of the plume during in-situ treatment (Sims et al, 1992). Infiltration galleries percolate oxygen and nutrient amendments through the unsaturated zone as well as the saturated zone, providing the potential for treatment of contaminants still contained within the unsaturated zone. Injection wells are usually coupled with a series of withdrawal wells to form a dynamic system in which a desired hydraulic gradient is induced, thereby controlling the direction of flow and the movement of contaminants and nutrients (Wilson and Brown, 1989). Nutrient solutions and oxygen may be delivered in batch or continuous format (Lee and Ward, 1985). The withdrawn water can be recirculated into the injection well (or infiltration gallery) after being replenished with oxygen and nutrients, or it may be discharged to a sewer or other receiving body, provided contaminant concentrations are sufficiently low.

A modification of the delivery systems described above involves the use of an above-ground reactor used in sequence with the withdrawal wells. This approach may be necessary for particularly recalcitrant compounds. In this case, withdrawn water is pumped to activating tanks where nutrient amendments and oxygen are added to the water; the water is pumped from the activating tanks to settling tanks from where it is introduced back to the subsurface. At the point where it is reinjected, the water is enriched with an acclimated culture, nutrients and oxygen (Lee and Ward, 1985).

Variations on the general format for in-situ ground water treatment include anaerobic treatment, with alternative electron acceptors, and inoculation with specialized microorganisms. Anaerobic in-situ treatment has not been widely demonstrated. Nitrate has been suggested as a likely electron acceptor for some applications. Because nitrate is more soluble in water than oxygen, it is easier to deliver to the subsurface. Its use has been demonstrated in the field, particularly for gasoline and phenolic compounds (Thomas and Ward, 1989). Lee et al. (1988) suggest that sequential anaerobic/aerobic treatment could be useful for in-situ aquifer treatment. The aquifer would act essentially like a large sequencing batch reactor. Initially, deoxygenated water would be introduced to create anaerobic conditions, then oxygenated water would be introduced to create aerobic conditions. The system would require hydraulic control. Obviously, treatability studies would be needed to determine the appropriate duration of each sequence. It must be emphasized that this system has not been demonstrated in the field. However, given the apparent promise of sequenced anaerobic/aerobic treatment for many pesticides (see section 4.7), this may be a likely treatment method for ground water contaminated with those pesticides.

In cases where the native microbial population does not demonstrate the necessary degradative capabilities, inoculation with specialized or acclimated microorganisms may be desirable, although regulatory constraints may be prohibitory. However, inoculation is subject to the problems discussed in section 4.3. The introduced microbes must be able to compete with the native population and must be able to thrive in the environment to which they are introduced. Introduced cells may be sorbed, die off or diffuse from the point of application (Lee et al, 1988). The largest logistical problem is that the introduced cells and contaminants must make physical contact (Lee et al, 1988). Microbial movement depends upon characteristics of both the subsurface and the microbes. Treatability studies must include appropriate controls to demonstrate that it is the introduced culture and not the native populations which are responsible for any observed degradation. Lee et al. (1988) cite a number of aquifer remediation histories using introduced organisms; the contaminants addressed include acrylonitrile, phenol, chlorophenol, ethylene glycol, propyl acetate, dichlorobenzene, dichloromethane, trichloroethane, and formaldehyde.

In-situ treatment of aquifers does have some potential disadvantages. Sites with complex, highly irregular geology are more difficult to address because it is difficult to design an adequate distribution (inject/pump) system for such settings (Stroo, 1992). For sites with

mul[illegible] and undefined sources of contamination, it is diffic[illegible]o desing and operate an effective in-situ system. Aquifers with low permeabilities may not be good candidates for treatment because of the difficulty of perfusing and circulating nutrients and oxygen (Thomas and Ward, 1989). Bacteria can plug aquifer formations and well screens, impeding the introduction of additional nutrients and oxygen (Lee and Ward, 1985). The introduction of nutrients to the subsurface, particularly nitrate, is held as suspect by most regulatory agencies unless it can be demonstrated that sufficient hydraulic control is provided to the system such that additional nutrients are being captured, or that the added nutrients are being consumed by the system.

There have been few field demonstrations of in-situ treatment of aquifers contaminated with pesticides. The North Dakota site discussed earlier in this chapter included in-situ ground water treatment as one component of the site biotreatment scheme (Bourquin, 1989). Contamination at this site was due to a fire at a pesticide storage facility. The fire had been extinguished using water; the water had transported pesticides into the ground water and to a nearby creek. Ground water contamination was identified in three areas: the burn site, a subsurface area removed from the burn site and an impoundment area created when a blockade was erected to contain runoff from the fire. The pesticide concentrations downgradient of the burn area were low enough that only monitoring was required. An in-situ treatment system was designed and built to address the remaining two areas. The in-situ treatment was used in conjunction with carbon filtration. Injection wells were placed up-gradient of the individual plumes. Nutrients and treated water were circulated through the injection wells. Down-gradient recovery wells and trenches were located and operated to recover treated water. Granulated activated carbon filters were used in conjunction with the in-situ treatment; this helped to maintain successful treatment in winter temperatures. The site was successfully restored to meet State standards; ground water quality goals were not discussed in the material on the site.

Chapter 6: Treatability Studies and Pesticide Treatability Classes

Bioremediation has been applied successfully to petroleum hydrocarbons, wood preserving wastes, selected solvents and explosives but has not been extensively applied to agricultural pesticides. Many pesticides have been traditionally believed to be recalcitrant to biodegradation (Alexander, 1981), although ongoing research is continually discovering microorganisms capable of degrading compounds which were once considered non-biodegradable (USEPA, 1991a). Furthermore, the conditions at most agricultural chemical facilities, including high concentrations and complex mixtures of pesticides, present additional challenges to the successful application of bioremediation. Due to these possible limitations and to the lack of available data on the application of bioremediation to agricultural pesticides, well designed and carefully conducted treatability tests are required prior to the selection of bioremediation to as an appropriate technology for any given site. The necessity of conducting treatability studies for proposed bioremediation systems at ag-chem facility sites should be

balanced w[illegible]he scale of the projects and the total costs associated w[illegible]he site investigation and clean up. Most ag-chem facility site clean-ups are small in scale relative to the average Superfund site. For example, most agricultural chemical site clean-ups in Minnesota have involved less than 1500 cubic yards of contaminated soil while an Illinois study of 48 ag-chem facility sites estimated that the average volume of contaminated soil at a single site would be approximately 900 cubic yards (IL Dept. of Agriculture and IL State Geological Survey, 1993). It is not uncommon for Superfund sites to involve tens to hundreds of thousands of cubic yards of contaminated soil. The economies of scale are very different for smaller sites. With smaller volumes of soil, the cost of extensive treatability tests may be greater than the costs to transport and incinerate or place in a licensed land-fill. Thus the risk of failure of the full scale project must be balanced against the time and cost of the treatability studies.

6.1 Treatability Tests

Treatability testing provides data to aid in the selection of an appropriate remediation technology and to design the treatment technology to meet site-specific constraints or needs (USEPA, 1991a). The results of treatability tests are combined with site data to determine whether bioremediation is likely to be successful, to identify possible limitations to bioremediation and to provide guidance in the design and operation of a bioremediation system for the specific site (Sims et al, 1989). The primary questions answered by treatability tests are:

1. Will the contaminants be adequately degraded in order to meet expected clean-up goals? and

2. What are the appropriate design and operating parameters needed to optimize bioremediation performance?

The USEPA has identified three tiers of treatability testing for use in the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA or "Superfund") process; which and how many of these tiers are necessary is site- and contaminant-specific (USEPA, 1991a):

1. Laboratory scale studies -- (remedy screening)
2. Bench scale studies-- (remedy selection)
3. Pilot scale studies (remedy design)

Some or all of these tiers may be needed on a case-by-case basis, and it may be possible to combine tiers. Tiers 1 and 2 can be combined; alternatively, tiers 2 and 3 can be combined in a pilot scale study.

Remedy screening determines whether the contaminants are biodegradable and identifies features which must be investigated further in the remedy selection tier. Remedy screening also identifies whether biotic or abiotic processes are predominant for any observed removal of the contaminant. Remedy screening studies are usually performed in small batch reactors (40 ml to 10 liters in size) which contain the contaminated media (USEPA, 1991a). The term "reactor" in this context encompasses shake jars, bottles, beakers, and designed reactors (USEPA, 1988). Generally, multiple reactors are used in parallel to allow for comparison of pH, loading rates, nutrient adjustments, and other variables in the treatment process. Indigenous microbes, acclimated microbes, selectively cultured microbes or proprietary mixtures may be used or tested in parallel. Sterilized controls are run in which microbial populations are eliminated or greatly reduced by the addition of sterilizing agents. The reactors are designed to allow for replicate or sacrificial sampling at desired time intervals. Mean contaminant concentrations in the test reactors are compared to the sterilized control reactors for each sampling event. In general, disappearance of the contaminant is measured. A mass balance approach is generally not used in this phase. Incubations for aerobic processes should run from 3 - 6 weeks or up to 2 - 4 months for solid phase processes. Anaerobic incubations should be longer. A contaminant concentration reduction of from 20% to 60% is considered successful; although if the study is not well designed analytical variability can possibly account for observed reductions at the lower end of this range. If degradation is not this extensive, the results should be scrutinized for possible causes of failure (USEPA, 1991a). Controls must be carefully designed at the outset of the study; without adequate controls the question of whether or not the technology will work can not be answered.

The goal of the *remedy selection* tier is to determine how well the chosen technology will work in a designed system (USEPA, 1991a). Although the above scheme matches remedy selection with bench scale studies, pilot scale studies are sometimes needed for this determination. Remedy selection testing may take from several weeks to months to complete. Generally, a mass balance approach is used to quantify all biotic and abiotic loss pathways. Toxicity testing of residual contaminants and/or identification of selected degradation products may be required, at the discretion of the regulatory staff. A successful test at this stage would result in degradation to the clean-up goals over a 1-3 month period for aerobic process. Anaerobic processes are generally slower and should be allowed a longer incubation time. Initial cost estimates and time predictions can be made based upon the results of remedy selection testing (USEPA, 1991a).

Remedy design testing which generally involves a pilot scale project conducted on-site allows for fine-tuning the operating conditions to maximize degradation (USEPA, 1991a). Remedy design data provides quantitative performance, cost and design information. The testing period may range from 2 to 6 months. Treatment times and ability to meet clean-up goals are determined. Cost estimates are refined based on remedy design data.

These three tiers are defined to fit into the Superfund process. They are consequently fairly comprehensive and do not balance costs with risks. The tiers are site-specific and should be tailored to meet the scale of the project. Determination of the extent of testing necessary requires that a balance be struck between the cost and time needed for the studies and the risk of selecting an inappropriate or ineffective technology (USEPA, 1991a). Treatability tests can be expensive; the USEPA (1991) estimated costs for each of the tiers of testing under the Superfund process: $10,000-$50,000 for remedy screening, $50,000-$250,000 for remedy selection and $100,000-$250,000 for remedy design. For sites involving relatively small soil volumes, the full scale project may be equivalent in size to a pilot project conducted at a large Superfund site, although the scope of testing required will generally be much less than that needed for most Superfund sites. With this in mind, an approach combining comprehensive laboratory and bench scale testing may be sufficient for many ag-chem sites, although a greater level of risk may be incurred for the full scale project.

Currently, there are not standardized, widely accepted methods and criteria available for treatability assessments (Center for Agricultural Molecular Biology, 1991). Consequently, type and quality of data generated will vary between projects. The goals of each phase of treatability testing should be clearly defined and clean-up goals should be discussed in advance. Since it is not possible to perform exhaustive pesticide fate studies for each treatability test, it is necessary to identify the expected degradation pathways from the literature for each pesticide involved so that potential toxic intermediates can be identified and tested during the treatability study. During laboratory and bench scale testing, analysis of relevant compounds should be performed and a mass balance approach should be used to determine mineralization, transformation, volatilization and residual concentration (Center for Agricultural Molecular Biology, 1991).

In determining the extent of testing required, some pre-screening should be performed (USEPA, 1991a). A literature search should be conducted on the microbial degradation of the pesticides of concern and on other pesticides in the same family. Aerobic and anaerobic processes should be considered. A literature search on the chemical/physical properties of the pesticides should also be performed so that bioavailability can be estimated. If information is available on treatability studies conducted on the selected pesticides, it should be considered too. Experts with experience with the selected pesticides should be consulted; these experts may come from academia, various USEPA laboratories and offices, or private industry.

6.2 Pesticide Treatability Classes

The term, "pesticide" unites a large and diverse body of organic and inorganic chemicals based solely on function rather than on the basis of chemical structure. This useful term falsely implies a singularity in chemical behavior and environmental fate. In fact pesticides represent a wide range of chemical families, and even members within the same family may have widely divergent environmental fate characteristics. It is impossible to make a single, general

state... ...t about the biodegradability or bioremediation poten... of all pesticides. Some pesticides are relatively amenable to biological degradation while others are highly recalcitrant, or resistant to degradation. For some pesticides, the degradation pathways have been well studied and documented. For others, the routes of microbial degradation are relatively poorly understood. The body of literature available on the microbial degradation pathways for the various pesticides is immense; a review of this literature goes well beyond the scope of this report.

In order to provide some information on the probable success of bioremediation technologies for pesticide contaminated media, the precedent set by Des Rosiers (1990), USEPA (1989) and the Illinois Department of Agriculture and The Illinois Geological Survey (1993) has been used to divide the pesticides commonly found at Minnesota sites into treatability classes (Table 6.1). The treatability classes are taken from USEPA (1989) and Des Rosiers (1990) and were originally prepared for the Office of Solid Waste of the USEPA to classify wastes under the Resource Conservation and Recovery Act (RCRA) (USEPA, 1989). The final column in table 6.1 ranks bioremediation potential for the different treatability groups, based upon conclusions in USEPA (1989) and Des Rosiers (1989). This information is admittedly useful only on a very coarse scale. Ideally, more detailed information will become available as ongoing studies; and remediation projects provide the information needed to judge effectiveness of the different bioremediation strategies for individual pesticide-families or compounds.

Table 6.1

Pesticide Treatability Classes For MDA List 1 Pesticides -Adapted from Illinois Department of Agriculture and Illinois Geological Survey (1993).

Treatability Class	Representative Pesticides	Corresponding Family	Predicted Bioremediation Effectiveness*
Non-Polar Halogenated Aromatics (W01)			
Halogenated Phenols, Creosols, Amines, Thiols, and other Polar Aromatics (W03)	Alachlor Atrazine Chlorpyrifos Cyanazine Metolachlor Propachlor Propazine Simazine (Pentachloro-phenol--non-ag-ricultural)	Acetanilides s-Triazines Heterocyclic Organo-P s-Triazines Acetanilides Acetanilides s-Triazines s-Triazines	PE (Des Rosiers, 1990) Literature review indicates that bioremediation should be successful for triazines and acetanilides. Acetanilides may be problematic. Pentachlorophenol appears to be very amenable to treatment. Limited data on other compounds.
Nitrated Aromatics (W06)	Ethalfluralin Pendimethalin Trifluralin	Dinitroanilines " "	DE (Des Rosiers, 1990) Literature review inconclusive; composting results promising but may have been due to abiotic mechanisms.
Heterocyclics (non-halogenated) and Simple Non-Halogenated Aromatics (W07)	Metribuzin Prometon	non-symmetric Triazines s-Triazines	DE (Des Rosiers, 1990) Literature review inconclusive. May be similar to other triazines.
Other Polar Non-Halogenated Organic Compounds (W09)	Butylate EPTC Linuron Metribuzin Phorate Prometon Triallate	Thiocarbamates Thiocarbamates Substituted Ureas non-symmetric Triazines Organophosphates s-Triazines Thiocarbamates	DE (Des Rosiers, 1990) Literature review inconclusive.

*PE=Potential Effectiveness DE=Demonstrated Effectiveness NEE=No expected effectiveness; Treatability conclusions are from desRosiers (1990) and USEPA (1989b). Conclusions (PE, DE, NEE) are for treatability class and may be based on non-pesticide compounds from same treatability group. Additional notes based upon this literature review.

Chapter 7: Summary and Recommendations

The use of bioremediation technologies on pesticide contaminated media appears promising but remains largely untested at the field scale. Bioremediation is an evolving technology which has been applied most commonly to specific wastes for which there is an established commercial market (petroleum-related, wood preservative and explosive-related wastes). The treatment strategies and technologies which are currently available have been tested to varying extents on pesticides in laboratory studies and in bench-scale systems. Laboratory scale studies have widely demonstrated the biodegradability of many pesticide families under specific and well defined conditions. However, it is important to recognize that the demonstration of biodegradation of a contaminant in the laboratory does not directly translate to the successful bioremediation of that contaminant in the field.

Since very few of the existing technologies have been applied to pesticides, and since pesticide degradation is very compound specific, it is impossible to make conclusive statements regarding the use of the individual technologies on all pesticides. No single bioremediation approach will act as a "silver bullet" for all remediation needs. However, of the currently available treatment strategies and technologies, several stand out as especially promising. Although more difficult to implement than strict aerobic treatment, sequential anaerobic/aerobic treatment appears to be applicable for a broad range of pesticides and makes particular sense for complex pesticide mixtures. Strict anaerobic treatment may be suitable for the older, heavily chlorinated pesticides, although sequenced treatment again appears equally appropriate. Composting appears promising for many of the compounds commonly found at sites in Minnesota; this is true not only from a technical viewpoint but also because the low-tech characteristics of composting are ideally suited to the small scale of most agricultural chemical dealership sites.

At this point in the development of the technology, each bioremediation project requires systematically designed treatability studies to test the application of a specific technology on site-specific contaminant and environmental conditions. Treatability studies guide in the selection of the most applicable biotreatment strategy and are necessary to optimize the selected strategy for the site specific conditions. Treatability study requirements for pesticide contaminated sites are currently not standardized for the industry. It would help the development of the technology if treatability study requirements were standardized. It should not be necessary to perform exhaustive pesticide fate studies as part of each treatability study. However, the expected degradative pathways of each contaminant must be identified from the literature so that potential toxic intermediates can be identified and tested during the treatability study.

Research should be promoted in two distinct directions. First, there is a great need for pilot- and field-scale demonstration projects of the existing strategies and technologies on pesticide contaminated media. Although many potential constraints may be addressed at the treatability

study stage, some problems will only become evident in a "real world" situation. Secondly, basic research is also needed. As the general understanding of specific pesticide degradation mechanisms and the factors affecting them grows, bioremediation technology will become more available for practical applications to pesticide contaminated soils and waters.

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Appendix 1

Guidance Documents for Ag-Chem Facility Investigations

Minnesota Department of Agriculture • Agronomy Services Division
90 West Plato Boulevard • St. Paul, Minnesota 55107
Telephone: (612) 297-3490

REIMBURSEMENT OF COSTS FOR AGRICULTURAL CHEMICAL INCIDENT CLEANUPS

AGRICULTURAL CHEMICAL RESPONSE AND REIMBURSEMENT ACCOUNT (ACRRA)

February 4, 1993 — Factsheet ACRRA.001

THE AGRICULTURAL CHEMICAL RESPONSE AND REIMBURSEMENT ACCOUNT (ACRRA)

The Agricultural Chemical Response and Reimbursement Account (ACRRA) was created by the 1989 Minnesota Groundwater Protection Act. The ACRRA fund was established primarily to reimburse persons for costs incurred after July 1, 1989, in cleaning up agricultural chemical (pesticide and fertilizer) incidents.

The account is funded by annual surcharges on pesticide and fertilizer manufacturers, distributors, applicators and dealers. The amount of sere-charges levied will largely be determined by the current ACRRA fund balance; the account has a required statutory minimum balance of $1,000,000 and a maximum balance of $5,000,000. The Commissioner of Agriculture determines if the surcharge must be increased.

Monies from the ACRRA fund can be used for reimbursement of costs resulting from cleanup of sudden incidents, such as fire or transportation accidents, or it can reimburse persons for cleaning up sites contaminated with agricultural chemicals. At present, it is the only program of its kind in the country. ◘

AGRICULTURAL CHEMICAL RESPONSE COMPENSATION BOARD (ACRRA BOARD)

The ACRRA fund is administered by the Agricultural Chemical Response Compensation Board (ACRRA Board). The ACRRA Board will determine and order reimbursements or payments from the fund to eligible persons. An eligible person is defined as a responsible person or an owner of real property, but does not include local, state or federal government, or agencies. ◘

REIMBURSEMENT OF CORRECTIVE ACTION COSTS

Before any reimbursement can be made, the Board must determine the following:

- The Minnesota Department of Agriculture (MDA) was given proper notice of the incident as required under Minnesota Statutes, Chapter 18D;
- The costs of investigation and cleanup were reasonable and necessary; and
- The eligible person complied with corrective action requests or orders issued by MDA or the eligible person took all reasonable action necessary to minimize and abate the incident, such as a spill, and the corrective action was subsequently approved by MDA.

If the conditions listed are met, the Board may reimburse an eligible person for:

- 90 percent of the total reasonable and necessary corrective action costs greater than $1,000 and less than $100,000; and
- 100 percent of the costs equal to or greater than $100,000 and less than $200,000.

(Continued on reverse side, see "REIMBURSEMENT")

REIMBURSEMENT

(Continued From Page 1)

The Board will not provide reimbursement until it has determined that the costs on the reimbursement request actually were incurred and were reasonable. The Board has authority to reduce reimbursement if the incident was caused by a violation of Minnesota Statutes, Chapters 18B, 18C, or 18D. ◘

PAYMENT OF CORRECTIVE ACTION COSTS

By request from an eligible person, the Board may pay the reasonable and necessary corrective action costs to the eligible person as they are incurred. Before any payments can be made, the Board must determine that the eligible person has complied with the following conditions:

- MDA was given proper notice of the incident as required in Minnesota Statutes, Chapter 18D;
- The responsible person must pay the first $1,000 of corrective action costs;
- The eligible person provides the Board with a sworn affidavit and other convincing evidence that the eligible person is unable to pay additional corrective action costs; and
- The eligible person continues to assume responsibility for carrying out the requirements of corrective action requests or orders.

An eligible person must refund any amounts paid and is not eligible for payment or reimbursement if false statements or misrepresentations are made regarding the eligible person's financial status. ◘

HOW TO PARTICIPATE IN THE ACRRA PROGRAM

1) Send a written request for an application to: ACRRA Program, Minnesota Department of Agriculture, Agronomy Services Division, 90 West Plato Blvd., 4th Floor, St. Paul, MN 55107

2) Fill out the application completely, attach appropriate documents, and submit to the ACRRA Program. The completed application must be received at least 30 days prior to the next Board meeting to be considered for reimbursement at that meeting.

3) The Board reviews the application, decides whether to order payment, and determines the amount to be reimbursed.

4) The Commissioner of Agriculture releases the reimbursement payment. The anticipated turn-around time is about 60 days. ◘

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For further information, call:
Sharon Haber, ACRRA Administrator, MDA (612) 297-3490

INELIGIBLE COSTS

Ineligible costs include, but are not limited to:

- All costs associated with actions that do not minimize, eliminate, or clean up an incident;
- Costs related to the repair or replacement, or upgrading of facility structures or equipment;
- Loss of income;
- Attorney's fees;
- Permanent relocation of residents;
- Decreased property values;
- Reimbursement for the eligible person's own time spent in planning and administering a corrective action design;
- Costs for third-party review of proposed investigative and corrective action or work plans;
- Aesthetic improvements;
- Any work, except for emergency corrective actions not in compliance with safety codes, including, but not limited to OSHA requirements, well codes, and fire codes;
- Costs for providing alternative sources for drinking water;
- Corrective action costs covered or payable under an insurance or other contract;
- Costs incurred in response to requests or orders issued to a person under authorities contained in Minnesota Statutes, Chapter 115B, or federal CERCLA–Superfund. ◘

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GUIDANCE FOR SUDDEN AGRICULTURAL CHEMICAL INCIDENT CLEANUP

This guidance document is one of a series that have been prepared by the Minnesota Department of Agriculture (MDA). To request a list of the guidance documents and the documents themselves, please call (612) 297-1975.

When a person discovers a leak, an overfill, a spill, or other signs of an agricultural chemical incident, the following steps should be taken to clean up the release and to comply with state and federal laws regarding agricultural chemical incident cleanups and to remain eligible for reimbursement of cleanup costs.

1. REPORT THE INCIDENT IMMEDIATELY

Under state law, anyone who has control of, custody of, or responsibility for an agricultural chemical is considered to be a responsible party and must notify the Minnesota Department of Agriculture (MDA) immediately when an incident involving that agricultural chemical occurs. Notify MDA of an incident as soon as possible by calling the 24-hour duty officer at the Department of Public Safety at *649-5451* (metro) or *1-800-422-0798* (non-metro). The MDA staff on duty will call you back to explain what steps to take.

Also notify MDA of suspected incidents including the discovery of product-contaminated soils, contaminated wells or surface water, product inventory loss and failed tank or pipeline tests.

Follow up your initial call with a confirming letter.

2. STOP ANY FURTHER CONTAMINATION OR HAZARD

If there is a release, take immediate action:

- Prevent further release of agricultural chemicals into the environment;
- Reduce the conditions that might present a public health risk or hazard from exposure or fire;
- Remove any other potential sources of further contamination; and
- Sometimes escaped product must be pumped out or contaminated soils must be excavated and safely stored or treated.

3. TAKE CORRECTIVE ACTION

Corrective action includes cleaning up or minimizing the impact of a release to public health and the environment. By taking immediate action to clean up a release and prevent further contamination, the corrective action may be completed in little time. Corrective actions for more extensive or complex site contamination usually take extended periods of time to complete.

4. SEND FOLLOW-UP REPORT TO MDA

After the cleanup has been completed, send a follow-up report to MDA. The report is necessary to document that the site cleanup has been adequately addressed so the Agricultural Chemical Response Compensation Board can determine your eligibility for reimbursement. If the cleanup is judged to be inadequate, no reimbursement of costs can be made.

5. APPLY FOR REIMBURSEMENT

Upon completion of steps 1-4 listed above, a responsible person who has investigated and taken corrective actions in response to a agricultural chemical incident may apply to the ACRRA board (which administers the Agricultural Chemical Response and Reimbursement Account) for partial reimbursement of cleanup costs incurred after July 1, 1989. Before you can receive reimbursement, the board must determine the following:

- You notified MDA of the incident as required in Minnesota Statutes Chapter 18D;
- Your costs for the investigation and corrective action were reasonable and necessary; and
- You complied with corrective action request or order issued by MDA; or you took all reasonable action necessary to minimize and abate an incident, such as a spill, and the corrective action was subsequently approved by MDA.

If all these conditions are met, the board may reimburse you for up to 90 percent of the total reasonable and necessary corrective action costs greater than $1,000 and less than $100,000, and 100 percent of costs between $100,000 and $200,000.

The board will not provide reimbursement until it has determined that the costs on the reimbursement request actually were incurred and were reasonable. The board has the authority to lower the amount of reimbursement if the conditions were not adequately met or the board determines that the incident was caused by a violation of Minnesota Statutes Chapter 18B, 18C, or 18D.

FOR MORE INFORMATION on the Reimbursement Program call *(612) 297-3490.*

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SEVEN STEPS TO AN AGRICULTURAL CHEMICAL INCIDENT CLEANUP AT A STORAGE FACILITY

Under state law, the person who has custody of, control of, or is responsible for an agricultural chemical container or agricultural chemical rinsate at the time of an incident is generally considered the responsible person. An incident is a release or the threatened release of an agricultural chemical into the environment that may cause adverse environmental effects.

When a leak, an overfill, a spill, or other sign of an agricultural chemical incident is discovered, seven steps must be taken to comply with state and federal laws regarding agricultural chemical incident cleanups and to remain eligible for reimbursement of cleanup costs.

1. REPORT THE INCIDENT IMMEDIATELY

Anyone who has control of, custody of, or responsibility for an agricultural chemical must notify the Minnesota Department of Agriculture (MDA) immediately when an incident involving that substance occurs. Call the 24-hour duty officer at the Department of Public Safety at *649-5451* (metro) or *1-800-422-0798* (non-metro). The MDA staff on duty will call you back to explain what steps to take.

Also notify MDA of suspected releases including the discovery of product-contaminated soils, contaminated wells or surface water, product inventory loss and failed tank or pipeline tests. Follow up the call with a confirming letter.

2. STOP ANY FURTHER CONTAMINATION OR HAZARD

If there is a release, take immediate action:

- Prevent further release of agricultural chemicals into the environment;
- Reduce the conditions that might present a public health risk or hazard from exposure or fire;
- Remove any other potential sources of further contamination; and
- Sometimes escaped product must be pumped out or contaminated soils must be excavated and safely stored or treated.

3. PERFORM A SITE INVESTIGATION

To conduct most agricultural chemical incident investigations, you need to hire an environmental consultant to do the necessary investigative and cleanup work. Before hiring a consultant, contact your insurance company. If your policy covers this type of incident, your insurance company may hire the consultant. The consultant typically will design and implement a preliminary investigation that will define the area of contamination and determine effects on ground and surface waters and soil. The information from this investigation will help do the following:

- locate and confirm the source of the incident;
- estimate the volume and type of product released;
- assess the magnitude of soil, ground water and surface water contamination; and
- identify migration routes, exposure points and impacts.

For more detailed information about what is typically required in a site investigation refer to the factsheet "Work plans for Remedial Investigations", available from MDA. The MDA must approve the plan prior to implementation.

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4. SUBMIT A REMEDIAL INVESTIGATION REPORT

When the site investigation is complete, submit an RI Report to MDA containing 1) site history and background information; and 2) site investigation results.

Much of the background information can be gathered by you, including specific information of the site such as names, addresses and phone numbers of current and past owners, general construction and agricultural chemical use history, and the cause of the incident.

Your consultant will provide all the information gathered during the site investigation for the initial report.

5. PREPARE A CORRECTIVE ACTION PLAN

After the investigation is completed, prepare and submit a Corrective Action Plan to the MDA for review and approval. The plan should describe how the contaminated soil and/or ground water will be cleaned up. The plan should adequately address impacts on and protection of public health and the environment.

The Corrective Action Plan must be approved by the MDA prior to implementation.

6. APPLY FOR INITIAL REIMBURSEMENT

Steps one (1) through five (5) listed above are collectively referred to as a Corrective Action Design. After you have completed these steps, which includes all necessary MDA approvals, you are eligible to apply to the ACRRA Board which administers the Agricultural Chemical Response and Reimbursement Account, for initial reimbursement of your costs.

7. TAKE CORRECTIVE ACTION

Take corrective action as approved in the Corrective Action Plan. Corrective action includes cleaning up or minimizing the impact of a release on public health and the environment. Many corrective actions may be implemented before or during the initial investigations. Some corrective actions will take little time to complete. Others are design to operate for extended periods to complete cleanup of more difficult or more extensive parts of the contamination. Corrective actions may include, but are not limited to the following;

- excavation of the contaminated soil;
- on and off-site treatment and disposal of contaminants;
- installation of ground water recovery systems;
- ground water treatment;
- biological treatment of soil;
- surface water/drainage controls; and
- restoration of contaminated water supplies and utility/sewer lines.

Submit a follow-up report to the MDA. This report is necessary to document the cleanup progress and indicate whether the site cleanup has been adequately addressed. An eligible person may subsequently apply at periodic intervals for reimbursement of additional costs incurred during the cleanup.

FOR MORE INFORMATION about ACRRA reimbursement call MDA at *(612) 297-3490.*

FOR MORE INFORMATION on MDA's Incident Response or to request a list of Environmental Consultants, call *(612) 297-1975.*

TO REPORT A SPILL OR LEAK call the Department of Public Safety's 24-hour duty officer at *649-5451* (metro) or *1-800-422-0798* (non-metro).

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AGRICULTURAL CHEMICAL INCIDENT REMEDIAL INVESTIGATION REPORT

A Remedial Investigation (RI) is generally required when an agricultural chemical (pesticide or fertilizer) incident occurs. An incident is a release or threatened release of an agricultural chemical into the environment that may cause adverse environmental effects. All agricultural chemical incidents must be reported ***immediately*** to the Minnesota Department of Agriculture (MDA) at *649-5451* (metro) or *1-800-422-0798* (non-metro).

An RI at an agricultural chemical incident site must accomplish the following:

- Identify the source;
- Define the extent and magnitude of contamination in both the soil and the ground water;
- Identify all actual or potential impacts resulting from the release; and
- Provide adequate information for designing any required corrective actions.

The amount of work necessary to accomplish these objectives varies according to the complexity of the site, the type and the amount of contamination. For example, an assessment of all potential sources of agricultural chemical contamination on a site is necessary if there is evidence of general site contamination. Potential areas of contamination include: Pesticide loading, bulk chemical load out, impregnated fertilizer load out, tank cleanout/rinsate disposal, unrinsed pesticide container storage, water fill sites, refuse burn piles and application equipment parking. The environmental consultant, and ultimately, the responsible party, is responsible for adequately investigating the site and recommending additional investigation or corrective actions as appropriate.

All field investigations must be conducted in accordance with all federal, state and local laws, rules, regulations and ordinances, with approval, if required, from the appropriate state and local authorities.

Guidance documents on a number of technical subjects related to pesticide incidents are available from MDA. A list of the available guidance documents also can be obtained from MDA.

Work plans for all RI activities must be approved by the MDA staff prior to the initiation of any RI work. (For more information about RI Work plans refer to the Guidance Document *"Work Plans for Remedial Investigation."* This approval is necessary in part, if the RP intends to apply to the ACRRA Board (which administers the Agricultural Chemical Response and Reimbursement Account) for partial reimbursement of investigation and cleanup costs. Only the costs that are considered reasonable and necessary will be eligible for reimbursement. For more information about reimbursement, refer to Minn. Stat. Chapter 18E or call MDA at *(612) 297-3490.*

After the field work is completed, an Agricultural Chemical Incident Remedial Investigation Report (RI Report) must be submitted to MDA.

The following information in this document is designed to provide guidance for drafting an RI Report. Although the following RI Report format is not required, MDA staff have the option to reject incomplete reports. If RI activities do not accomplish the RI objectives, additional activities and reports may be necessary.

The RI Report should be a comprehensive document. All data used in the RI such as geologic logs or well construction diagrams should be reproduced in the appropriate tables and appendices rather than simply referenced to a previous report.

REMEDIAL INVESTIGATION REPORT

1. **INTRODUCTION** - This section of an RI Report should address the following:

   - the purpose for the investigation;
   - when and by whom the work was authorized;
   - the scope of services for the project;
   - a brief summary of the report; and
   - the dates the work was performed.

2. **BACKGROUND INFORMATION** - This section should contain:

   - a brief chronology of events related to the incident, including the source of the release and the estimated volume of the release;
   - a description of the site, the surrounding area and the anticipated hydrogeology;
   - a description of known or suspected contaminants or sources in the area, such as petroleum tanks or dispensers, non-agricultural chemicals or other potential agricultural chemical sources; and
   - previously reported incidents or releases at the site.

3. **RESULTS** - This section should describe:

   - the results of all work conducted during this investigation, such as (but not limited to) soil borings, monitoring wells, trenches, laboratory analyses, water level measurements and tank testing results;
   - a summary of the results of calculations made during the investigation including those used to determine hydraulic conductivity, hydraulic gradient, and ground water flow direction; and
   - If ground water has been affected by the release, an Impact Survey should be conducted (see attachment) and the results reported in this section.

4. **DISCUSSION** - This section should describe:

   - the results of all work performed;
   - the site geology and hydrogeology;
   - a comparison of results of current work with the results of previous work;
   - any difficulties experienced during the investigation;
   - unanticipated or questionable results; and
   - any details the author wishes to emphasize.

5. **CONCLUSIONS** - This section should summarize the findings of the investigation including:

   - the source of the contamination;
   - the extent and magnitude of contamination in soil and/or ground water; and
   - an evaluation of the potential impacts and receptors from the contamination.

6. **RECOMMENDATIONS** - This section should present:

   - recommendations for any additional investigation or corrective actions;
   - rationale for the recommendations;
   - If corrective action is recommended, a "*Corrective Action Plan Proposal*" must be submitted; and
   - If "*no further action*," is recommended, it must be justified.

7. **SIGNATURE AND DATE** - The author's name, address, telephone number, signature, and date of signature should be in the report.

8. **FIGURES**

   A. **Maps**

   - All maps must include a north arrow, scale, and a legend.
   - Site Location Map - Adapt from a U.S. Geological Survey 7.5 minute quadrangle.
   - Site Map - Present all significant features of the site and adjacent properties including, when applicable, the following information:

     (a) Physical layout including buildings, roads, paved areas and water bodies. Identify features by name or owner;
     (b) All potential sources of contamination, including non-agricultural sources;
     (c) Location of all wells and soil borings conducted to date including private wells, tile lines and sumps;
     (d) Approximate extent of soil excavation;
     (e) Soil sampling locations and depths; and
     (f) Utility lines, storm and sewer lines, and tile lines.

   - Agricultural Chemical Incident Impact Survey Results - Identify and label the locations of all private wells or other potential receptors.
   - Ground water Contour Map - Show all well locations and differentiate the wells constructed in different aquifers. Label ground water contours and elevations at each data point used for contouring. List the date the water level measurements were collected.

   B. **Cross-sections** - Two perpendicular cross-sections are recommended. Include vertical and horizontal scales and a key. Identify the location and direction of the endpoints.

   C. **Water Level Measurements and Water Quality Diagrams** - Use of a graphical presentation of water level measurements and water quality results is recommended.

9. **TABLES**

A. **Soil Analysis Results** - Include the laboratory results for all soil analyses, the laboratory name, sampling date, date the samples were analyzed, and identify the sample location and depth of each sample.

B. **Water Level Measurements** - Include all data (depth and elevation) collected at the site to date, presented in chronological order by measurement date for each monitoring point.

C. **Water Quality** - Include all data collected at the site to date, presented in chronological order by sampling date for each monitoring point.

D. **Well Construction** - Summarize monitoring well information. Include the elevations of the top of riser, ground surface, top of well screen, bottom of well and/or well screen, top of filter pack, and top of seal (if used). Include the depth of the top and bottom of the screened interval or open hole. Reference elevations to the National Geodetic Vertical Datum (NGVD) or to a local benchmark which is identified. List Minnesota unique well numbers for all wells.

E. **Agricultural Chemical Incident Impact Survey Results** - Prepare a table in conjunction with a figure to identify potential receptors of contamination from the site.

10. **APPENDICES** - This section should contain sufficient information to document all activities conducted during the investigation and corrective actions. All data must be legible. At a minimum, this information must include the following (when applicable):

A. Descriptions of all methods and procedures (with references, when appropriate) used in the investigation or corrective actions, including soil borings, soil sampling, well installation, water sampling and a list of laboratory methods used for soil and water analyses;

B. Geologic logs for each well or soil boring including logs from previous investigations. Geologic logs should contain the date drilled, name of drilling firm, drilling method, the surveyed elevation of the ground surface, interval sampled, blow counts, classification of soils (ASTM method D 2487/D 2488), and observations during drilling such as staining or odors;

C. Monitoring well construction diagrams for all monitoring wells, and copies of Minnesota Department of Health Water Well Records for each well;

D. Copies of laboratory or subcontractor reports;

E. Copies of water well records obtained for the Agricultural Chemical Incident Impact Survey;

F. Field data and calculations performed for the investigation;

G. Copies of permits or approvals required for all work; and

H. All information requested in the guidance document "*Ground Water Sample Collection Protocol*" and "*Soil Sample Collection Protocol.*"

AGRICULTURAL CHEMICAL INCIDENT IMPACT SURVEY

An Agricultural Chemical Incident Impact Survey should be conducted at every site where an agricultural chemical incident has affected ground water (this may be preceeded by a general survey of the area during the reconnaissance site visit). The results of the Impact Survey should be included in the Agricultural Chemical Incident Remedial Investigation (RI) Report that is sent to the Minnesota Department of Agriculture (MDA). The purpose of the survey is to aid the evaluation of potential impacts from the incident. The following activities should be done for the survey:

1. Identify all surface water that potentially may be affected.
2. If ground water has been affected, complete the following activities:
   a. Determine whether a municipal water supply is in the site vicinity. Identify any present or potential municipal water supply located within a one-mile radius;
   b. Obtain copies of all well logs within a one-mile radius of the site that are on file with the Minnesota Geological Survey. If more than 50 well logs are present, contact MDA project staff to determine which well logs to obtain and include in the RI Report. If the affected water source is a regional aquifer, obtain all well logs from wells constructed in the affected and connected aquifers within a two-mile radius of the site;
   c. Contact appropriate local authorities, county water planning officials and local well drillers to determine whether any unregistered or abandoned wells are located within a one-mile radius of the site. Report the location, construction, depth and use of any identified wells if the information is available;
   d. Contact the site owner and owners of all property that adjoins the site to determine whether existing or abandoned wells are located on their properties. Report the location, construction, depth and use of any identified wells if the information is available;
   e. Summarize the data on all identified wells in a table, including the following information: Minnesota unique well number or other identifier; the ground surface elevation; the well base elevation; the casing base elevation; water level elevation; and aquifer use. Elevations should be referenced to the National Geodetic Vertical Datum, if possible. Produce a map showing the location of all wells with the map labels corresponding to the information on the tables;
   f. Contact appropriate local authorities and property owners to determine whether any ground water development is scheduled up to one mile down-gradient of the site;
   g. Provide legible copies of all well logs obtained with the Minnesota unique well number or other identifier corresponding to the table discussed in number 2d clearly marked;
3. Identify all other receptors potentially impacted by the agricultural chemical incident (e.g., tile lines, utilities, storm and sewer drainage system, air quality).

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WORK PLANS FOR REMEDIAL INVESTIGATIONS

The preliminary objectives of a remedial investigation (RI) are to: identify the contaminate and contamination sources; define extent and magnitude of contamination in both soil and groundwater; identify impacts relating to the contamination; and provide adequate information for designing any required corrective actions.

A work plan generally is required for all remedial investigations. Work plans for RI activities must be approved by the MDA staff prior to the initiation of any RI work. The purpose of the work plan is to present details pertaining to how the information required for the RI report will be obtained. Review of MDA site files, site visits and interviews with facility personnal may be needed prior to developing a detailed work plan.

The following information provides a general framework for submittal of RI work plans. Additional site specific information should be included where appropriate.

INTRODUCTION

- the purpose for the investigation
- when and by whom the work was authorized

SCOPE OF WORK

- brief description of services to be performed at the site
- if the investigation is to be performed in phases, briefly describe the scope of each phase

BACKGROUND INFORMATION

- brief site chronology (including dates)
- brief description of site operations, current and historical (for example: fertilizer impregnation; pesticide mixing and loading; bulk pesticide storage)
- description of previous releases (date, product and amount)
- list areas of known or suspected contamination (cite references)
- brief description of work performed to date
- list of references

SITE DESCRIPTION

- soil type(s)
- estimated depth to ground water
- anticipated regional ground water flow direction
- anticipated hydrogeology
- topography
- list of references

SAMPLE COLLECTION

- soil sample plan (types and depths)
- sample locations (locate on site map)
- number of borings to be completed
- number of samples to be collected
- number of wells and locations (locate on site map)
- sample analysis parameters
- field screening methods, supporting documentation and how they are to be used
- sample analysis plan describing which samples will be analyzed and which will be held (frozen)
- grouting

SAMPLE COLLECTION PROCEDURES (consult guidance documents for details)

- General:
  - sampling equipment
  - equipment cleaning
  - sample storage, containers, transport, preservation, handling and chain of custody
- Soil:
  - drilling method and borehole diameter
  - sample intervals (analytical and lithologic)
  - how samples will be collected
  - lithologic classification methods
  - sample type (composites, discretes)
  - how and when samples will be split
- Ground Water:
  - water level measurement
  - well stabilization
  - how samples will be collected
  - sample preservation
- Surface Water:
  - sample location and depth
  - how samples will be collected and characterized

LABORATORY QA/QC

- name of laboratory performing analysis (must have QA/QC and analytical methods approved and on file with MDA)
- sample holding time
- approximate sample turnaround time

WELL DRILLING and INSTALLATION METHODS

- well locations and explanation of location selection
- construction methods (drilling method, well diameter, screen length and placement, well protection)
- construction materials (screen and riser materials, filter pack and interval, seal materials)
- compliance with Minnesota Department of Health Water Well Code
- collection and disposal of cuttings
- well development
- equipment cleaning
- grouting methods
- well abandonment

MAPS

- topographic map with site location indicated (adapted from USGS quads)
- soil map and legend, if available
- site map showing all permanent structures (labeled), legend and north arrow (scale helpful but not required)
- outline of investigation areas, approximate boring locations and well locations

IMPACT SURVEY (if groundwater is affected)

- how and when it will be conducted (this may be preceded by a general survey of the area during the reconnaissance site visit)

WORK SCHEDULE

- proposed timetable for work to be performed

COSTS

- outline of costs associated with proposed work

Attachment 1

GUIDELINES FOR REMEDIAL INVESTIGATIONS AT AGRICULTURAL CHEMICAL FACILITIES

The following general procedures apply to agricultural chemical incident investigations.

1. Investigation activities should focus on those areas where contamination is most likely. This includes: the mix and load area; the fertilizer impregnation tower area; the interior of earthen dikes; equipment parking areas; bulk storage areas; any areas where bulk pesticides and fertilizers were loaded or unloaded; areas with pesticide staining; dead or barren vegetation areas; pesticide container storage areas; small package loadout areas; scale pits; areas where pesticide containers have been burned; areas where runoff ponds; water fill sites; facility wells; areas associated with spills at the facility.

2. In general, investigations at agricultural chemical facilities should be conducted in a phased approach. The first phase, the site reconnaissance, should consist of collecting a composite sample(s) from each high risk area at a facility to identify areas that require more detailed investigation. The second phase of a facility investigation, the detailed remedial investigation, should consist of collecting sufficient analytical data to determine the horizontal and vertical extent of contamination in each area of concern. The data should be adequate to design and conduct a site cleanup (corrective action). These two phases may be combined where practical.

3. Reconnaissance sampling may be conducted by collecting composite samples. Each composite sample for reconnaissance purposes should consist of three (3) to six (6) evenly spaced subsamples from an area roughly fifteen (15) feet in diameter. The subsamples should be taken at the same depth. For surface composites in non-graveled areas, samples should be taken from the zero (0) to six (6) inch layer. In graveled areas samples should be taken from one (1) to one and one half (1½) feet below grade.

   MDA staff recommends that one discrete "grab" sample be collected during the site reconnaissance from the 2-3 feet and the 5-6 feet depth intervals in each high risk area (in obviously stained areas, surface grab samples should also be collected) by hand auger boring or test trenching during the site reconnaissance. The grab samples should be stored frozen under proper chain of custody and analyzed sequentially from the surface downward in the event that the composite sample is contaminated. The actual depth of sampling and sequence of sample analysis may vary depending on site conditions.

4. Applicable field screening methods may be approved and are encouraged for remedial investigations as long as documentation relating to their use in similar investigations is provided. If adequate documentation is not available, the use of field screening methods may be approved if the field data is supported by laboratory data. Field nitrate determination and immunoassay kits for pesticide detection are examples of potentially useful field screening methods.

5. For lithologic classification, soil borings should be advanced using hollow stem auger methods with split spoon samples (ASTM D 1586) collected every two (2) feet for the first ten (10) feet below ground surface and at every five (5) feet and at changes in lithology thereafter. Classify soil samples in accordance with ASTM methods D 2487 or D 2488. Solid stem auger borings or less frequent sampling intervals may be conducted with prior approval from MDA staff.

6. Collect analytical soil samples from soil borings at appropriate, site specific intervals to define the potential area for excavation. Selected soil samples from the borings can be frozen for future analysis and analyzed in a phased approach, pending interpretation of the data from the first round of analysis.

7. One deep hollow stem auger boring (to 25 feet or the water table whichever is less) generally will be required in each contaminated area (see precautions in item 9 of this document). At least one (1) boring to the water table (for lithological and analytical sampling) should be completed at each site, unless MDA staff approve otherwise (Ex: excessive depth to water table).

8. Soil borings not completed as monitoring wells or in areas that will not be excavated should be immediately grouted from the bottom of the boring to the ground surface to prevent the potential vertical migration of contamination.

9. Soil borings should not be a conduit for vertical migration of contamination. Soil borings should not penetrate through confining layers below saturated zones or connect aquifers.

10. If monitoring wells are installed, a minimum of three wells is necessary to define the ground water flow direction. At most large facilities, more than 3 wells may be required. Locate monitoring wells to adequately document ground water impacts. Construct monitoring wells using stainless steel construction materials (PVC may be allowed for nitrogen investigations if pesticides haven't been handled at the site) below the water table unless other construction material are approved by MDA staff. MDA staff recommends that monitoring wells be protected from galvanic corrosion. Any monitoring wells designed to intercept the water table or perched water should be constructed to allow for seasonal fluctuations in water levels.

11. For pesticide investigations, bailers must be made of stainless steel or teflon. PVC bailers may be used for nitrate sampling. Disposable bailers meeting the above qualifications may be used.

12. When collecting surface water samples, the water temperature and flow rate should be determined for the sample location to document conditions at time of sampling.

13. Guidance documents on collecting soil and ground water samples are available from the MDA.

14. Do not excavate contaminated soils without prior approval from MDA staff. Store excavated contaminated soils on an impermeable surface, contour the pile to prevent infiltration from precipitation or surface runoff and cover with plastic. Weigh down the plastic covering the pile with clean soil or other suitable material to prevent it from blowing off or getting damaged. Damaged plastic coverings must be repaired and replaced immediately. Keep soil piles from each excavation area separate. Sampling of the pile may be required prior to land application and should be done according to the following schedule. Once composition of the pile is known, an application to land apply contaminated soil should be filled out.

Volume of Soil (cubic yards)	Minimum number of Samples
< 200	1
200 - 500	2
500 - 1000	3
1000 - 2000	4
Each additional 2000 yds.	1

15. The MDA has prepared standard analytical lists for use in pesticide incident investigations. These lists should be adapted to site specific conditions. All samples should be collected, transported and stored in accordance with EPA approved procedures. QA/QC plans and analytical methods for commercial laboratories must be approved by MDA. Lists of commercial laboratories with approved QA/QC plans are available from MDA on request.

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ANALYTICAL LISTS
FOR PESTICIDE INCIDENT INVESTIGATIONS

The Minnesota Department of Agriculture (MDA) has prepared the following standard analytical lists for soil and ground water analyses at pesticide incident investigations. At a typical pesticide incident site, MDA staff will require that soil and ground water samples be analyzed for the pesticides listed on one or more of the following lists plus any pesticides not on a list which are a concern at that site. The actual MDA requirements for each incident investigation are always site specific.

The Quality Assurance/Quality Control (QA/QC) plan and analytical methods used by the proposed commercial laboratory must be pre-approved by MDA **for each pesticide incident investigation**. A QA/QC plan on file at MDA and which has been approved within the last two years generally will be acceptable for fulfilling this requirement. Please note that the analytical methods must be pre-approved by MDA for each pesticide not on one of the analytical lists.

Pesticide residue samples should be collected, stored and transported using EPA approved methods and chain of custody procedures. Lists of commercial laboratories with approved QA/QC plans, and guidance documents on soil and ground water sample collection protocols are available from MDA on request.

Additional analytical lists are currently under development including many of the newer pesticides.

These lists will be reviewed and updated periodically:

List 1 Pesticides - (Neutrals)

1. alachlor (Lasso)
2. atrazine (Aatrex)
3. butylate (Sutan)
4. chlorpyrifos (Lorsban)
5. cyanazine (Bladex)
6. EPTC (Eptam/Eradicane)
7. ethalfluralin (Sonalan)
8. fonofos (Dyfonate)
9. linuron (Lorox)
10. metolachlor (Dual)
11. metribuzin (Lexone/Sencor)
12. pendimethalin (Prowl)
13. phorate (Thimet)
14. propachlor (Ramrod)
15. prometon (Pramitol)
16. propazine (Milogard)
17. simazine (Princep)
18. terbufos (Counter)
19. triallate (Far-go)
20. trifluralin (Treflan)

List 2 Pesticides - (Acids)

1. 2,4-D
2. 2,4-DB (Butyrac)
3. dicamba (Banvel)
4. chloramben (Amiben)
5. MCPA
6. MCPB
7. MCPP
8. picloram (Tordon)
9. 2,4,5,-T
10. 2,4,5,-TP (Silvex)
11. triclopyr (Garlon)

Analytical List for Pesticide Investigation (con't.)

List 3 Pesticides - (Carbamates)

1. aldicarb (total), including aldicarb sulfoxide, aldicarb sulfone, (Temik)
2. carbaryl (Sevin)
3. carbofuran (total), including 3-OH carbofuran, (Furadan)

Unique Chemistry -

1. bentazon (Basagran)
2. bromoxynil (Buctril)
3. clomozone (Command)

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INSTRUCTIONS FOR PROPOSAL TO LAND APPLY SOIL FROM AGRICULTURAL CHEMICAL INCIDENTS

This fact sheet contains instructions for completing the attached *Proposal to Land Apply Soil from Agricultural Chemical Incidents* form. At the discretion of Minnesota Department of Agriculture (MDA) staff, completion of the land application form, or selected portions thereof, may not be required for an emergency cleanup of spills, for small quantities of soil, or for other unusual circumstances.

GENERAL INSTRUCTIONS FOR PROPOSAL FORM

- Answers which cannot be completed in the space provided on the form may be continued on the back of the form or as a separate attachment.
- Attach to the proposal form legible copies of all analytical data for the soil to be land applied. Also attach all calculations necessary for determining the rate of application.
- Attach to the proposal form legible copies of the appropriate topographic maps, plat maps and soil maps (with legend) showing the location(s) of the proposed application(s).
- If you have questions regarding the completion of this form, please contact the MDA staff familiar with your site or phone the number listed at the top of this page.

GENERAL INSTRUCTIONS FOR EXCAVATION AND LAND APPLICATION OF SOIL

- Do ***not*** consolidate soil excavated from different areas of a facility unless prior approval to do so is obtained from MDA staff. Proposals to land apply individual soil piles should be prepared and evaluated separately.
- The pounds of pesticide applied in the land-spread soil must be accounted for when determining pesticide applications at the spreading site in the same season. Specifically, the sum of the land-spread application and subsequent applications in the same season (or following season, in the case of fall land-spreading) must not exceed label rate restrictions for any pesticide applied. In instances involving nitrogen spills or high soil-nitrogen content, the applied nitrogen should be considered when calculating nitrogen credits for the receiving acreage. The owner of the proposed application property must be informed of the amount and type of pesticide, and/or the pounds of nitrogen to be spread.
- A safety factor must be built into the proposed spreading rate to account for hot spots in the soil pile or the effects of pesticide mixtures. There are several ways to create a safety factor; these are described in points 8C, and 8F of the instructions.

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- Pesticide contaminated soil must be spread on a currently labeled site or crop in a manner consistent with the label directions. In the case of annual crops, the crop must be present on the application area during the current season. If land-spreading takes place in the fall, the crop must be present on the application area the following season.

LAND APPLICATION NOTES

1. Additivity: Certain pesticide families display similar biological activity; members of these families must be summed for application calculations. This policy is based upon information from pesticide manufacturers and pesticide labels (example: the Prowl label states that it is additive with trifluralin). The products which must be added are products which are not usually used together in the same crop year.

   Based on this information, you must add the following compounds:
   - Acetanilide Herbicides (alachlor, propachlor and metolachlor)
   - All Triazine herbicides (atrazine, cyanazine, etc.)
   - All Nitroaniline herbicides (trifluralin, pendimethalin, ethalfluralin, etc.)
   - Nitrate nitrogen and total Kjeldahl nitrogen (TKN)

   The application credit on additive compounds is based on the total pounds of all combined products divided by the application acreage. This amount is credited equally for all compounds that were added. For example, a total of 10 pounds of alachlor+metolachlor applied to 5 acres results in a 2#/acre application credit for alachlor and a 2#/acre application credit for metolachlor.

2. For post-emergence applications, application credits must include any pre-plant or pre-emergence applications of the same active ingredient.

3. Except in cases where incorporation is prohibited by the label (example: Prowl label prohibits pre-plant incorporation on corn) or in cases where a crop is present, land applied soil must be incorporated as soon as possible after application.

4. Atrazine application: The MDA actively promotes voluntary best management practices (BMPs) for atrazine. Currently, the MDA BMPs recommend no fall applications of atrazine; additionally, many atrazine labels prohibit fall application. For this reason, fall applications of contaminated soil piles containing atrazine will not be approved. Exceptions may be made in cases where the atrazine contamination is very low in comparison to the other contaminants or where extenuating circumstances prevent spring application. MDA staff should be contacted for approval prior to proposing fall application of soils containing atrazine.

5. Land spreading incompatible products may be approved in cases where the application rate will not result in crop injury or illegal crop residues. Since each pesticide mixture is unique, please contact the MDA staff familiar with your site or phone the number listed on page "i" of this form, for assistance with piles containing cancelled products or products which are incompatible with the crop selected.

6. Trifluralin application to corn is only labeled for Post-Emergent applications. Pre-emergent applications of trifluralin to corn may be approved by MDA staff in cases where trifluralin is not the limiting pesticide, as long as the application rate will not exceed 0.18#/acre, which is ½ of the phytotoxic level for a pre-emergent application on corn.

INSTRUCTIONS FOR COMPLETION OF PROPOSAL TO LAND APPLY SOIL

These instructions correspond to specific lines on the proposal form.

Item Instructions:

4 If the site has been used for waste disposal activities in the past, another site should be selected.

5 Soil from agricultural chemical incidents may not be land applied after the excavated soil pile is frozen. Excavated soil may be land applied to a frozen ground surface *only* if the applied soil can be incorporated and if there are no label restrictions against incorporation for the limiting (see Land Application Note #3) product.

8 In most cases, soil samples must be collected from the excavated soil pile and analyzed for a list of parameters which is approved by MDA staff. The resultant concentrations are then used to calculate the quantity of pesticide and/or nitrogen present. The pile should be sampled in a manner which represents the entire pile, including the suspected area of highest concentration. (See MDA Soil Sampling Protocol Guidance Document for information on sampling contaminated soil piles).

An alternative to sampling the soil pile is to use in-situ analytical data (see item 8C) obtained during the remedial investigation (prior MDA approval is needed to use in-situ data).

8A List all contaminants detected in the soil individually, unless the products are to be combined due to additivity (see Land Application Note #1). Additive compounds should be lsited together on the same line.

8B For pesticides, consult current EPA product labels for completion of this column. The label selected for each pesticide should be for the product spilled, if known. If the product is not known, select the label for the product most commonly handled at the spill site. In cases where additivity must be considered, you must choose the label for the contaminant which was detected at the highest level.

8C In cases where additivity must be considered, concentrations of additive compounds should be added together (not averaged). For completion of the rest of this table, the total will be treated as a single detected compound.

If only 1 sample was collected from the soil pile, circle "Max." at the top of the column and list the concentration detected for each contaminant. If more than 1 sample was collected from the soil pile, circle "Avg." and list the average concentration for each contaminant detected.

If in-situ data is to be used, you should circle "Max." and list the highest detection for each compound found in the excavation area. This calculation method will build in a safety factor and allow for excavation and land spreading without mixing the soil. An alternative to this approach is using a weighted average concentration for the contaminants detected (care must be taken to ensure that the pile is thoroughly mixed prior to spreading if a weighted average concentration is used). To use the weighted average concentration, circle "Avg." at the top of the column and list the weighted average concentration (in ppm), calculated as indicated below for each contaminant detected.

a) multiply: contaminant concentration (in ppm) x proportional extent of sample (% of excavation area represented by sample ÷ 100) = proportionate concentration (in ppm)

b) add all proportionate concentrations = weighted average concentration (in ppm)

8D Calculate the total quantity (in pounds) for each contaminant, using the concentration from column C (as described below). This calculation assumes a soil density of 2376lb/yd^3; alternatively, the site specific soil density may be used.

a) multiply:
volume of excavated soil (cubic yards) x 0.002376 million lb/yd^3
= millions of pounds of soil

b) for pesticides, multiply:
(millions of pounds of soil) x (concentration of pesticide in ppm)
= pounds of pesticide

c) for nitrogen, multiply:
(millions of pounds of soil) x [(concentration of TKN) + (concentration of NO_3-N)]
in ppm in ppm
= pounds of nitrogen

8E If the pesticide is not labeled for corn or soybeans, cross out the unlabeled crop and write in the labeled crop(s) selected.

8F For the soil texture and soil organic matter content at the proposed application site (listed in item 7), and each crop listed, enter ½ of the lowest application rate listed on the label for each pesticide present. If a pesticide is not labelled for a listed crop, fill in "N/A". The use of ½ of the lowest application rate builds in a safety factor. Prior MDA approval must be obtained for higher application rates.

For nitrogen assume an application rate of 100 pounds per acre. Alternatively, consult *"Fertilizer Recommendations for Agronomic Crops In Minnesota,"* 1990 Minnesota Extension Service Bulletin AG-MI-3901 (or current version), for crop-specific application rates.

8G For each crop listed and contaminant present, calculate the area required (in acres) to land apply the excavated soil using the total quantity (item 8D) from the excavated soil pile and the application rate (item 8F).

To calculate the minimum area required to land apply soil:
a) divide: (total quantity)/(application rate in pounds per acre) = *minimum acreage required*

9 At this point you may stop and mail or fax a completed copy of page 2 (item 8) of this form to the MDA for preliminary screening. MDA staff highly recommend this approach because the most common reason for application rejections or changes involve the selection of the appropriate crop and the proposed application acreage. (See Land Application Note #5)

9a Using the information in item 8, select the crop to be planted at the identifield site. The selected crop should be the one that all of the contaminants are labeled for. If all contaminants are not labeled for the same crop, select the crop which will result in the best fit for the products detected, the total quantity of pesticides and the label application rates. *Note:* If the application site initially identified in item 3 will not be planted to the crop you select, a new site must be selected and the information contained in item 3 of this form must be changed to reflect the newly selected site.

9b After selecting the crop, find the pesticide in item 8 which requires the greatest number of acres (column 8G) for the crop selected. This is the limiting pesticide for the selected crop.

9c The minimum application acreage required is the number of acres in column 8G for the crop selected in 9a and for the limiting pesticide identified in 9b. When selecting the appropriate acreage for the soil pile, adjustments should be made for incompatible products, cancelled products and hot spots in the pile. To make these adjustments, you may need to increase the application acreage (see Land Application Notes 5 and 6).

10 It is important to thoroughly mix the soil pile before it is loaded into the spreading equipment. Special attention should be directed at eliminating hot spots (mixing zones of suspected greater contamination within the pile with zones of lesser contamination). Crushing, pulverizing and/or sorting may be required.

11 MDA staff will not generally approve applications below 1 yd^3/acre, because the spreading equipment generally available is incapable of uniformly spreading below this rate. If the application rate is not reasonable for the equipment proposed, MDA staff will request equipment testing and calibration using clean soil, prior to approval of land spreading for contaminated soil.

15a Fill in the selected crop from item 9a of this form.

15b Fill in the application acreage from item 9c of this form.

15c Fill in the legal description of the proposed application site from item 3f of this form.

15d List each contaminant detected in this column, starting with the limiting pesticide and continuing with pesticides requiring progressively smaller application credits. With the exception of nitrogen, pesticides which were listed together due to additivity must be listed separately (see land application note #1). If more than 7 compounds were detected, list only the contaminants which will result in an application credit greater than 0.01 pounds/acre for the number of acres proposed.

15e For all compounds listed, including those combined in Table 8 due to additivity, the Application Credit = total quantity (item 8D) ÷ acres to be used (item 15b).

MINNESOTA DEPARTMENT OF AGRICULTURE • AGRONOMY SERVICES DIVISION
90 WEST PLATO BOULEVARD • ST. PAUL, MINNESOTA 55107-2094
TELEPHONE: 612/297-1975 • FAX: 612/297-2271
TDD METRO AREA 612/297-5353 • TDD GREATER MINNESOTA 1/800/627-3529

PROPOSAL TO LAND APPLY SOIL FROM AGRICULTURAL CHEMICAL INCIDENTS

Refer to the corresponding general instruction sheet prior to completing this form.

1. Facility or Spill Site Information:
   a) Site Name/MDA Case File Number:
   b) Street Address:
   c) Mailing Address:
   d) City/Zip:
   e) Contact Person:
   f) Relationship to Site:
   h) Phone: ( ) -

2. Person Preparing This Form:
   a) Name:
   b) Title:
   c) Company:
   d) Mailing Address:
   e) City/Zip:
   f) Phone: ( ) -

3. Proposed Land Application Site:
   a) Property Owner:
   b) Mailing Address:
   c) City/Zip:
   d) County:
   e) Phone: ( ) -
   f) Legal Description of Property (township, range, section):

4. Have there been past waste disposal activities at the proposed application site? If so, describe them in detail: _____

5. When do you propose that the soil will be spread at the site? (MDA approval of this form *must* be received prior to application.) _____

Determination of the Quantity of Pesticide in Excavated Soil:

6. Volume of soil to be land applied (in cubic yards): _____

7. Soil characteristics of proposed spreading area.
   a) Soil texture: _____
   b) Soil organic matter content: Low Medium High
      (circle one)

IN ACCORDANCE WITH THE AMERICANS WITH DISABILITIES ACT, AN ALTERNATIVE FORM OF COMMUNICATION IS AVAILABLE UPON REQUEST.

GD09 - Revised February 16, 1993

CONTAMINANT QUANTITY AND APPLICATION RATE CALCULATIONS

8.

	A	B	C	D	E	F	G
	Contaminant	Label Used (product name & EPA reg. no.)	Avg. or Max. Concentration (ppm)	Total Quantity (pounds)	Labeled Crops (if other, change name)	Application Rate (% of the lowest label rate - pounds/acre)	Minimum Acreage Required (total qty./rate)
					Corn:		
1)					Soybeans:		
					Corn:		
2)					Soybeans:		
					Corn:		
3)					Soybeans:		
					Corn:		
4)					Soybeans:		
					Corn:		
5)					Soybeans:		
					Corn:		
6)					Soybeans:		
					Corn:		
7)					Soybeans:		
					Corn:		
8)					Soybeans:		
					Corn:		
9)					Soybeans:		
					Corn:		
10)					Soybeans:		

2

Additional Considerations:

9. Using the results from number 8 and the instructions on page ii, what is the:
   a) selected crop? _____
   b) limiting pesticide for the selected crop? _____
   c) minimum application acreage required? _____ acres

10. What equipment will be used to spread the soil and how will this equipment be calibrated? _____

11. Is the application rate for the limiting contaminant physically feasible with the equipment to be used? If not, propose an alternative approach. _____

12. List any other label and/or environmental considerations which may restrict land application of the excavated soil at the calculated rate for the limiting contaminant. Examples include restricted seasonal use of some pesticides; best management practices, the proximity of wells, rural residences, wetlands, surface water or sinkholes to the application site and shallow depth to bedrock. Also, locate the listed physical environmental factors on an attached site map. _____

Documentation of Approval:

13. Appropriate local units of government (County, City and Township) at the application site must be notified of the proposal to land apply soil from an agricultural chemical incident at least two weeks prior to spreading soil at the site (this may be less in emergency situations, with MDA approval).

    Provide some form of documentation that the appropriate units of government (City, County, and Township) have been notified of the proposal to land apply soil from an agricultural chemical incident within their jurisdiction. At a minimum, identify the name of the contact person, phone number and title. Also, list the date and method of notification for each appropriate unit of government.

14 Complete the application summary (item 15) on page 4 of this form. After the application summary is completed, the contaminant owner and the landowner/operator must sign and date page 4 of this form. Copies of the completed land application form, notification of approval from local units of government and all requested documentation must be given to the landowner/operator and must be submitted to the MDA for review and approval before land application may begin.

3

15.

APPLICATION SUMMARY

a) Crop: _____ b) Acres to be used: _____

c) Application Location: _____

_____

d) Contaminant	e) Application Credit (lbs./acre)
1.	
2.	
3.	
4.	
5.	
6.	
7.	
8.	
9.	
10.	

The parties _____ (Soil/Contaminant Owner) and _____ (Landowner/Operator), agree to the terms in this proposal. The parties understand the MDA may monitor compliance with the terms of this proposal by conducting on-site inspections, sampling, and other oversight activities.

The parties understand that this proposal shall be considered an agreement between MDA and the parties. MDA reserves the right to modify or rescind this proposal for just and reasonable cause upon written notification to the parties.

If a party violates this agreement, MDA reserves the right to enforce the agreement pursuant to Minn. Stat. § 18D.325 (1992).

_____ / /
Signature of Soil/Contaminant Owner *Date*

_____ / /
Signature of Landowner/Operator *Date*

☐ _____ ☐ _____ / /
MDA approval as Proposed or *MDA Approval as Modified* *Date*

4

Appendix 2

Pesticide Physical/Chemical Properties

Pesticide Physical/Chemical Properties

Pesticide	Molecular Formula	Molecular Weight (g/mole)	Solubility* (mg/l)	Vapor Pressure* (mm Hg)	Henry's Law Constant* atmxm3/mole	Soil Adsorption Coefficient, Koc	Octanol-Water Partition Coefficient, Kow
MDA LIST 1							
alachlor	C14 H20 Cl N O2	269.8	242	3.10E-05	6.12E-08	43 - 190	436; 794
atrazine	C8 H14 Cl N5	215.6	28	3.0E-07 @ 20	3.04E-07 @ 20-25	89 - 513	214 - 631
butylate	C14 H21 N3 03	279.38	45	1.30E-02	5.5E-6 @ 20-25	20 - 540	
chlorpyrifos	C9 H11 C13 N O3 P S	350.59	2	1.87E-05 @ 20	4.16E-06	5888 - 13,490	2,042 - 184,926
cyanazine	C9 H13 Cl N6	240.73	171	1.6E-09 @ 20	2.78E-02	38 - 426	63; 174
EPTC	C9 H19 N O S	189.32	375	3.4E-02 @ 20	1.0E-05 @ 20-25	240	1585
ethalfluralin							
fonofos	C10 H15 O P S2	246.32	13	2.10E-04	5.20E-06	1072	7762; 7943
linuron	C9 H10 Cl2 N2 O2	249.1	75 - 81	1.5E-05 @ 20	5.10E-08	501 - 602	155; 1000
metolachlor	C15 H22 Cl N O2	283.81	530 @ 20	1.3E-05 @ 20	9.2E-09 @ 20	120 - 309	851 - 2818
metribuzin	C8 H14 N O S	214.28	1050 @ 20	4.35E-07 @ 20	1.2E-10 @ 20	65 - 525	40; 50
pendimethalin	C13 H19 N3 04	281.31	0.3 @ 20	3.00E-05	8.56E-07	30 - 851	151,356
phorate	C7 H17 02 P S3	260.4	20 @ 24	8.4E-04 @ 20	6.4E-06 @ 24	324 - 631	813 - 8318
prometon	C10 H19 N5 O	225.3	750	2.3E-06 @ 20	8.9E-10 @ 20	83 - 174	489; 977
propachlor	C11 H14 Cl N O	211.67	613 - 700	2.25E-04	1.10E-07	117 - 129	41
propazine	C9 H16 Cl N5	230.09	8.5 @ 20	2.9E-08 @ 20	9.9E-09 @ 20	49 - 363	813; 871
simazine	C7 H12 Cl N5	201.66	3.5 - 5 @ 20	6.1E-09; 3.6E-08 @20	3.4E-09 @ 20	138	87 - 182
terbufos	C9 H21 02 P S	288	4.5 @ 27	2.62E-04 @ 20	2.2E-06 @ 20 - 27	288 - 1072	166 - 50,199
triallate	C10 H16 Cl3 N O S	304.7	4	1.2 @ 20	1.0E-05 @ 20 - 25	2042	19,498
trifluralin	C13 H16 F3 N3 O4	335.29	4 @ 27	1.10E-05	4.84E-05 @ 23	871 - 30,903	117,490 - 218,776

* Values for 25 deg. C unless otherwise stated

= estimated (calculated) value

Data from Montgomery (1993)

Pesticide Physical/Chemical Properties

Pesticide	Molecular Formula	Molecular Weight (g/mole)	Solubility* (mg/l)	Vapor Pressure* (mm Hg)	Henry's Law Constant* atmxm3/mole	Soil Adsorption Coefficient, Koc	Octanol-Water Partition Coefficient, Kow
MDA LIST 2							
2,4-D	C8 H6 Cl2 O3	221.04	890@25; 45@20	4.7E-03 @ 20	1.96E-02 @ 20-25	48 - 537	30 - 75858
2,4-DB							
dicamba	C8 H6 Cl2 O3	221.04	6500	3.38E-05 @ 20	1.2E-09 @ 20-25	0.4 - 2	3
chloramben	C7 H5 Cl2 N O2	206.02	700	7E-03 @ 100		190	13
MCPA	C9 H9 Cl O3	200.63	730 - 825	1.5E-6 @ 20		107-117	23 - 27
MCPB							
MCPP							
picloram	C6 H3 Cl3 N2 O2	241.48	400 - 430	6.16E-07 @ 35	3.4E-10 @ 25-34	26	2
2,4,5-T	C8 H5 Cl3 O3	255.48	220 @ 20	3.75E-05 @ 20	4.87E-08 @ 20	52, 186	4 - 2512
2,4,5-TP							
triclopyr							
MDA LIST 3							
aldicarb	C7 H14 N2 O4 S	222.29	6.00E+03	3.47E-05	1.45E-09 @ 20-25	7.0 - 47	5.0 - 13
carbaryl	C12 H11 N O2	201.22	104 @ 20	6.58E-06	1.27E-05 @ 20	105 - 389	204 - 646
carbofuran	C12 H15 N O	221.26	320 @ 20	2E-05 @ 33	3.89E-08 @ 30-33	95 - 209	40 - 209
* Values for 25 deg. C unless otherwise stated							
	= estimated (calculated) value						
Data from Montgomery (1993)							

The literature review will be available for distribution to the public in the fall of 1993.

A6. **Benefits:** The review and evaluation of information regarding bio- remediation techniques will enable contaminated soil clean-ups to be conducted more cost efficiently and effectively than currently possible. Broadening the understanding of the available technology base will provide more and better clean-up of soils, thereby protecting ground water.

B. Degradation and Leaching of Atrazine and Alachlor Under Simulated Spill Conditions.

B1. **Narrative:** The mineralization (degradation) and movement of atrazine and alachlor will be determined on one vulnerable and one non-vulnerable soil to determine the degradation and leaching potential at elevated herbicide concentrations common with point source contamination events in Minnesota. Methodologies to accurately document pesticide degradation (metabolites and parent compound) and movement will be determined building on existing knowledge and research on field-use rate levels. In-field leaching studies will be established at two sites which have been under controlled conditions for 3-4 years and which have been used for previous field-use rate studies on parent compounds and associated metabolites. This will allow application of the degradation and transport knowledge obtained at field-use rates to the elevated spill level work. Some actual Ag dealership spill site soil cores for which there is existing analytical data may be investigated along with the controlled sites if funding allows.

^{14}C will be used to allow metabolite work. In addition to allowing more efficient mass flow characterization, this is the only reasonable approach to a truly comprehensive "balance sheet" study which includes metabolite characterization.

B2. **Procedures:** The experiment will utilize two field sites which have been used for ongoing research on atrazine and alachlor at field-use rates; one site is hydrogeologically vulnerable and the other not considered vulnerable to pesticide leaching. In-field leaching studies will be conducted in which elevated levels of atrazine and alachlor simulating spill conditions will be applied and monitored (these study areas receive regulatory oversight). Leachate from the columns will be analyzed for parent compound and associated degradation products. After leaching, the columns will be sub-sectioned into depth increments and the herbicide will be extracted from the soil and quantified. The movement of parent herbicide and metabolites will be determined. Pesticide mineralization and movement at these elevated levels will be compared with ongoing work at field-use rates at these sites.

B3. Budget:

a. Amount Budgeted: $130,000.00
b. Balance: $- 0 -

B4. Timeline for Products/Tasks:

	July 91	Jan 92	June 92	Jan 93	June 93
Field Sample Collection/Methods Development	**********				
Sample Collection/Extraction/ Analysis		**********	**********	**********	
Data interpretation/Extend Results Interpretation to Other Soils and Pesticide Problems				**********	**********

B5. Status:

ABSTRACT

The behavior of atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) and alachlor [2-chloro-2',6'-diethyl-*N*-(methoxymethyl)acetanilide] at elevated levels common with spill and waste disposal sites, their persistence, degradation and impact on soil microbial activity were determined in a Webster clay loam (fine loamy, mixed mesic Typic Haplaquoll) and an Estherville sandy loam soil (sandy, mixed, mesic Typic Hapludoll). Atrazine and alachlor were studied over a concentration range of 5 to 5,000 mg kg^{-1} and 10 to 10,000 mg kg^{-1} respectively, in laboratory experiments. Field studies approximating 5 and 5000 mg kg^{-1} of each herbicide in the plow layer were conducted to validate laboratory results and determine movement. Preliminary work on amendments to remediate atrazine spill sites was conducted.

The persistence of atrazine under laboratory conditions did not differ due to concentration but was dependent on soil type. The amount of atrazine degraded increased proportionally with increasing concentration in the clay loam soil, and in the sandy loam soil at concentrations below 500 mg kg^{-1}. The proportional amount of atrazine degraded decreased slightly at 500 to 5000 mg kg^{-1} in the sandy loam soil. Degradation and mineralization of atrazine was stimulated at higher concentrations in both soils. Mineralization was the most important pathway for the dissipation of atrazine at all concentrations in the clay loam soil, and from 5 to 500 mg kg^{-1} in the sandy loam soil. It was postulated that some soil microorganisms were able to use the N and/or C from the *s*-triazine ring, and the addition of atrazine stimulated soil microbial growth and activity and thus the degradation of atrazine. This assumption was supported by an observed increase in soil respiration in the clay loam at the 500 and 5,000 mg kg^{-1} atrazine concentration. Introduction of high concentrations of atrazine into soil did not show any inhibitory effect on soil microbial activity. Degradation pathways in both soils were not influenced by concentration. Ring cleavage and hydrolysis were the major metabolic pathways in both soils, with dealkylation of less importance.

The persistence of alachlor increased with increasing concentration. The effect of concentration on the overall behavior of alachlor was similar in both soils. At 10,000 mg kg^{-1}, alachlor became virtually nondegradable, with estimated 50% dissipation times of 12.6 and 13.5 years in the clay loam and sandy loam soil, respectively. Based on percent of applied alachlor, mineralization and formation of various degradation products and bound residues decreased at higher concentrations. However, on an absolute amount basis, mineralization at 1,000 and 10,000 mg kg^{-1} was similar to that at 100 mg kg^{-1}. It appeared that ~100 mg kg^{-1} was the maximum capacity for alachlor to be degraded or mineralized in these two soils. Limited water solubility and the specific biodegradation mechanisms of alachlor are postulated as the rate limiting factors for the degradation of alachlor at elevated levels. The overall soil microbial activity was slightly reduced at high concentrations, but it was assumed, however, that the microorganisms involved in alachlor degradation were not affected. Land spreading with proper dilution was postulated as an effective remediation means to detoxify alachlor contaminated soil.

Field studies showed that alachlor, and to a lesser extent, atrazine, have the potential for greater impacts on ground water when present at high concentrations. Higher concentrations of atrazine were detected in soil layers throughout the profile at various time intervals after the introduction of high concentrations to the soil profile as compared to the introduction of low concentrations to the soil profile. Only slight differences in the time interval before detection of atrazine at the 71 to 85 cm soil depth occurred comparing the high and low atrazine concentrations, although the concentration of atrazine was higher at each given depth at each point in time when exposed to high concentrations.

Alachlor varied greatly in the potential for movement below the crop rooting zone as a function of concentration compared to atrazine. Low concentration applications of alachlor did not pose any environmental risk for alachlor movement below the rooting zone. However, at high concentrations, alachlor moved to the lowest depth sampled and presumably below the rooting zone posing a threat to ground water. Alachlor was detectable at lower depths in the soil profile, often at concentrations an order of magnitude higher than present with similar high concentration applications of atrazine. Preliminary field data and corroborative laboratory studies indicate that the persistence of alachlor will be increased several orders of magnitude at high concentrations as well, compounding concern for potential environmental impacts.

Degradation rate differed little in soil amended with corn meal and ammonium phosphate, but degradation was increased by the addition of manure. Adaptation and stimulation of atrazine degradation by soil microorganisms was involved. Atrazine and its nonpolar metabolites degraded rapidly in initial phases of degradation in manure amended soil, likely due to the

abu[illegible] microorganisms present in the manure. Mineraliz[illegible] of atrazine was greatly stimulated by the addition of dairy manure.

1.0 INTRODUCTION

High concentrations of pesticides enter the soil environment threatening water resources from spillage during pesticide mixing, loading and rinsing operations, and through direct dumping of pesticide wastes into soil disposal pits, ditches and ponds. An EPA database cited the detection of 74 pesticides in ground water in 38 states, with 46 detections attributed to normal agricultural use and 32 attributed to point sources or misuse (Ritter, 1990). Accidental and incidental agricultural chemical spillages have been identified as a major point source for ground water contamination (Habecker, 1989). Eight case studies conducted in Iowa found that pesticide concentrations in ground water in the vicinity of farm-chemical supply dealerships were much higher than local background (Hallberg, 1986). In twenty mixing and loading facility sites examined in Wisconsin, 17 different pesticides were found in soil, and 19 different pesticides were found in ground water. A total of 720 sites contaminated with elevated levels of pesticides were reported in Minnesota, California, Florida, Michigan and Wisconsin in a recent survey (Buzicky et al., 1992).

Concentrations of pesticides ranging from a few mg kg^{-1} soil to that of undiluted commercial product have been reported at sites where sudden or chronic release of high concentrations of pesticides have occurred. Thirteen pesticides totaling 5,420 to 19,330 mg kg^{-1} soil were found in the top 7.5 cm soil layer in a soil pit used for pesticide disposal in California (Winterlin et al., 1989). Though extensive studies and surveys have evaluated the behavior and impact of pesticides in the soil environment, they have generally focused on normal agricultural field use rates. Pesticide concentration was identified as the single most important factor influencing pesticide degradation (Schoen and Winterlin, 1987). The few reported studies of elevated concentrations generally found that pesticides behaved differently at elevated concentrations compared to normal field use rates. Parathion [O,O-dimethyl S-(alpha-ethoxycarbonylbenzyl)-phosphorodithioate] was detected at 13,800 mg kg^{-1} in the top 10 cm of soil five years after treatment (Wolfe et al., 1973). The persistence of azinphosmethyl [O,O-dimethyl S-(4-oxo-1,2,3,-benzotriazin-3 (4H)-ylmethyl)phosphorodithioate] in a sandy loam soil following gross topical contamination with 18.1% liquid emulsifiable concentrate formulation was significantly prolonged, with 361.0, 1054.0 and 62.3 mg kg^{-1} found at depths of 0 to 7.5, 7.5 to 15.0 and 15.0 to 22.5 cm, respectively, eight years after application (Staiff et al., 1975). Persistence of atrazine, methyl parathion, trifluralin [2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)benzenamine] and 2,4-D [(2,4-dichlorophenoxy)acetic acid] increased significantly at high concentrations compared to low concentrations (Davidson et al., 1980). For instance, less than 0.1% of ^{14}C-methyl parathion was mineralized to $^{14}CO_2$ at 10,015 mg kg^{-1} during 52 days of incubation, compared to 75 to 85% mineralization at 24.5 mg kg^{-1}.

Alachlor [illegible] of the herbicides most frequently detected in ground w[illegible] (Cohen et al., 1986). Contamination of ground water from spills and mishandling was often much more severe than contamination caused by normal agricultural practice. For instance, detections from the normal agricultural application averaged 0.02 - 17 μg L^{-1} (Chesters et al., 1989), while at an agrichemical facility in Illinois, alachlor concentration was 24,000 mg kg^{-1} in the top 10 cm of soil, and 100 mg kg^{-1} at the depth of 60 cm (Felsot and Dzantor, 1990).

The mechanisms and factors controlling degradation of pesticides at elevated levels in soils must be understood to assess the behavior and environmental impact of pesticides at elevated concentrations common to spill sites, and to develop effective remediation methods. The objective of this study was to quantitatively determine the influence of the concentration of atrazine and alachlor on their persistence, degradation, and movement, and on soil microorganisms. Laboratory incubation studies were conducted to determine the effects of concentration on persistence and degradation for atrazine and alachlor. The experiments are reported in Sections 2 and 3. These studies showed that, while high concentrations of alachlor are considerably more persistent than low concentrations, atrazine was degradable in soil at high concentrations (5,000 mg kg^{-1}), although atrazine biodegradation was dependent upon soil type. Atrazine degradation was related to the stimulation of microbial activity, as indicated in the increase of CO_2 evolution.

Section 4 reports upon field studies which evaluated the leaching behavior of atrazine and alachlor in high concentration situations. Finally, in Section 5, the results of preliminary laboratory studies on the use of amendments to enhance atrazine degradation in contaminated soils are presented. The results of section 2 indicated that soil microorganisms were capable of using atrazine as a N and/or C source; the amendment experiments tested the hypothesis that proper nutrient additions could increase active microbial populations and activity and concurrently accelerate the biodegradation of atrazine.

2.0 Effect of Concentration on Persistence and Degradation of Atrazine in Soil - Laboratory Studies

2.1 Materials And Methods

2.1.1 Materials

^{14}C-atrazine was ring labeled, with a specific activity of 381.1 MBq $mmol^{-1}$ and a radiochemical purity >99.0 %. The technical grade atrazine used in this study had a purity > 99.0 %. The commercially formulated atrazine wettable powder (Aatrex 80W, Ciba-Geigy Corp., Agricultural Division, Greensboro, North Carolina 27419) had an a.i. content of 40.23% as determined by HPLC.

Samples of a Webster clay loam and an Estherville sandy loam soil were collected from Waseca and Westport, MN., respectively. Properties of the clay loam include organic carbon (OC) content = 4.09%; pH 7.0; clay content = 35%;and cation exchange capacity (CEC) = 36.2 meq 100 g soil^{-1}. Properties of the sandy loam include OC content = 2.49%; pH 4.8; clay content = 17%; and CEC = 18.5 meq 100 g soil^{-1}. Soils were sieved through a 2 mm screen after collection from the field.

2.1.2 ^{14}C-atrazine Treatment

Thirty grams of soil (24.5 g oven-dried weight) were weighed into 150-mL glass Erlenmeyer flasks. Atrazine stock solutions of different concentrations were made by dissolving the appropriate amount of commercial and ^{14}C-labeled atrazine in deionized water. Two mL of each stock solution were then added evenly onto the soil surface with a pipette and the soil flasks left overnight before mixing thoroughly. Soil was brought to a water content equivalent to a water potential of 0.32 bar by adding distilled water and remixing. The initial concentration of atrazine in soil was 5, 50, 500 or 5,000 mg kg^{-1} soil (based on oven-dried weight). Each flask contained 16.7 kBq ^{14}C-activity. One set of flasks did not receive any treatment and were used as controls.

The flasks were stoppered tightly with aluminum foil wrapped rubber stoppers. A 7-mL vial containing 4 mL 1.0 M NaOH was suspended from the stopper into the flask to trap released $^{14}CO_2$ and CO_2. The flasks were incubated in the dark at 25.0 ± 0.2 C in an incubator and soil moisture checked and maintained at 0.32 bar throughout the experiment.

2.1.3 Sampling and Analysis

Soil in the flasks was aerated each week by rotating the flask by hand. At the same time, sodium hydroxide vials in the flasks were replaced with vials containing 4 mL newly prepared 1.0 M NaOH. Aliquots (0.5 mL x 2) of the NaOH samples were assayed for radioactivity in the form of $^{14}CO_2$ by liquid scintillation counting (LSC) using a Packard Tri-Carb 1500 Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL). The cumulative release of $^{14}CO_2$ was used as an index of atrazine mineralization in soil. At selected time intervals, NaOH traps were also analyzed for the total inorganic carbon content using a Dorhmann DC-80 Automated Laboratory Total Organic Carbon Analyzer (Dorhmann Div., Xertex Co., Santa Clara, CA). This concentration was used as an index of soil respiration.

At 2, 6, 12, 20 and 40 weeks after treatment, two soil flasks were removed for each soil-concentration combination. All the soil in the flask was transferred into a 250-mL centrifuge tube, mechanically shaken with 60 mL methanol-water (4:1, v/v) for 1 h, centrifuged, and the supernatant decanted. The soil was resuspended using a vortex mixer and the extraction procedure repeated a second time. After the second extraction, 60 mL methanol-water (4:1, v/v) was added to the soil. The soil was resuspended and allowed to equilibrate overnight.

The three supernatants were combined, the total volume measured, and aliquots (2 mL x 2) of the extracts assayed for radioactivity by LSC.

One hundred mL of the combined extracts were transferred into an evaporation flask and methanol was removed with an air stream at 40 ^{o}C using a Turbo-Vap II evaporator (Zymark Co., Hopinton, MA). The aqueous sample was acidified to pH 1-2 with 1.0 M HCl, saturated with sodium chloride, and partitioned (3 x) with an equal volume of dichloromethane. Activity remaining in the aqueous phase after extraction was determined by LSC. The organic extracts were combined and further concentrated to 1 mL. Aliquots from the organic extracts were spotted on precoated TLC plates [Si250F-PA(19), J. T. Baker Inc., Phillipsburg, NJ] and co-chromatographed with standards of parent and metabolites in hexane-acetone-acetic acid (70:30:1, v/v/v). Polar metabolites remaining at the original position after development and activity remaining in aqueous phase were considered together as hydroxylated metabolites. The positions and radioactivities of parent and metabolites were determined with a Berthold Automatic TLC-Linear Analyzer (Berthold Instruments, Pittsburgh, PA). The samples from the 5000 mg kg^{-1} treatments were assayed by HPLC to identify and quantify atrazine and metabolites. Soil was air-dried after extraction and duplicate 0.5 g soil samples oxidized in a Packard Tri-Carb Sample Oxidizer (Packard Instrument Co., Downers Grove, IL) to determine the non-extractable (bound) residues. The released $^{14}CO_2$ was trapped in 8 mL Carbo-sorb (Packard Instrument Co., Inc., Meriden, CT) and mixed with 12 mL toluene-based scintillation cocktail. Recovery was determined to be >98%.

2.2 RESULTS AND DISCUSSION

2.2.1 Persistence of Parent Atrazine

Atrazine dissipated rapidly in the Webster clay loam soil (Figure 1). Concentration had negligible influence on the persistence of atrazine. Less than 2% of the applied atrazine was still in the parent form 20 weeks after application at all concentrations. Atrazine persisted longer in the Estherville sandy loam than in the clay loam at all concentrations. In the sandy loam, persistence increased slightly at higher concentrations. The estimated 50% dissipation times (DT_{50}) for atrazine in the sandy loam soil were 8.4, 9.3, 9.8 and 13.3 weeks, at 5, 50, 500 and 5,000 mg kg^{-1} respectively. The estimated 50% DT_{50} values for atrazine in the clay loam soil were 5.2, 6.5, 7.5, and 8.1 weeks, at 5, 50, 500, and 5,000 mg kg^{-1} resctively. In both soils, parent atrazine was not as persistent as reported previously for atrazine or other chemicals at elevated levels (Wolfe et al., 1973; Staiff et al., 1975; Davidson et al., 1980).

On the basis of the absolute amount of atrazine degraded, more atrazine was degraded at higher concentrations than at lower concentrations in both soils. At the end of 40 weeks incubation, the amount of atrazine degraded in the clay loam was 0.119, 1.20, 11.99, and 118.2 mg per flask at 5, 50, 500 and 5,000 mg kg^{-1}, respectively. The corresponding amounts in the sandy loam were 0.116, 1.15, 11.32, and 106.6 mg per flask. This stimulation pattern for

atrazine degradation at higher concentrations is characteristic of microbial process. It is likely that atrazine was used as a N and/or C source by some soil microorganisms, and the abundant amount of atrazine enhanced the population and activity of those atrazine degraders.

At the end of the incubation, the actual remaining concentration of atrazine was 0.015, 0.05, 0.35, and 74.0 mg kg^{-1} for the clay loam soil, and 0.176, 2.16, 28.25, and 556.5 mg kg^{-1} for the sandy loam soil at the 5, 50, 500, and 5,000 mg kg^{-1} treatment level, respectively. At At high concentrations, the small percentage of applied atrazine that remains is still high relative to field use rates, and could impose environmental risks. Even though relative persistence did not increase, atrazine potentially may cause more negative environmental impacts at elevated levels than at normal field use rates because higher concentrations of residues are available to leach through the soil profile to ground water during the same time period.

Soil properties affected atrazine degradation via both microbial and chemical degradation pathways. The clay loam had higher OC, clay content, and pH than the sandy loam. The different properties of the two soils resulted in different microbial ecology. Also, organic matter could serve as an energy source for soil microorganisms and therefore the clay loam should support a larger microbial population and higher activity.

The major degradation pathways of atrazine in soil have been identified as dehalogenation and hydrolysis, dealkylation, and ring-cleavage - mineralization (Kaufman and Kearney, 1970). In addition, atrazine and its degradation intermediates complex with soil organic matter and minerals to form solvent-nonextractable or bound residues (Capriel et al., 1985; Sorenson, 1992). To better understand mechanisms of the concentration effect, it is necessary to examine the interactions of concentration with each specific degradation pathway.

2.2.2 Mineralization of Atrazine

Since ^{14}C-atrazine was labeled on the *s*-triazine ring, any production of $^{14}CO_2$ would be attributed to ring cleavage and subsequent mineralization. Ring cleavage of atrazine and its metabolites is considered to be a microbial degradation process (Kaufman and Kearney, 1970). The pattern of mineralization of atrazine as a function of concentration differed between the clay loam and sandy loam soils (Figure 2). More mineralization occurred in the clay loam than in the sandy loam soil. The mineralization of atrazine was generally more extensive than previously reported (Kaufman and Kearney, 1970; Wolf and Martin, 1975), and more significant than previously reported for atrazine or other pesticides at elevated levels (Stojanovic et al., 1972; Wolfe et al., 1973; Davidson et al., 1980). At the end of 40 weeks of incubation, 43.7 to 72.0% of the applied atrazine was mineralized to $^{14}CO_2$, the exception being the 5,000 mg kg^{-1} treatment in the sandy loam with 6.3% mineralized.

Mineralization of atrazine in the clay loam was slow during the first 5 to 7 weeks, and then increased rapidly for 5 to 10 weeks before reaching a plateau (Figure 2). Different

concentrations, though varying in the order of 3 magnitudes, had very similar kinetics of mineralization. On an absolute basis, the difference in the amount of mineralization at varying concentrations was proportional to the difference in the amount of atrazine initially applied (Figure 3). Significant stimulation was apparently involved. Two distinct phases existed across concentrations: a phase representing rapid mineralization (phase 1) followed by a phase representing limited mineralization (phase 2). Phase 1 was 9 to 10 weeks for the 5 and 50 mg kg^{-1} treatments, and 12 to 14 weeks for the 500 and 5,000 mg kg^{-1} treatments. Most of the mineralization occurred during phase 1, with the accumulative mineralization during phase 2 accounting for <10% of total mineralization. The calculated rate constants for mineralization were 5.2, 57.9, 431 and 3348 μg $week^{-1}$ $flask^{-1}$ for phase 1, and 0.4, 5.1, 53.4, 340 μg $week^{-1}$ $flask^{-1}$ for phase 2, at the concentrations of 5, 50, 500, and 5,000 mg kg^{-1} respectively. Rate of mineralization in both phases increased proportionally to the increase in initial concentration. Rate constants for phase 2 were only about one tenth of the respective constants for phase 1, indicating much slower mineralization. Degradation products were mineralized in phase 1 and/or became more strongly bound to soil. Reduced mineralization in phase 2 could be attributed to the depletion of partially degraded products available for further microbial ring-cleavage (data not shown).

Mineralization in the sandy loam was not proportional to concentration at 5000 mg kg^{-1} (Figure 2). Only 6.3% of ^{14}C-atrazine decomposed to $^{14}CO_2$ in 40 weeks in soil treated with 5,000 mg kg^{-1}, while 44.3 to 53.7% was mineralized at the lower concentrations. On an absolute basis, the two phase phenomenon was less distinctive in the sandy loam compared to that of the clay loam (Figure 3). Though a lower percentage of atrazine was mineralized at 5,000 mg kg^{-1}, the absolute amount mineralized was still greater than that at 500 mg kg^{-1}. Thus, no inhibitory effect of high concentration of atrazine to the microbial mineralization process was observed at any of the soil-concentration combinations.

Since ring-cleavage is considered mainly a microbial degradation process, different soil properties may affect mineralization by affecting soil microbial structure, population and activity. Higher organic matter and clay content, and more neutral pH conditions may have contributed to the development of populations that can more rapidly mineralize atrazine in the clay loam. The addition of atrazine apparently stimulated mineralization at high concentration in both soils. The results indicate that some soil microorganisms can use the N and/or C from the *s*-triazine ring, causing ring cleavage. Soil generally is rich in atrazine biodegraders (Grant and Williams, 1982). Numerous species of bacteria and fungi that grew on various triazines as the only carbon or nitrogen source have been isolated (Kaufman and Kearney, 1970; Giardina et al., 1980 and 1985; Cook and Hutter, 1981; Behki and Khan, 1986).

If atrazine was used as a N source for some soil microorganisms, N-containing nutrients could also supply N thereby slowing microbial degradation of atrazine. Stimulation of the growth of original soil microflora would accelerate the breakdown of high levels of atrazine, and has the

potential for use in remediation to detoxify atrazine contaminated soil. This could be through direct enhancement of soil microbial populations by introducing microbial enrichments, or indirect enhancement by adding soil amendments. Since soil can be rich in atrazine degrading microorganisms, addition of nutrient amendments should be a reasonable choice due to its low cost and easy feasibility.

2.2.3 Formation of Degradation Intermediates

Deethyl and deisopropyl atrazine were the main nonpolar metabolites in both soils, with the production of deethyl atrazine always slightly higher than that of deisopropyl atrazine. Examples at 20 weeks after treatment are given in Tables 1 and 2. Neither of these two dealkylated products accumulated to > 6.5% of the total ^{14}C applied, indicating that microbial dealkylation was not the most important degradative pathway. It is also likely that these two dealkylated intermediates went through dehalogenation and hydrolysis to form their hydroxylated derivatives. The fact that the formation of dealkylated products as a percentage of applied atrazine remained approximately at the same level across concentrations, demonstrated that microbial dealkylation of atrazine was not affected by concentration.

The level of hydroxylated metabolites increased and then decreased with time (data not shown), accounting for a large proportion of the total ^{14}C-residues at specific times. Hydroxylated metabolites were more persistent at higher concentrations. At the end of 40 weeks of incubation, the combined hydroxylated metabolites accounted for 15.01 and 26.46% of the initial 5,000 mg kg^{-1} atrazine applied in the clay loam and the sandy loam soil, respectively, while the percentage dropped to <1.5% in lower concentrations. The presence of abundant hydroxylated products in soil at high concentrations may have occupied most reactive sites on organic matter and mineral surfaces, resulting in a larger percentage available for extraction with methanol and water than at low concentrations. Also, the formation of hydroxylated products was often followed by a surge in $^{14}CO_2$ evolution. Thus, hydrolysis of atrazine occurred prior to ring cleavage. The formation of hydroxylated metabolites has been reported in numerous studies and is widely considered as a chemical rather than a biological process (Armstrong et al., 1967; Skipper et al., 1967: Skipper and Volk, 1972; Best and Weber, 1974; Obien and Green, 1967).

Nonextractable or bound residues formed a major part of the residues for all soil-concentration combinations. More bound residues were found in the sandy loam than clay loam, 30.10 to 46.04% compared to 24.38 to 28.22% respectively, at 20 weeks after treatment (Tables 1 and 2). The increased bound residue formation in the sandy loam may be caused by the lower pH of that soil. Hydrolysis of atrazine increased as soil pH decreased (Armstrong et al., 1967; Skipper et al., 1967), and hydroxylated atrazine was more easily bound to soil than atrazine and its dealkylated metabolites (Brouwer et al., 1990; Clay and Koskinen, 1990). The percentage of bound residues at any given time (data not shown) did not differ significantly among concentrations in the same soil, indicating no influence of concentration. In the clay

loam, the highest level of bound residue was reached 12 weeks after treatment followed by a slight decrease at all concentrations, indicating that a small percentage of bound residues were released.

Table 1

Mass Balance of Atrazine in a Webster Clay Loam 20 Weeks After Application

Compound	Parent atrazine and degradation products			
	5[a]	50[a]	500[a]	5000[a]
	----------% of applied----------			
Atrazine	1.78	0.60	0.31	1.01
Deethyl atrazine	1.43	0.41	0.08	0.17
Deisopropyl atrazine	0.32	0.09	0.02	ND
Hydroxylated products[b]	1.53	1.07	2.39	14.00
Nonpolar metabolites[c]	0.23	0.14	0.20	0.38
Bound Residues	27.39	24.52	24.38	28.22
CO_2	62.25	69.46	61.29	51.55
Total	94.93	96.29	88.67	95.33

[a]initial atrazine concentration at application (mg kg^{-1})
[b]Sum of hydroxylated metabolites.
[c]Sum of unidentified nonpolar metabolites.

Tab.

Mass Balance of Atrazine in an Estherville Sandy Loam 20 Weeks After Application

Compound	Parent atrazine and degradation products			
	5[a]	50[a]	500[a]	5000[a]
	----------% of applied----------			
Atrazine	6.26	17.62	17.58	40.52
Deethyl atrazine	1.54	2.56	2.25	1.71
Deisopropyl atrazine	0.68	0.52	0.32	0.27
Hydroxylated products[b]	4.69	2.43	8.98	12.15
Nonpolar metabolites[c]	0.67	0.55	0.34	2.80
Bound Residues	40.34	40.43	46.04	30.10
CO_2	42.60	33.33	21.03	3.90
Total	96.79	97.44	96.54	91.45

[a]initial atrazine concentration at application (mg kg^{-1})
[b]Sum of hydroxylated metabolites.
[c]Sum of unidentified nonpolar metabolites.

The existence of a high percentage of aged atrazine residues as bound residues in soil has been widely reported (Capriel et al., 1985; Sorenson et al., 1992). Though the nature of bound residues is not clearly understood, formation of bound residues is often considered a natural detoxification process (Khan, 1982; Kovacs, 1986; Calderbank, 1989). However, at elevated levels, bound residues would have more environmental importance. As found with many pesticides, bound atrazine residues could be released back into the environment under certain circumstances. The release of bound pesticides residues at elevated levels may result in a significant amount of parent and/or metabolites in the environment.

2.2.4 Impact on Soil Microorganisms.

Soil microbial activity was enhanced at higher concentrations in the clay loam as indicated by CO_2 evolution. This enhancement became more significant with time (Figure 4). Enhancement of soil microbial activity confirmed the observed stimulation in mineralization and degradation of atrazine at higher levels in the clay loam soil. The effect of concentration on soil respiration in the sandy loam was not as significant. These results also confirmed that atrazine could be used as a N and/or C source, and that the introduction of a large amount of atrazine can stimulate soil microbial growth and activity, with atrazine itself in turn used or degraded through this stimulation.

3.0 Effect of Concentration on Persistence and Degradation of Alachlor in Soil - Laboratory Studies

3.1 Materials And Methods

3.1.1 Materials

Uniformly-ring-labeled ^{14}C-alachlor was used with a specific activity 999.0 MBq $mmol^{-1}$ and a radiochemical purity >99.0 %. Technical grade alachlor used had a purity > 99.0 %. Alachlor EC formulation had an active ingredient content of 0.511 kg L^{-1}. Metabolites 2-chloro-2',6'-diethylacetanilide, 2,6-diethylaniline, [2-(2,6-diethylphenyl) (methoxymethyl) amino]-2-oxoacetic acid (sodium salt), and [2-(2,6-diethylphenyl) (methoxymethyl) amino]-2-oxo-ethanesulfonic acid (sodium salt), were provided by Monsanto Agricultural Co., 800 N. Lindbergh Blvd., St. Louis, MO. Soils were the same clay loam and sandy loam described previously in the atrazine study.

3.1.2 ^{14}C-alachlor Treatment

Thirty grams of soil were treated with alachlor stock solutions of different concentrations of the appropriate amount of formulated and ^{14}C-labeled alachlor. The initial concentrations of alachlor in soil were 10, 100, 1,000 or 10,000 mg kg^{-1} soil. To determine the effect of formulation on alachlor degradation at high concentrations, the sandy loam was also treated with ^{14}C-alachlor-fortified technical grade alachlor at a concentration of 1,000 mg kg^{-1}. Flasks with untreated soil were used as controls. Flasks were maintained and sampled as previously described in the atrazine study.

At 2, 6, 12, 20 and 40 weeks after treatment, two soil flasks were removed from each soil-concentration combination. The soil was extracted with methanol:water. The methanol was removed and the water was acidified, saturated with NaCl, and partitioned with diethyl ether and ethyl acetate. Activity remaining in the water was determined by LSC. The organic extracts were concentrated to ~1 mL. Aliquots from the organic extracts were analyzed for alachlor and metabolites by TLC. For samples from the 10,000 mg kg^{-1} treatment, HPLC was used to identify and quantify alachlor and metabolites. Soil after extraction was oxidized to determine the nonextractable (bound) residues. The $^{14}CO_2$ released was trapped and counted by LSC.

3.2 Results And Discussion

3.2.1 Persistence of Alachlor

The dissipation of alachlor in both soils followed first-order kinetics (Figure 5). Concentration showed similar effects on alachlor persistence in the clay loam and sandy loam soil with the

persistence of alachlor increasing at higher concentrations. A maximum capacity for degradation of alachlor existed in both soils with concentrations up to 100 mg kg^{-1} readily degraded. Above this concentration, the persistence of alachlor increased significantly. Alachlor became extremely persistent in both soils at concentration above 1,000 mg kg^{-1}, and virtually nondegradable at 10,000 mg kg^{-1}.

The estimated times for 50% dissipation (DT_{50}) of alachlor for initial concentrations of 10, 100, 1,000 and 10,000 mg kg^{-1} were 8.7, 21, 88, 654 weeks in the clay loam and 2.8, 8.3, 97, and 700 weeks in the sandy loam, respectively. At the end of 40 weeks incubation, the absolute amount of alachlor degraded with the initial concentrations of 10, 100, 1,000, and 10,000 mg kg^{-1} was 0.24, 2.37, 12.66, and 23.45 mg per flask in the clay loam, and 0.23, 2.25, 11.49, and 18.72 mg kg^{-1} for the sandy loam.

3.2.2 Mineralization of Alachlor

The pattern of mineralization of alachlor as a function of concentration was similar in both soils (Figure 6). Mineralization decreased as initial concentration increased based on percent of applied alachlor. Mineralization at 100 mg kg^{-1} was reduced compared to the 10 mg kg^{-1} treatment, but was still significant. Above 100 mg kg^{-1}, mineralization was significantly decreased. At 10,000 mg kg^{-1}, only 0.14 % and 0.32% of the initially applied alachlor was mineralized at the end of 40 weeks incubation in the clay loam and the sandy loam, respectively. Most of the mineralization at 10,000 mg kg^{-1} occurred during the first 15 weeks with virtually no evolution of $^{14}CO_2$ detected thereafter.

The absolute mineralization for 100, 1,000 or 10,000 mg kg^{-1} was similar (Figure 7), indicating that the soils had a maximum mineralization capacity for alachlor. The amount of alachlor mineralized did not increase at concentrations above 100 mg kg^{-1}. The adjuvants and other chemicals in the commercial alachlor EC formulation did not affect the mineralization rate at 1,000 mg kg^{-1}.

3.2.3 Formation of Degradation Intermediates.

There were no major differences in the degradation of alachlor patterns between the two soils, with numerous metabolites formed during degradation (Tables 3 and 4). No effect of alachlor formulation was observed on the overall metabolic pathways of alachlor at 1,000 mg kg^{-1} (Table 4). The level of the four major metabolites generally increased and then decreased with time with no accumulation of any specific metabolite. The major nonpolar metabolite in the concentration range of 10 to 1,000 mg kg^{-1} was 2-chloro-2',6'-diethylacetanilide, comprising <8.1% of the initially applied activity at any sampling time. Another nonpolar metabolite was identified as 2,6-diethylaniline, present at < 1.4%. Neither metabolite was detected at 10,000 mg kg^{-1}.

Acetate and sulfonate derivatives of alachlor were the major polar metabolites identified in the concentration range of 10 to 1,000 mg kg^{-1}. The concentration of acetate was always was much higher than that of sulfonate (Tables 3 and 4). Formation of both polar products was reduced at 1,000 mg kg^{-1} and was not detectable at 10,000 mg kg^{-1}.

Table 3

Mass Balance of Alachlor in a Webster Clay Loam 20 Weeks After Application

Compound	Parent alachlor and degradation products			
	10[a]	100[a]	1000[a]	10000[a]
	----------% of applied----------			
Alachlor	1.90	7.88	62.52	91.95
2-chloro-2',6'-diethylacetanilide	3.72	3.51	3.74	ND
2,6-diethylaniline	0.44	0.52	0	ND
2',6'-diethyl-*N*-(methoxymethyl)oxanilic acid	6.90	24.22	3.18	ND
N-[(2,6-diethyl)phenyl]-*N*-methoxymethyl-2-amino-2-oxoethanesulfonic acid	1.86	0.74	0.95	ND
Unidentified nonpolar metabolites	5.06	4.06	2.44	ND
Unidentified polar metabolites	6.14	3.89	3.48	ND
Bound Residues	40.28	38.64	18.35	6.13
CO_2	24.38	10.59	1.47	0.13
Total	90.68	94.05	96.13	98.21

[a]initial alachlor application rate (mg kg^{-1})

Tab

Mass Balance of Alachlor in an Estherville Sandy Loam 20 Weeks After Application

Compound	Parent alachlor and degradation products				
	10[a]	100[a]	1000[a]	10000[a]	1000(TB)[ab]
	% of applied				
Alachlor	1.09	2.00	52.32	96.45	60.17
2-chloro-2',6'-diethylacetanilide	3.27	0.53	8.06	ND	3.82
2,6-diethylaniline	ND	ND	ND	ND	ND
2',6'-diethyl-*N*-(methoxymethyl)oxanilic acid	9.23	27.23	3.48	ND	3.61
N-[(2,6-diethyl)phenyl]-*N*-methoxymethyl-2-amino-2-oxoethanesulfonic acid	1.26	2.07	3.7	ND	0.98
Unidentified nonpolar metabolites	3.24	5.09	3.7	ND	3.15
Unidentified polar metabolites	5.21	2.99	1.68	ND	0.79
Bound Residues	45.14	43.02	27.73	5.31	25.27
CO_2	28.35	15.17	1.61	0.30	1.79
Total	96.78	98.10	100.49	102.06	99.58

[a]initial alachlor application rate (mg kg^{-1})
[b]Technical alachlor treatment of 1,000 mg kg^{-1}

Formation of nonextractable or bound residues increased with time and comprised a major proportion of the total ^{14}C-residues at low concentrations (Figure 8). At the end of incubation, bound residues were 47.7 and 37.4% in the clay loam, and 44.1 and 42.4% in the sandy loam with the initial application of 10 and 100 mg kg^{-1}, respectively, based on percent of applied alachlor. However, the formation of bound residues as a percent of applied alachlor decreased significantly above 100 mg kg^{-1}. Only 8.52 and 7.43% bound residues were found at 10,000 mg kg^{-1}, after 40 weeks incubation in the clay loam and the sandy loam soil, respectively.

3.2.4 Impact on Soil Microorganisms

Soil microbial activity was slightly reduced at higher concentrations in both soils as indicated by CO_2 evolution (Figure 9). An inhibitory effect has been observed with other chemicals at elevated levels (Davidson et al., 1980). Although overall microbial activity was inhibited,

microorga...s related to alachlor degradation and mineralizatio... ...ay have remained unaffected, as the absolute amount of mineralization and degradation were not reduced at higher concentrations in either soil. Increased respiration in soil treated with 1,000 mg kg^{-1} technical alachlor may have been caused by mineralization or volatilization of the small amount of methanol used to apply the chemical.

The persistence of alachlor increased at higher concentrations in both soils. Many factors could contribute to this effect. Limited water solubility and the specific mechanism of alachlor biodegradation were probably the rate limiting factors. For instance, alachlor has a water solubility of 260 mg L^{-1} at 25 °C. Theoretically, the applied amounts of alachlor at 1,000 and 10,000 mg kg^{-1} were 13.2 and 132 times their solubility, respectively (water content of soil was 30%). Assuming no sorption taking place, 7.6% or 0.76% of the applied chemical would be present in soil solution when the soil received 1,000 or 10,000 mg kg^{-1} of alachlor, respectively. Thus, only a fraction of pesticide residues in soil solution would be available to microorganisms.

Chemical degradation of alachlor in soil is not an important dissipation pathway (Beestman and Deming, 1974). Alachlor is primarily metabolized by soil microorganisms. Bacteria or fungi capable of using alachlor without any other supplements have not been successfully isolated to date (Kaufman and Blake, 1973; Lee, 1984, 1986). Degradation of alachlor produced numerous intermediates (Chesters et al., 1989). Soil properties, such as the type and content of organic matter and minerals, and soil pH, may affect the degradation of alachlor by affecting microbial biomass and activity. It appears soil property effects were minimal since there were no differences between the two soils at the high alachlor concentration.

Adjuvants and other chemicals in formulated alachlor did not contribute significantly to increased persistence at elevated concentrations. Persistence of alachlor in the sandy loam soil treated with 1,000 mg kg^{-1} did not differ due to formulation, with a DT_{50} of 100 and 97.2 weeks for technical-grade alachlor and commercially formulated alachlor, respectively. Thus, it appears that the observed inhibitory effects of high concentrations of alachlor on soil microorganisms are not attributable to formulation ingredients.

Soil properties did not significantly influence the persistence and degradation of alachlor at elevated levels. Concentration did not affect the degradation pathways, as similar degradation products were identified at the various concentrations. Formation of bound residues decreased on a percentage basis at high concentrations. The effect of concentration mainly resulted from alachlor itself, and formulation showed minimal contribution.

The nature of bound pesticide residues is not well understood; formation of bound residues isen considered as a natural detoxification process (Khan, 1982). Formation of bound pesticide residues is commonly considered as a complexation process of pesticides and/or their metabolites with soil organic matter and minerals. Chemical, biochemical or physical

reactions could be involved (Khan, 1982). It was often found, however, that bound pesticides could be released back into soil solution in certain circumstances. Bound residues at elevated concentration may have considerable environmental importance, since the release of bound residues may result in a significant amount of parent and/or metabolites in the environment.

4.0 Comparative Studies on the Persistence and Movement of Atrazine and Alachlor in Soils at Normal and Elevated Levels under Field Conditions

4.1 Materials and Methods

Alachlor and atrazine were surface applied at 11 and 11,000 kg ha^{-1} to approximate a 5 and 5,000 ppm concentration when distributed throughout the plow layer. Atrazine was applied as a flowable formulation, and alachlor an emulsifiable concentrate. Treatments were applied to 0.9 by 3.0 m plots, replicated three times, and the trial repeated at Waseca and Westport, MN. The Waseca site was a Webster clay loam and the Westport site an Estherville sandy loam soil. Soil cores were removed before and immediately after treatment (0 MAT, months after treatment), and at 1 and 3 MAT, and approximately 1 year after application. Two subsample soil cores were taken from each plot at each sampling date. Soil cores consisted of one5.1 cm diameter surface core taken to a depth of 10 cm followed by the removal of one 2.5 cm diameter core to a depth of 75 cm from the bottom center of the surface core hole. Soil cores were kept at -15 C until analysis. The frozen 2.5-cm diameter soil cores were cut into 15 cm sections, and all cores thawed at room temperature. Soil water content was determined for each individual sample.

Two different methods were used for extraction and analysis of soil samples for the high and low rate treatments. Soil samples from the top 0 to 10-cm layer of the high concentration treatments were extracted manually and analyzed using HPLC to avoid carry-over contamination in the robotic and GC systems caused by the extremely high pesticide content. Twenty grams of soil (x 2) were weighed into a 200 mL centrifuge bottle and then extracted with 80 mL methanol-water (4:1, v/v) by shaking for 1 h. Supernatant was collected after centrifugation at 2,000 rpm for 5 min. The same extraction procedure was repeated a second time after resuspending the soil. After the second extraction, the sample was left overnight, and resuspended in 80 mL methanol:water (4:1, v/v). All supernatants were combined and the total volume measured. Aliquots (20 µl x 2) were taken and dissolved in 1000 µl HPLC mobile phase for injection with HPLC flow rate 1.0 mL min^{-1}, solvent methanol:water (80:20, v/v), wavelength 220-240 nm, and injection volume 100 µl.

Soil samples from high concentration treatments below 10 cm, and from all low concentration treatments were extracted by robotics and analyzed by GC. Ten grams of soil (x 2) were weighed into a 50 mL centrifuge tube and extracted and cleaned using the robotic system. The final samples were prepared in 1 mL methanol with metribuzin as an internal standard.

4.2 Results and Discussion

Soil samples from 10 to 100 cm were taken after the top layer soil was removed in the plots treated at high concentrations to determine if cross-contamination was occurring by this sampling method. No cross contamination occurred for atrazine, and slight contamination occurred for the alachlor treatment (Table 5). The concentration of alachlor and atrazine in the 0 to 10 cm soil layer after the application were within limits of application targets (Table 6).

Results indicate that atrazine and, to a greater extent, alachlor, have the potential for greater impacts on ground water when present at high concentrations. The residual levels of atrazine and alachlor 1 and 3 MAT are given in Tables 7-10. All results are means of at least 8 replicates. At 1 MAT, atrazine was detectable deeper in the soil profile for 5000 mg kg^{-1} treatments compared to 5 mg kg^{-1} treatments. For both locations, atrazine was detected in the 71 to 85 cm layer under the 5000 mg kg^{-1} treatmentbut only in the 56 to 70 cm layer for the 5 mg kg^{-1} treatment (Table 7). By 3 MAT, atrazine was present in the deepest soil layer sampled at both concentrations (Table 9), and was present at higher concentrations in each layer with the 5000 mg kg^{-1} application treatments.

Alachlor varied greatly in the potential for movement below the crop rooting zone as a function of concentration. Alachlor was not detected below 25 cm 1 MAT in either soil in the 5 mg kg^{-1} treatments, but was present in the 71 to 85 cm layer in both soils in the 5000 mg kg^{-1} treatments (Table 8). By 3 MAT, alachlor still was not detected below 25 cm (Table 10). Atrazine levels in the high concentration treatment 1 MAT were higher in respective soil layers compared to alachlor levels in the high concentration treatment in the clay loam, but the reverse occurred in the sandy loam (Tables 7 and 8). By 3 MAT, the concentration of alachlor in the high concentration treatments in respective soil layers exceeded that of atrazine in both soils (Tables 9 and 10).

A final sampling event occurred in August, 1993 for both sites. The data for this sampling event will be submitted to the LCMR as an addendum when analysis is completed.

Tab

Sampling methodology contamination determination for high concentration treatments, 5000 mg kg^{-1}.[a]

Soil Depth	Atrazine	Alachlor
(cm)	(mg kg^{-1})	
11 - 25	0.0790 ± 0.0398	0.0023 ± 0.0012
26 - 40	ND[b]	0.0007 ± 0.0007
41 - 55	ND	0.0001 ± 0.0002
56 - 70	ND	0.0009 ± 0.0007
71 - 85	ND	0.0011 ± 0.0008
86 - 100	ND	0.0011 ± 0.0014

[a] Samples from Waseca site only. Average of 5 soil cores.
[b] ND = not detected within limits of the analysis used.

Table 6

Concentration in surface soil layers (0-10 cm) at application (0 MAT).

	Concentration measured in soil			
	Atrazine		Alachlor	
	5[a]	5000	5	5000
	(mg kg^{-1})			
Waseca (Clay loam)	6.03 ± 1.38	4544.47 ± 585.95	3.76 ± 1.19	4306.2 ± 1201.3
Westport (Sandy loam)	7.22 ± 3.38	6387.17 ± 1601.74	4.47 ± 1.27	6909.49 ± 2740.27

[a] Targeted initial application rate (mg kg^{-1}).

Table 7

Distribution of atrazine in the soil profile 1 MAT.

Soil Depth	Concentration of atrazine detected	
	5[a]	5000[a]
(cm)	(mg kg^{-1})	
Clay Loam		
0 - 10	0.8376 ± 0.3030	3698.5 ± 1245.6
11 - 25	0.0455 ± 0.0489	10.8588 ± 9.7332
26 - 40	0.0036 ± 0.0051	1.3446 ± 2.4606
41 - 55	0.0046 ± 0.0046	0.4548 ± 0.7688
56 - 70	0.0011 ± 0.0011	0.2603 ± 0.4917
71 - 85	ND[b]	0.1239 ± 0.1516
Sandy Loam		
0 - 10	1.2291 ± 0.5459	6175.2 ±1544.8
11 - 25	0.0527 ± 0.0225	1.1649 ± 0.5459
26 - 40	0.0152 ± 0.0090	0.1867 ± 0.1065
41 - 55	0.0041 ± 0.0039	0.0510 ± 0.0720
56 - 70	0.0229 ± 0.0404	0.0136 ± 0.0075
71 - 85	ND	0.0022 ± 0.0011

Table 8

Distribution of alachlor in the soil profile 1 MAT.

Soil Depth	Concentration of alachlor detected	
	5[a]	5000[a]
(cm)	(mg kg^{-1})	
Clay Loam		
0 - 10	0.3635 ± 0.1101	3698.5 ± 1245.6
11 - 25	0.0066 ± 0.0085	4035.2 ± 1862.1
26 - 40	ND[b]	0.0385 ± 0.0193
41 - 55	ND	0.0245 ± 0.0189
56 - 70	ND	0.0185 ± 0.0265
71 - 85	ND	0.0360 ± 0.0179
Sandy Loam		
0 - 10	0.1865 ± 0.0941	6557.7 ± 971.6
11 - 25	ND	1.3572 ± 0.7384
26 - 40	ND	0.6160 ± 0.4040
41 - 55	ND	0.2553 ± 0.2587
56 - 70	ND	0.1087 ± 0.1107
71 - 85	ND	0.0287 ± 0.0122

[a] Initial application rate (mg kg^{-1}).
[b] ND = not detected within limits of the analysis used.

Table 9

Distribution of atrazine in the soil profile 3 MAT.

Soil Depth	Concentration of atrazine detected	
	5[a]	5000[a]
(cm)	(mg kg^{-1})	
Clay Loam		
0 - 10	0.2859 ± 0.2040	3698.5 ± 1245.6
11 - 25	0.0557 ± 0.0288	4035.2 ± 1862.1
26 - 40	0.0202 ± 0.0131	0.0385 ± 0.0193
41 - 55	0.0057 ± 0.0088	0.0245 ± 0.0189
56 - 70	0.0048 ± 0.0057	0.0185 ± 0.0265
71 - 85	0.0040 ± 0.0067	0.0360 ± 0.0179
86 - 100	0.0057 ± 0.0067	
Sandy Loam		
0 - 10	1.2817 ± 0.5359	5752.9 ± 2043.6
11 - 25	0.2525 ± 0.1751	5.1424 ± 5.3921
26 - 40	0.0380 ± 0.011	0.9664 ± 0.8381
41 - 55	0.0092 ± 0.0083	0.0923 ± 0.0777
56 - 70	0.0251 ± 0.0448	0.1186 ± 0.1765
71 - 85	0.0064 ± 0.0111	0.0436 ± 0.0257

[a] Initial application rate (mg kg^{-1}).

Tab

Distribution of alachlor in the soil profile 3 MAT.

Soil Depth	Concentration of alachlor detected	
	5[a]	5000[a]
(cm)	---------- (mg kg^{-1}) ----------	
Clay Loam		
0 - 10	0.1455 ± 0.0964	4152.0 ± 2110.2
11 - 25	0.0390 ± 0.0574	11.1473 ± 7.5731
26 - 40	ND	2.0250 ± 2.7938
41 - 55	ND	0.4118 ± 0.9524
56 - 70	ND	0.2063 ± 0.4790
71 - 85	ND	0.0700 ± 0.1373
86 - 100	ND	0.1937 ± 0.2739
Sandy Loam		
0 - 10	0.5794 ± 0.3232	6355.9 ± 1798.5
11 - 25	0.0239 ± 0.0263	65.5133 ± 32.7519
26 - 40	ND	11.5222 ± 11.2711
41 - 55	ND	2.1352 ± 1.3179
56 - 70	ND	1.2447 ± 0.7354
71 - 85	ND	0.3638 ± 0.0352

[a] Initial application rate (mg kg^{-1}).
[b] ND = not detected within limits of the analysis used.

5.0 Enhancem... of Atrazine Biodegradation at Elevated Levels In So... ...h Amendments

5.1 Materials and Methods

Ten grams of a Webster clay loam soil were pre-treated with 5,000 ppm atrazine and incubated in a 50 mL centrifuge tube to simulate an incidental spillage. After 2 weeks, amendments were mixed into the soil. Amendments were ammonium phosphate fertilizer ($NH_4H_2PO_4$), dairy manure, and corn meal. The fertilizer contained N which was instantly available to the soil microorganisms. Manure and corn meal are naturally occurring and economical. Dairy manure is rich in different forms of N and other elements. Corn meal is an organic carbon source for microorganisms. One gram of air dried dairy manure or corn meal, and 0.1 g of ammonium phosphate were mixed into the soil homogeneously by hand. A vial containing four mL of 1.0 M NaOH solution was suspended into the tube to trap $^{14}CO_2$, and the evolved activity determined every week. The formation of $^{14}CO_2$ was used as an index of microbial mineralization of atrazine. Degradation and formation of bound residues were analyzed by sampling the soil at different time intervals. Methodology for assays were the same as developed for the previous incubation studies.

Samples were extracted in the centrifuge tubes by shaking for 1 h in 20 mL acidified methanol, and decanting the supernatant after centrifugation at 8,000 rpm for 10 min. The same extraction procedures were repeated a second time. After the second extraction, the soil was resuspended in 20 mL methanol/water (4:1) and shaken overnight and supernatant removed. The extracts were combined and aliquots (2 x 2 mL) were taken for radioactivity measurement. Methanol was then removed from the extract in a Turbo-Vap-II and the aqueous residues were extracted with equal volumes of diethyl ether (x 2) and ethyl acetate (x 2) consecutively after being saturated with sodium chloride and acidified with 1.0 mL 1.0 M HCl. Aliquots from the aqueous phase were taken for radioactivity measurement. The organic phase was further concentrated to less than 1.0 mL and an aliquot was applied on a TLC plate. The extracted soil was dried at 60 C, mixed well, and 0.5 g (x 3) oxidized in a Packard Sample Oxidizer.

5.2 Preliminary Results & Discussion

5.2.1 Dissipation of Parent and Nonpolar Metabolites

Degradation of atrazine was apparently affected by amendments. When soil was mixed with dairy manure, a very rapid dissipation of atrazine and its nonpolar metabolites occurred during 4 to 6 weeks after treatment (Figure 10). However, after 12 weeks, no further significant degradation was observed. Degradation rate differed little in soil amended with corn meal and ammonium phosphate. After 20 weeks of incubation, greater amounts of atrazine and nonpolar metabolites remained in soils amended with corn meal and ammonium phosphate than in soil amended with manure or in the control. In the control soil that did not receive any amendments, degradation was relatively slow the first 6 weeks, but increased until the end of

the incubation period. The control soil exhibited lowerlevels of nonpolar residues than the other treatments after 20 weeks incubation. Adaptation and stimulation of atrazine degradation were involved.

The initial rapid disappearance of atrazine and its nonpolar metabolites in manure amended soil could be attributed to the abundant microorganisms existing in the manure before its introduction. It is also likely that there was a large population of non-selective microorganisms that could degrade organic N-containing nutrients in manure, and atrazine was merely cometabolized by these microbes as an organic N source. Inorganic N-products such as nitrite were formed following decomposition of organic N-compounds, providing a source of N for microorganisms other than atrazine assuming the microbes used atrazine as an N source. Biodegradation of atrazine was inhibited, possibly when the accumulation of these inorganic N-products reached a certain level, resulting in a much slower phase of atrazine degradation.

5.2.2 Mineralization of Atrazine

Mineralization of atrazine was highly responsive to different types of amendments. Addition of dairy manure greatly stimulated mineralization during 4 to 8 weeks after treatment (Figure 11). As much as 57.15% of the initially applied atrazine was completely decomposed during this 4 week period. This stimulation could have been caused by the introduction of N-degrading microorganisms with the manure. Mineralization of atrazine in the control soil was slower compared to manure amendment, but increased steadily with time indicating stimulation as well. As found in the previous incubation studies, it is likely that the control soil contained microorganisms which could use N and/or C sources from the *s*-triazine ring. Mineralization of atrazine at such an extensive scale has not been reported to date.

The mineralization process was inhibited in the cornmeal amendment treatment. Fungi were found growing in the mixture. It is possible that the addition of cornmeal changed the ecological structure of soil microorganisms present such that species not capable of mineralizing atrazine outcompeted microorganisms which could mineralize atrazine. Based on this observation, organic carbon amendments such as cornmeal would not be effective in removing atrazine from the soil matrix.

The addition of ammonium phosphate completely stopped the mineralization of atrazine. It is possible that micro-organisms used the readily available N in this fertilizer in preference to using atrazine as a N source. Existence of inorganic N-fertilizers at atrazine spill sites would likely slow down the detoxification of atrazine by microbial remediation. Though mineralization was inhibited in the treatments with ammonium phosphate and corn meal, atrazine was nevertheless degraded to some extent (Figure 10). Therefore, degradation was only inhibited up to the step of mineralization.

5.2.3 Formation of bound residues

At the end of incubation, 18 to 28% of ^{14}C-residues were in the form of nonextractable or bound residues in all treatments except ammonium phosphate (Figure 12). In soil amended with ammonium phosphate, significantly more bound residues were formed. As discussed for mineralization, it is likely that degradation intermediates accumulated in soil as cleavage of the *s*-triazine ring was stopped due to the use of N-sources from fertilizer in preference to atrazine. Higher levels of intermediates, such as the hydroxylated metabolites, were bound to soil organic matter and minerals than parent atrazine.

5.2.4 Future Research Needs

The results of this study strongly support further research on the use of amendments to stimulate biodegradation of atrazine residues in soil. The effects of readily available carbon sources, such as glucose, should be investigated. The effect of C:N ratio in atrazine degradation and mineralization needs clarification. Finally, the effects of different sources of manure should be explored.

6.0 SUMMARY AND CONCLUSIONS

This study illustrates that the effects of pesticide concentration on persistence, degradation and transport are highly compound-specific and vary with soil type. While the persistence of atrazine is relatively unaffected by concentration, high concentrations of alachlor are considerably more persistent than low concentrations of alachlor. The difference between the effects of concentration on the environmental behavior of atrazine and alachlor have implications for the choice of remediation options for soils contaminated with these chemicals at high concentrations.

The overall behavior of atrazine in the soils tested was not greatly affected by concentration in the range of 5 to 5,000 mg kg^{-1}, though differences between soils were observed. Stimulation of degradation and mineralization at higher concentrations resulted in similar persistence across all concentrations with the exception of the 5,000 mg kg^{-1} treatment level in the Estherville sandy loam soil. Ring cleavage, hydrolysis and formation of bound residues were the main metabolic pathways for atrazine decomposition in soil at all concentrations. Soil microbial activity was stimulated at higher concentrations in the Webster clay loam, evidence that some microbial species may have used atrazine as a N and/or C source.

These results indicate that the degradation and persistence of atrazine at elevated levels in soil is dependent, to some extent, on soil type but independent of concentration. The presence of a biodegradable chemical such as atrazine can cause a proliferation of active microbial flora and concurrently increase the rate of the decomposition of the applied pesticide. Any remediation method which increases the population, and activity of the relevant biodegraders would

accelerate the dissipation of atrazine and thus could be effective in detoxifying contaminated sites. Addition of proper nutrient amendments to soil merits further investigation as a remediation technique for atrazine decontamination in soil. Preliminary results were discussed.

This research suggests that it may be possible to treat soils containing high levels of atrazine in-situ, provided that other conditions (oxygen concentration, pH, organic matter content, nutrient supplies) are optimized. This type of treatment would only be feasible at isolated sites with low potential for ground and surface water contamination. At sites which are vulnerable to ground or surface water contamination, this research supports treatment of soil contaminated with high concentrations of atrazine by stockpiling and treating ex-situ in an aerated compost format with appropriate safeguards to prevent leachate or runoff. For either of these treatments, appropriate amendments such as livestock manure may enhance degradation. These recommendations are based on atrazine degradation behavior observed in laboratory experiments. Behavior under field conditions may vary from that observed in the laboratory. For instance, there is conflicting evidence on the effect of "aged" residues (residues which have been contained in soils for extended periods ranging from months to years) on the extent and rate of degradation. It would be necessary to test either approach in a pilot scale study prior to implementation at an actual site. Treatment of atrazine-contaminated soil by landspreading at or below labeled rates on labeled sites is a feasible remediation alternative if adequate land area is available.

High concentrations of alachlor pose a more serious threat to ground and surface water resources. Degradation was severely retarded and leaching increased dramatically at elevated concentrations compared to low, field application rates of alachlor. Additionally, a low percentage of alachlor was in the form of bound residue when applied at high concentrations. This indicates that while most of the alachlor residues were still available for further biodegradation, they are also readily available for leaching. Based on this and on the fact that alachlor degraded rapidly at concentrations at and below 100 ppm, landspreading may be a viable alternative for treatment of alachlor-contaminated sites and to reduce leaching at the contaminated site. Exposure of diluted alachlor and its degradation products to abundant soil microorganisms after landspreading should enhance the degradation process. Soils containing high concentrations of alachlor would not degrade sufficiently if left in place or if stockpiled without additional treatment. Thus, based upon this research, in-situ treatment of soils containing high concentrations of alachlor is not recommended.

Field studies showed that atrazine, and, to a greater extent, alachlor have the potential for greater impacts on ground water quality when present at high concentrations. Higher levels of atrazine were detected in soil layers within the profile following various time interval after the introduction of high concentrations compared to low concentrations to the site. Only slight differences in the time interval before detection of atrazine at the 71 to 85 cm soil depth occurred between the high and low atrazine concentration applications, although the

concentration of atrazine was higher at each given depth at each point in time in higher concentration situations.

Alachlor varied greatly in the potential for movement below the crop rooting zone as a function of concentration compared to atrazine. The low concentration of alachlor did not pose any environmental risk for alachlor movement below the rooting zone. However, at high concentrations, alachlor moved to the lowest depth sampled and presumably below the rooting zone posing a threat to ground water. Alachlor was detectable at lower depths in the soil profile, often at concentrations an order of magnitude higher than present with similar high concentration applications of atrazine. Preliminary field data and corroborative laboratory studies indicate that the persistence of alachlor will be increased several orders of magnitude at high concentrations as well, compounding concern for potential environmental impacts.

Degradation of atrazine was affected by amendments. Degradation rate differed little in soil amended with corn meal and ammonium phosphate, but degradation and mineralization were stimulated by the addition of dairy manure. Adaptation and stimulation of atrazine degradation by soil microorganisms were involved. Atrazine and its nonpolar metabolites degraded rapidly in initial phases of degradation in manure amended soil, likely due to the abundancef microorganisms in the manure. There is a possibility that non-selective microorganisms capable of degradingrganic N-containing nutrients in manure cometabolized atrazine.. Further studies are needed to delineate effects of metabolism of inorganic and organic compounds in the soil in preference to atrazine as sources of carbon and/or nitrogen. The effects of various amendments on alachlor degradation should also be explored.

7.0 References

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Publications/Presentations Resulting From This Project -

Published or Presented:

1. Gan, J., W. C. Koskinen, R. L. Becker, D. D. Buhler, and L. J. Jarvis. 1992. Biodegradation of alachlor in soil as a function of concentration. Agron. Abstr. pp. 39-40.

2. Gan, J., R. L. Becker, W. C. Koskinen, D. D. Buhler, and L. J. Jarvis. 1992. Biodegradation of atrazine in soil as a function of concentration. Agron. Abstr. p. 40.

To be Published or Presented:

1. Gan, J., W. C. Koskinen, R. L. Becker, D. D. Buhler, L. J. Jarvis. Biodegradation of alachlor and atrazine in soils as a function of concentration. University of Minnesota and USDA-ARS, St. Paul, MN 55108. Proc. Fourth Nat. Pest. Conf., New Dimensions in Pesticide Research, Development, Management, and Policy. Richmond, Virginia. (accepted for presentation in November 1993).

2. Gan, J., R. L. Becker, W. C. Koskinen, D. D. Buhler, and L. J. Jarvis. (0000). Degradation of Atrazine in Soil as a Function of Concentration. To be submitted to J. Envir. Qual.

3. Gan, J., W. C. Koskinen, R. L. Becker, D. D. Buhler, and L. J. Jarvis. (0000). Effect of Concentration on Persistence and Degradation of Alachlor in Soil. To be submitted to Soil Sci.

B6. **Benefits:** The difficulties inherent in assessing the potential for pesticide movement at known or future pesticide spill sites, and the prohibitive costs for remedying such situations require a more thorough knowledge of the behavior of these compounds at elevated levels. Private and public resources will be conserved by utilizing more efficient and prioritized bioremediation methodologies. This project will provide the basis for making better management decisions regarding the clean-up or containment of existing and future point source pesticide contamination.

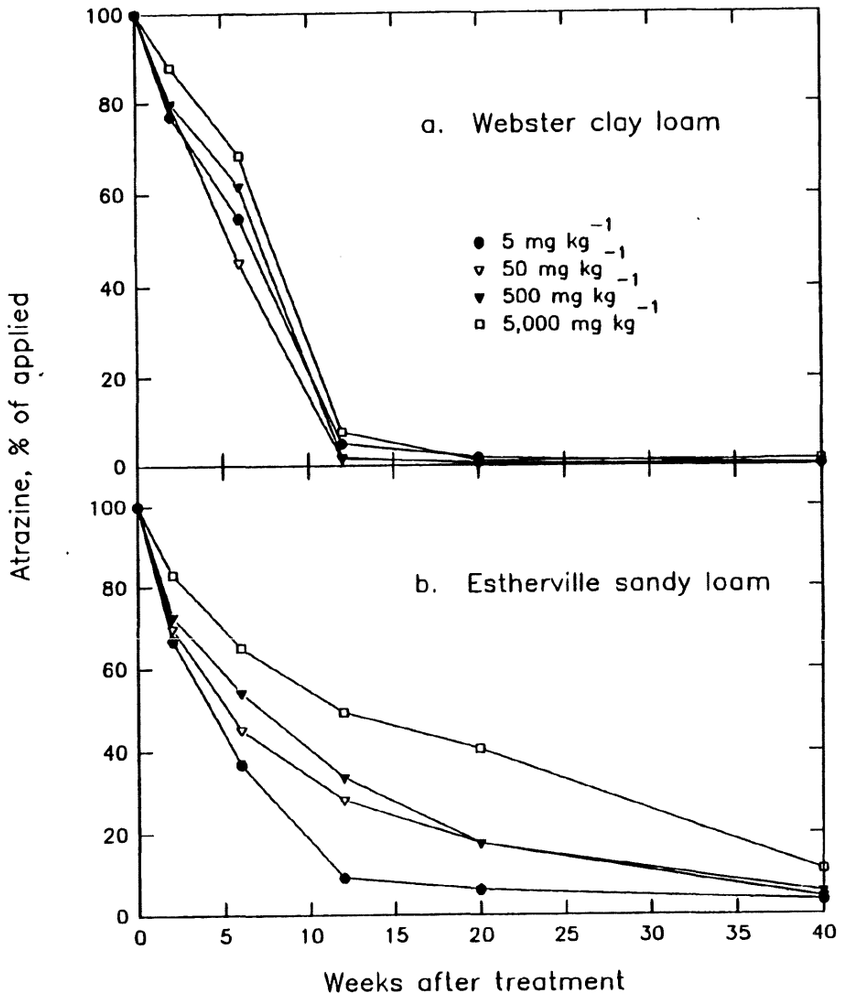

Figure 1. Dissipation of parent atrazine as a percent of initial application.

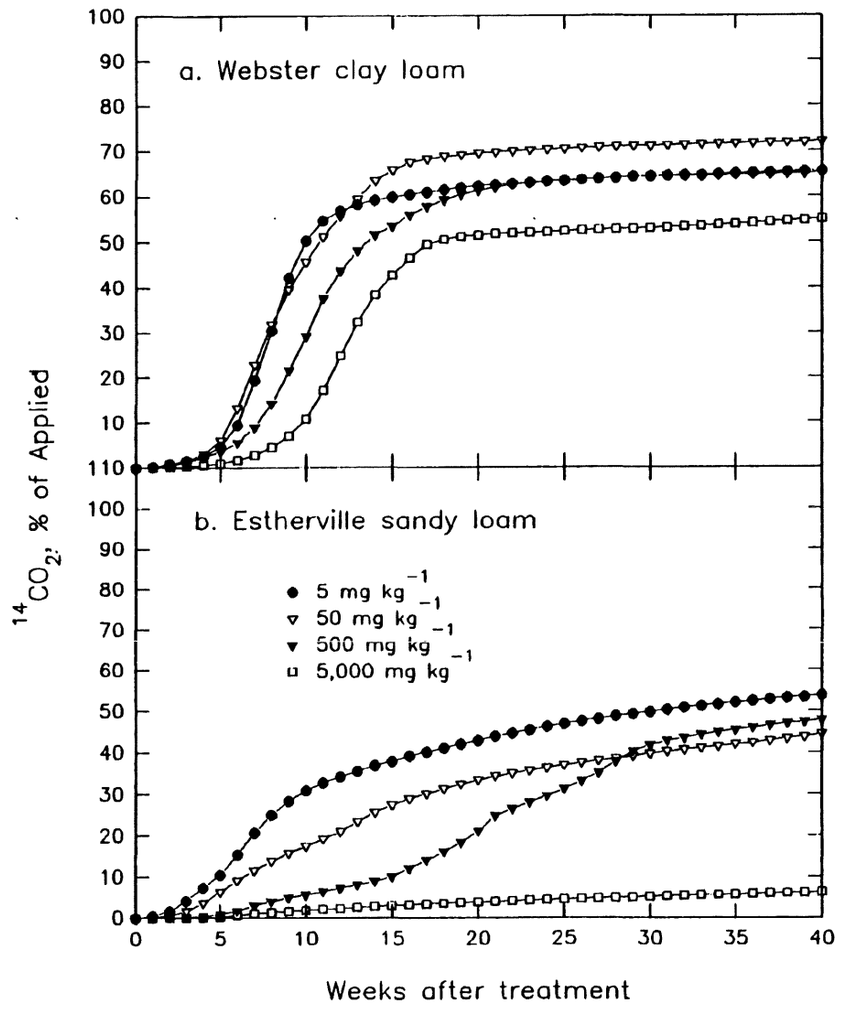

Figure 2. Mineralization of atrazine as a percentage of initial application.

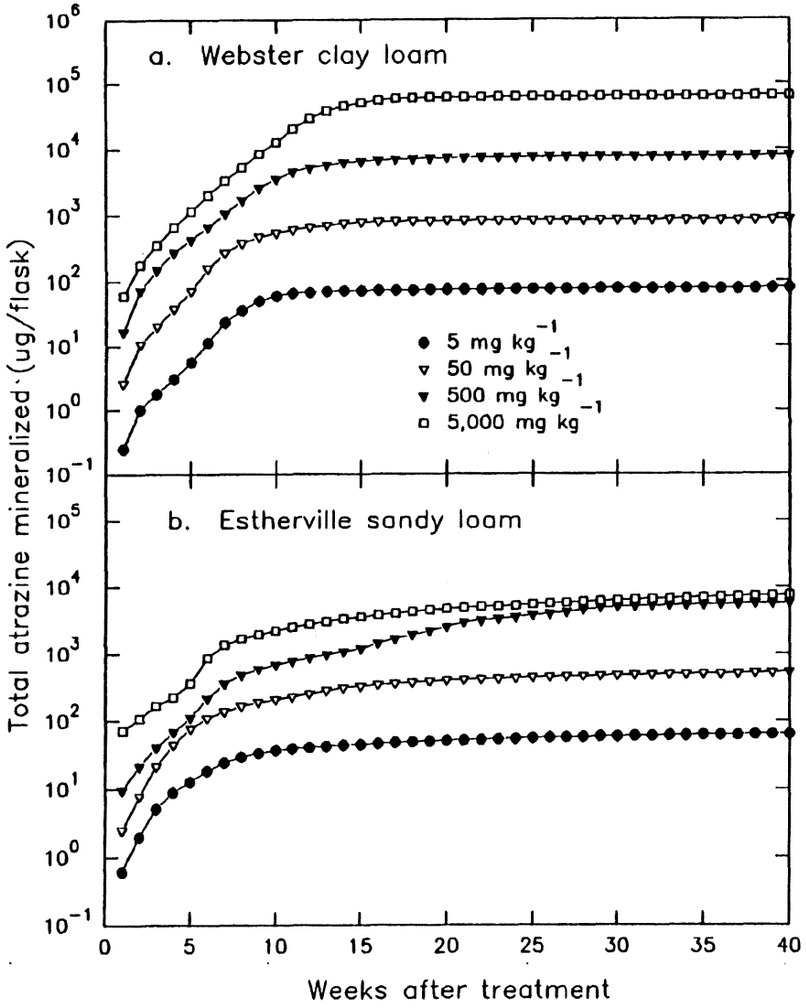

Figure 3. Mineralization of atrazine on an absolute amount basis.

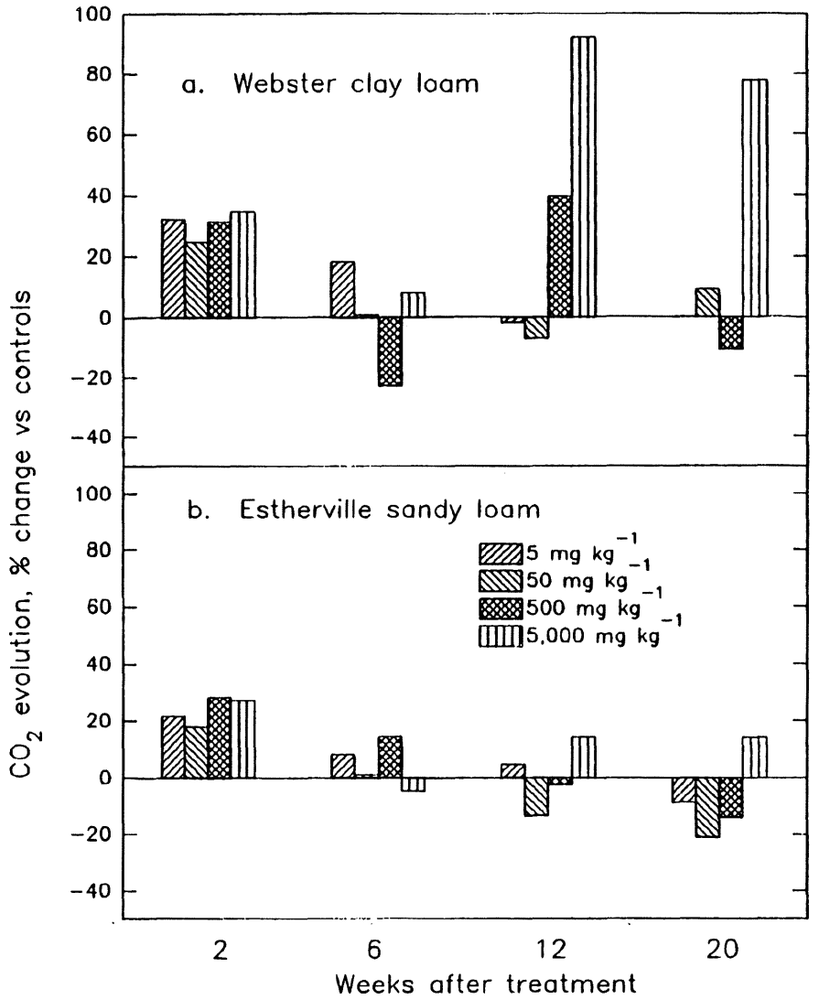

Figure 4. Effect of atrazine concentration on soil respiration.

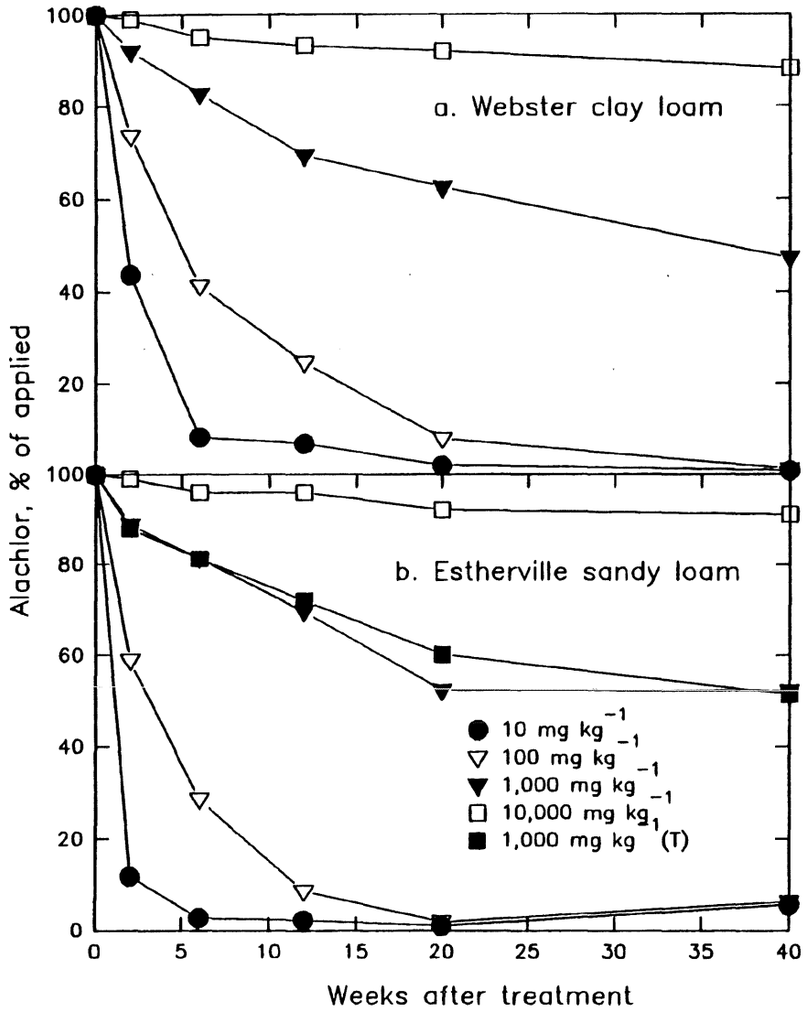

Figure 5. Dissipation of parent alachlor as a percent of initial application.

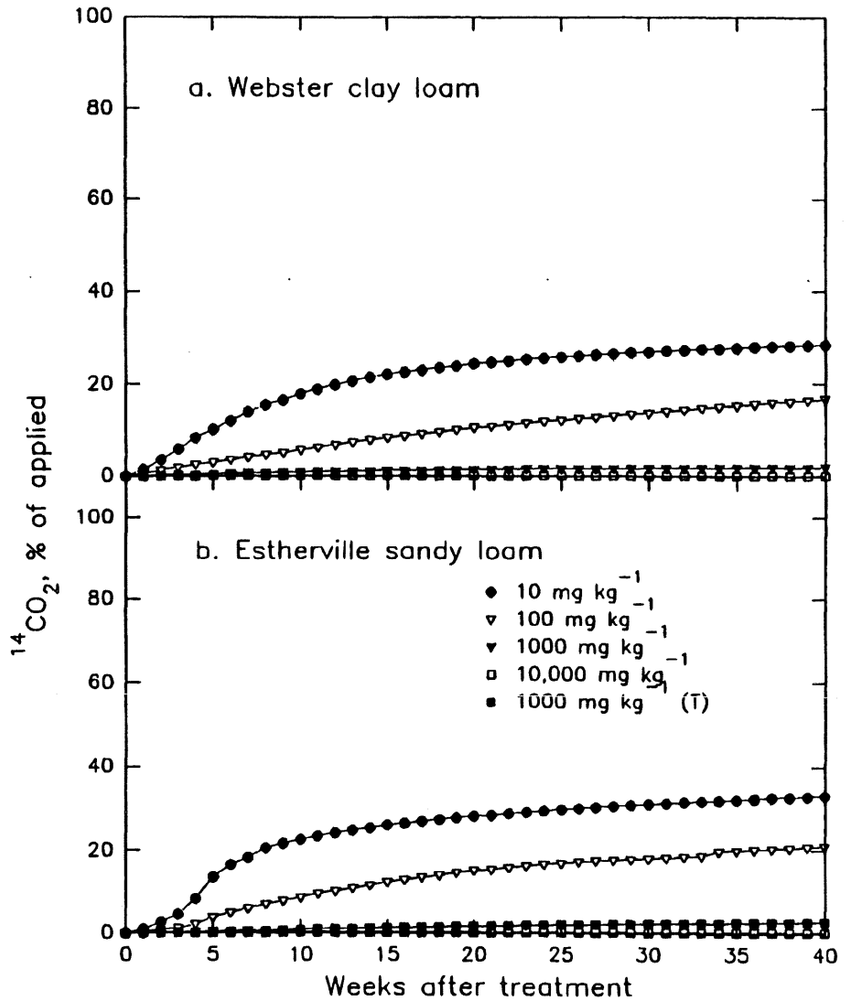

Figure 6. Mineralization of alachlor as a percentage of initial application.

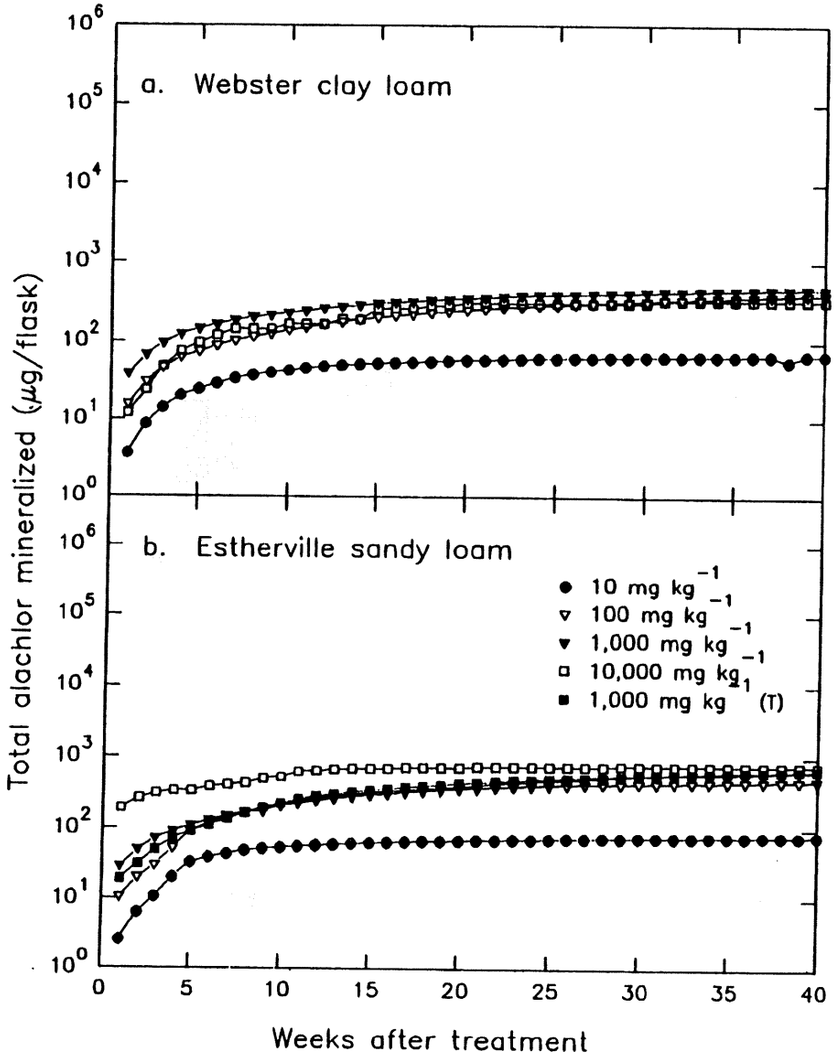

Figure 7. Mineralization of alachlor on an absolute amount basis.

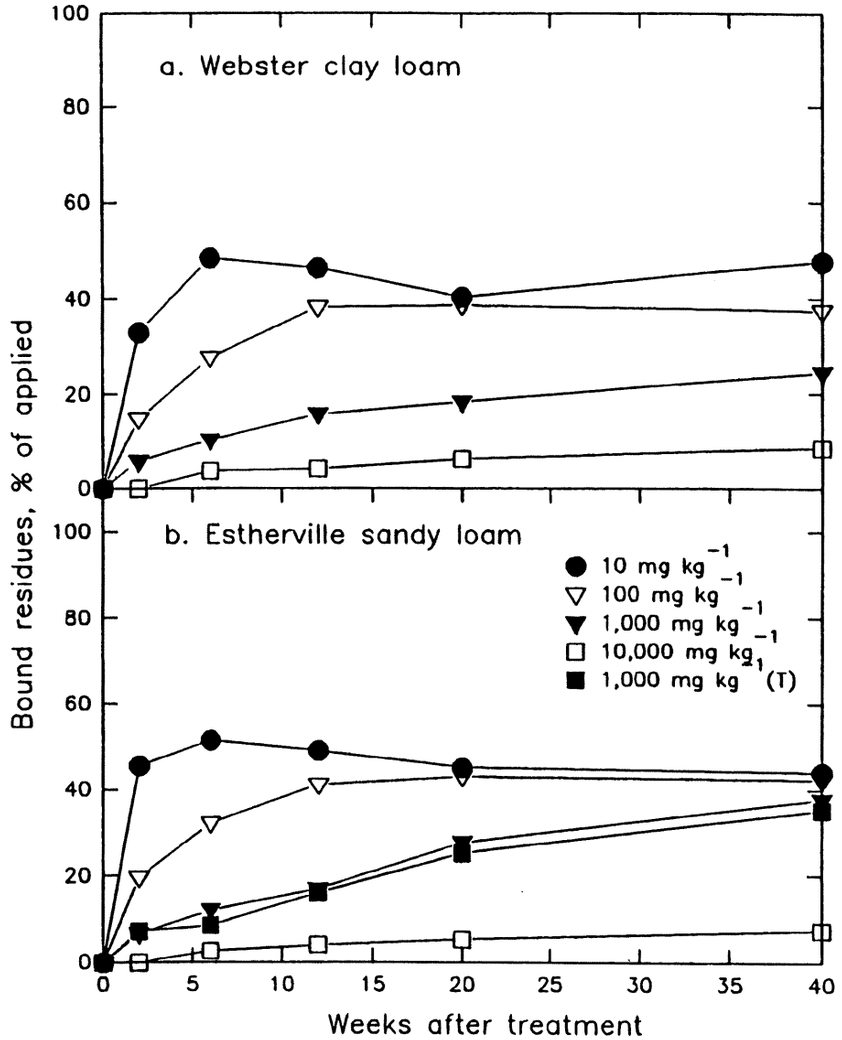

Figure 8. Formation of bound residues as a percentage of initial application.

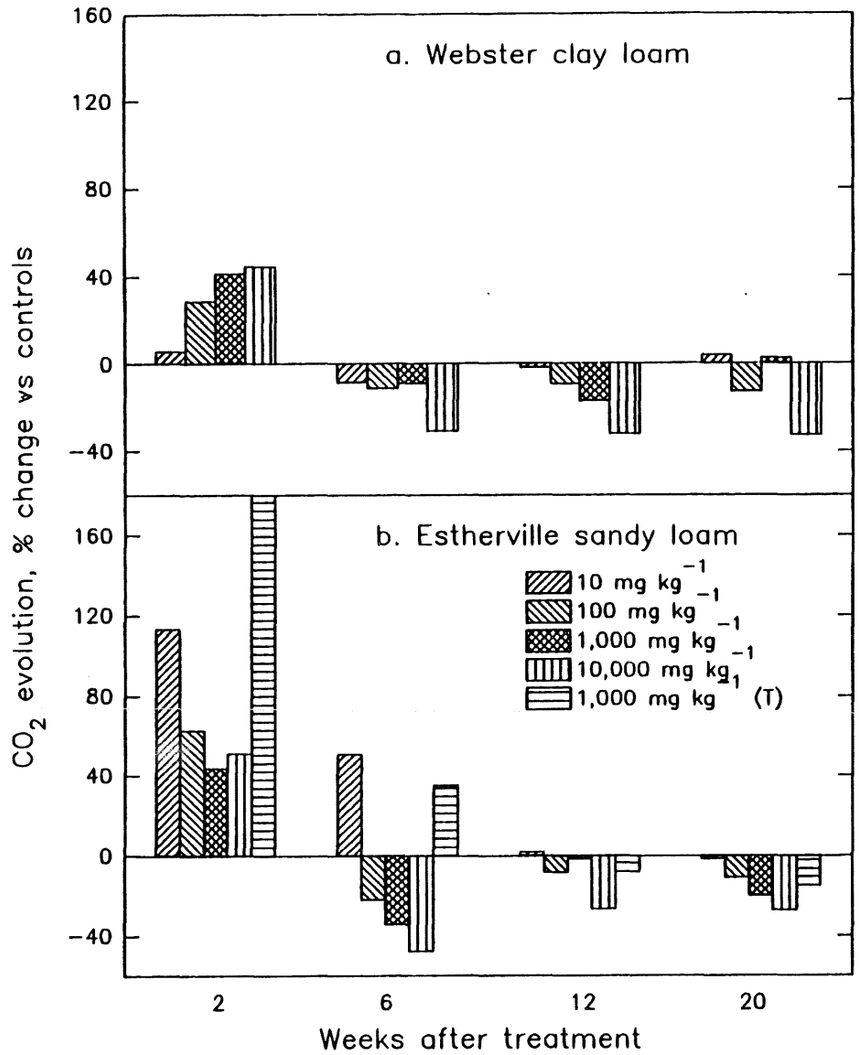

Figure 9. Effect of alachlor concentration on soil respiration.

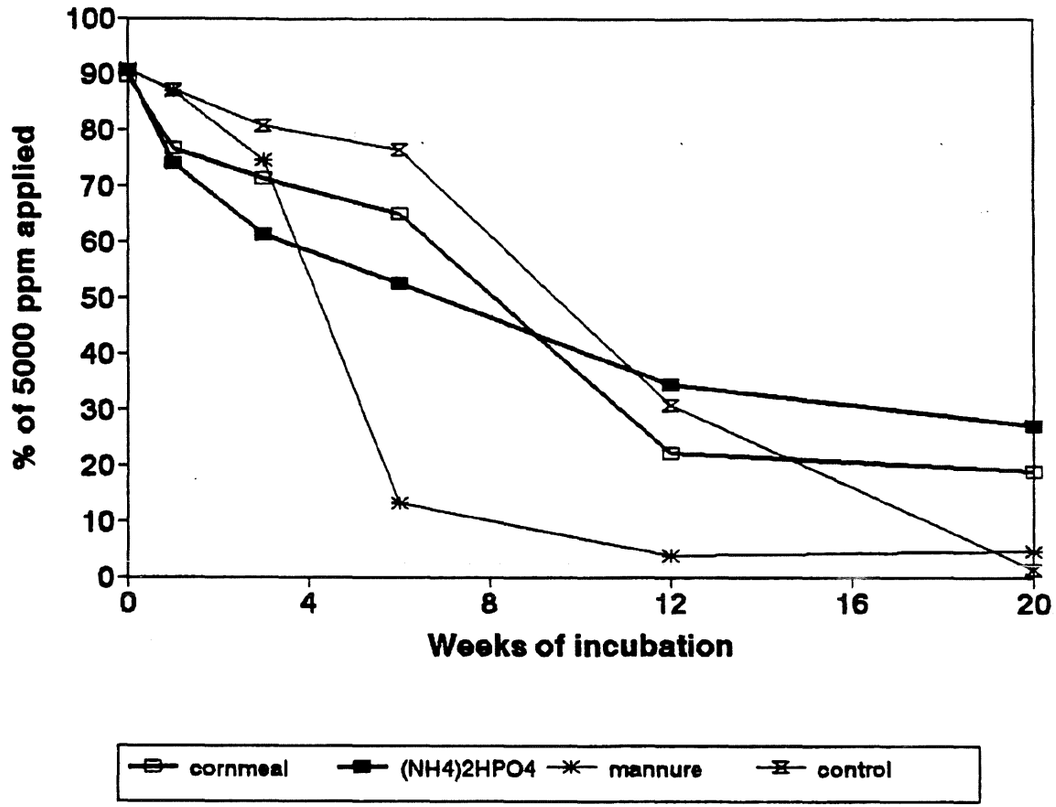

Figure 10. Effect of soil amendments on decline of parent atrazine and nonpolar metabolites in a Webster clay loam.

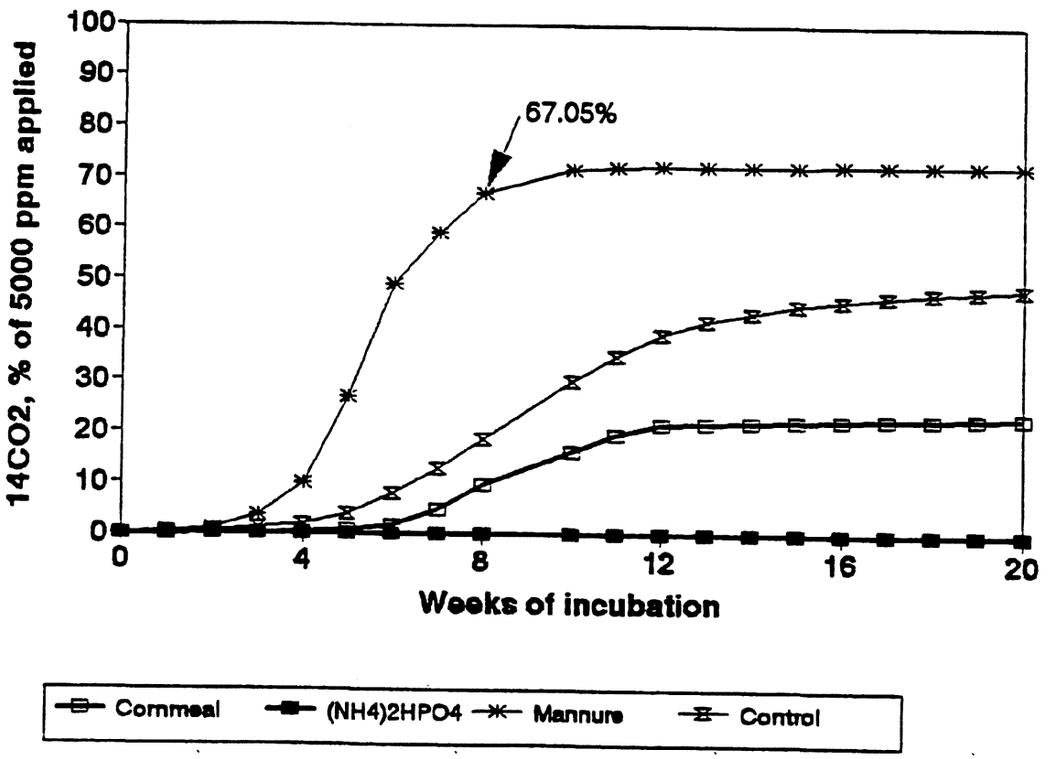

Figure 11. Effect of soil amendments on mineralization of atrazine in a Webster clay loam.

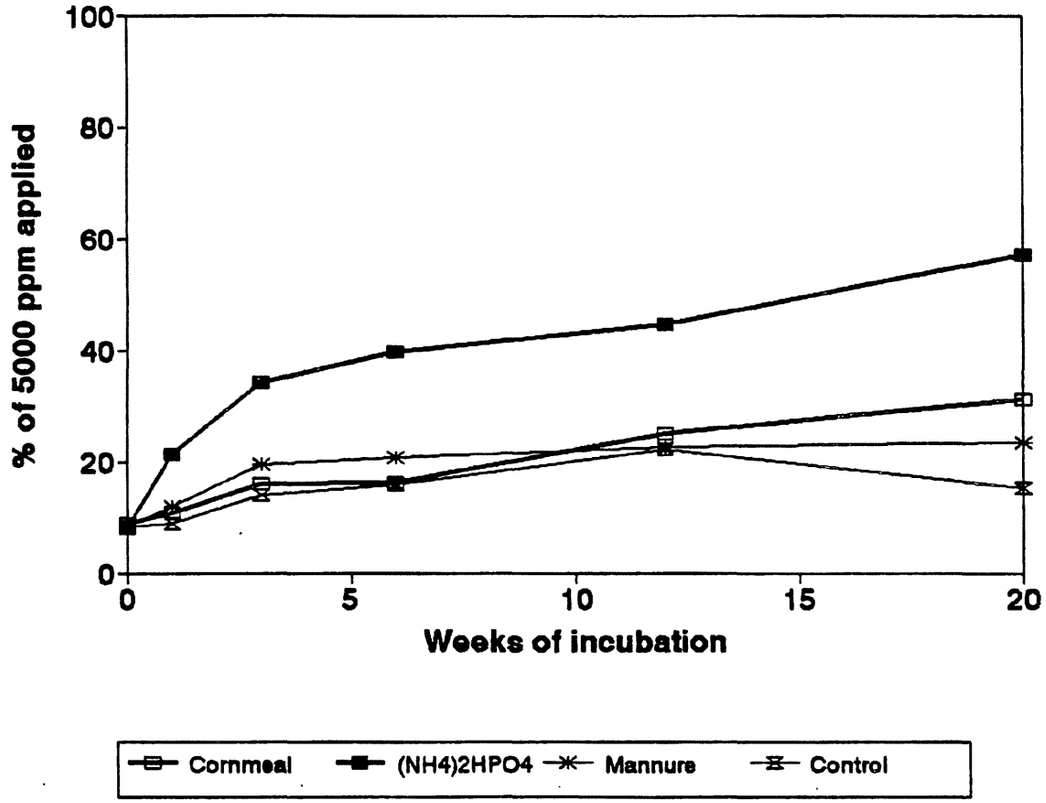

Figure 12. Effect of soil amendments on the formation of bound residues of atrazine in a Webster clay loam.

C. Investigate Innovative Bioremediation Techniques for Reduction of Elevated Pesticide Concentrations.

C1. Narrative: Atrazine is commonly detected in ground water and is commonly involved in pesticide spills. The focus of Objective C is to examine innovative approaches using plants and microbes to enhance biodegradation and removal of pesticides from spill sites. If enhanced degradation occurs in laboratory and greenhouse experiments, exploratory field studies may be undertaken as funding allows.

C2. Procedures: Atrazine is biotransformed by a number of reactions including oxidative dealkylation and conjugation with glutathione. It is proposed in this research to effect biotreatment of atrazine spills by enhancing the activities of microorganisms which catalyze these reactions. Two main approaches to the development of bioremediation methods will be attempted: investigation of enhanced degradation due to plant rhizosphere effects; and identification of microorganisms that metabolize atrazine. Plant species will be tested for tolerance of elevated levels of herbicide.

Previous work has shown that the conjugation product of atrazine with glutathione is tightly adsorbed to soil, mitigating against potential leaching of the pesticide into ground water (Clay and Koskinen, 1990, Weed Sci 38: 262-6). Typically, plants which display herbicide resistance manifest this resistance via oxidative and/or conjugative reactions to detoxify the pesticide. Some of these same types of biochemical reactions are known to be catalyzed by terrestrial and aquatic microorganisms. We have extensive experience studying bacterial oxygenases which are crucial in the biodegradation of aromatic compounds and chlorinated aliphatic compounds (Wackett and Gibson, 1988, Appl. Environ. Microbiol., 54: 1703-8; Wackett, et al., 1989, Appl. Environ. Microbiol., 55: 2960-4: Fox, et al., 1990, Biochemistry 29: 6419-27).

Furthermore, we have previously investigated the major group of conjugating enzymes, known as the glutathione S-transferase supergene family, from bacteria (Scholtz, et al., 1988, J. Bacteriol. 170: 5698-704), fungi (Wackett and Gibson, 1982, Biochem. J. 205: 117-22), and mammals (Blocki and Wackett, unpublished data). We have an E. coli strain that produces up to 50 mM glutathione in its immediate environment via overexpression of cloned glutathione biosynthetic genes. The ability of these and other well characterized soil isolates to effect high-level atrazine biotransformation will be assessed. We also will examine other well-determined biodegradation regimes for activity with atrazine.

Similar analytical methods will be used to monitor pesticide metabolism in the soil and in pure culture experiments. Extraction methods and HPLC analysis of metabolites will follow procedures developed by Bill Koskinen. Previously described dealkylated and conjugated

metabolites will be identified by comparison with authentic standard compounds. Heterocyclic ring side chain oxidation will be analyzed by trapping out ^{14}C - CO_2 using standard methods.

Selective enrichment cultures will be used to isolate bacterial populations (mixed or isolated) capable of growth on atrazine as a sole nitrogen source. All enrichment cultures will be subcultured into a homologous medium at two week intervals. Cultures will be successively subcultured under limiting conditions; subsequently, atrazine degradation will be quantified by HPLC analysis of the remaining atrazine in the medium. Positive enrichments will be harvested; attempts will be made to isolate pure cultures capable of degrading atrazine from the mixed cultures.

Atrazine mineralization assessment will be performed using uniformly ring-labeled [^{14}C]atrazine amended media inoculated with isolated cultures. Evolution of $^{14}CO_2$ will be measured; the atrazine medium will also be analyzed for residual radioactivity. Atrazine degradation pathways will be determined using ammonia 31 enzymatic analysis of ammonia in growth media and Thin Layer Chromatography analysis in addition to High Pressure Liquid Chromatography (HPLC) measurements of atrazine and its metabolites. Mass spectroscopy will also be used to further identify metabolites which coelute on the HPLC system.

Molecular probes to identify specific genes in microorganisms will be used to monitor potential enhancement of those microbial populations most active in pesticide detoxification. For example, gene probes are available for several bacterial oxygenases and they could be prepared from cloned genes that are known to be involved in conjugative reactions. We also have glutathione S-transferase gene family probes from bacterial, plant and mammalian sources. Higher levels of microbial populations containing these genes will be indicated by enhanced levels of atrazine detoxification. Since gene probe methodologies are relatively rapid, this will afford us a convenient tool for assessing the effectiveness of specific pesticide-detoxifying strains of bacteria.

Field testing will be exploratory and confined to one or two sites because of budget constraints. Based on previous experiments, the most likely candidates for field testing will be selected and inoculated in field plots at sites for Part B. These plots will be replicated and blocked. Soil samples at different depths in and below the root zone will be monitored for remaining parent compound and metabolites and compared to unplanted control plots.

C3. Budget:

a. Amount Budgeted: $100,000.00
b. Balance: $ - 0 -

C4. Tim[illegible] for Products/Tasks:

	July 91	Jan 92	June 92	Jan 93	June 93
Greenhouse and Growth Chamber Experiments	**********				
Cultures and Molecular Probe Analysis		**********	**********		
Test Cultures on Aged Soil				**********	
Final Report					******

C5. Status:

Abstract

The purpose of this research was to test innovative approaches using plants and microbes to enhance biodegradation and removal of pesticides from spill sites. The pesticides selected for study were atrazine and alachlor, because they are often detected in groundwater and are commonly involved in pesticide spills in Minnesota. Three major approaches were attempted in seeking remediation technologies: 1) enrichment cultures for microorganisms obtained from soils exposed to repeated spills, 2) conjugation of atrazine via glutathione-S-transferase, and 3) plant rhizosphere enhanced degradation. The most successful approach was enrichment for atrazine degrading microorganisms utilizing 100 ppm (0.46 mM) atrazine as a sole nitrogen source. Bacterial growth occurred concomitantly with formation of atrazine metabolites and subsequent biosynthesis of protein. With ring labeled [^{14}C]atrazine, > 80% of the s-triazine ring carbon atoms were liberated as $^{14}CO_2$. Over 200 pure cultures isolated from the enrichment cultures failed to utilize atrazine as a nitrogen source. Mixing pure cultures restored atrazine mineralizing activity. Repeated transfer of the mixed cultures led to increasing rates of atrazine metabolism. Degradation half lives for 100 ppm atrazine ranged from 0.5 to 2 days in liquid culture, which far exceeds the rates previously reported in the literature for soils, waters, mixed and pure cultures of bacteria. Hydroxyatrazine was found to be an intermediate in the atrazine mineralization pathway. Bacterial enrichment cultures in two soils and cell free protein extract from the bacteria produced hydroxyatrazine from atrazine, which was then further metabolized. Bacterial atrazine dechlorination was hydrolytic as demonstrated by ^{18}O incorporation from $H_2{}^{18}O$ into hydroxyatrazine. Preliminary experiments testing inoculation of soil bacterial mixed culture on spill site soils indicate significant (60%) degradation of atrazine after 28 days in the presence of added sodium citrate source.

1.0 Introduction

Atrazine (2-chloro-4[ethylamino]-6[isopropylamino]-1,3,5 triazine) is the most widely used s-triazine herbicide in the United States (Burkart et al., 1988). It has widespread use in the control of broad-leaf weeds in corn, sorghum, and certain other crops. Approximately 800

million lbs [illegible]e used in the United States between 1980 and 1990 (G[illegible]si, 1987). Atrazine is moderately persistent in the environment (Erickson and Lee, 1989). It has a water solubility of 33 mg L^{-1} (27°C) and a reported half-life in soils ranging from 4 to 57 weeks (Cohen et al., 1984). Its spillage at herbicide loading sites and subsequent runoff can cause crop damage and ground water contamination. As a result, atrazine is detected in ground water and soils in concentrations exceeding the current maximum contaminant level (MCL) of 3 mg L^{-1} (U.S. Environmental Protection Agency, 1991). In Kansas, atrazine has been detected in well water at several locations at levels up to 7.4 mg L^{-1}(ppb) (Koelliker et al., 1986). Point source spills of atrazine resulted in levels as high as 1000 ppb in some wells in Minnesota (DeLuca, 1992).

Herbicides containing an s-triazine ring are relatively persistent in the environment. This has stimulated investigations into their biodegradation with mixed success (Erickson and Lee, 1989). In one report, 33 mixed bacterial cultures were examined and all failed to degrade atrazine (Geller, 1980). Enrichment cultures from silty loam soil failed to mineralize atrazine to CO_2(Fernandez-Quintanilla et al., 1981). Generally, less heavily substituted and non-chlorinated s-triazines are more biodegradable than atrazine (Cook, 1987). For example, two soil fungi degrade cyanuric acid, but not atrazine to CO_2 (Wolf and Martin, 1975). Bacteria capable of utilizing s-triazine compounds as a sole nitrogen source have been isolated by enrichment culture. However, Pseudomonas and Klebsiella strains which degraded various s-triazine ring compounds were not capable of metabolizing atrazine (Cook, 1987; Cook and Hutter, 1981). Several microorganisms that can degrade atrazine have been isolated, but in most cases only N-dealkylation of the atrazine side chains occurred, and complete metabolism of the s-triazine ring was not demonstrated. Pseudomonas strains are described to N-dealkylate atrazine and use the side chains carbons for growth (Behiki and Khan, 1986).

Kaufman and Blake (1970) studied several soil fungi that were able to degrade atrazine by N-dealkylation as evidenced by $^{14}CO_2$ evolution from [^{14}C] ethyl or isopropyl atrazine. McMahon et al. (1992) reported that the ethyl side chain of atrazine, but not the s-triazine ring, was degraded by microbial processes in alluvial-aquifer sediments. Most recently, a very slow liberation of $^{14}CO_2$ from the atrazine ring was observed in soil bioreactors (Nair and Schnoor, 1992). Less than 10% of uniform ring labeled [^{14}C]atrazine was converted to $^{14}CO_2$ in 125 days. It is widely accepted that the atrazine dechlorination reaction in soils is a soil-catalyzed chemical process (Fig. 1), while N-dealkylation reactions are biologically mediated (Erickson and Lee, 1989; Armstrong et al., 1967; Skipper et al., 1967; Obien and Green, 1969; Kaufman and Blake, 1970; Skipper and Volk, 1972; Muir and Baker, 1978; Fernandez-Quintanilla et al., 1981; Adams and Randtke, 1992; Sorenson, 1992). While s-triazine compounds with less bulky side chain substituents undergo bacterially mediated dechlorination (Cook and Hutter, 1984), atrazine was not transformed to hydroxyatrazine in this or other studies of bacterial atrazine degradation (Erickson and Lee, 1989). Only a slow dechlorination of atrazine by soil fungi has been reported (Kaufmann and Blake, 1970; Couch et al., 1965). Other data are interpreted to support non-biological mechanisms of atrazine hydrolysis. Soils reported sterilized by sodium-azide or heat retained the capacity to form

hydroxyatrazine, presumably by organic matter catalysis (Armstrong et al., 1967; Harris, 1967; Agnihorti et al., 1976; Nearpass, 1972; Li and Felbeck, 1972). These chemical transformations are strongly pH dependent with both acid and alkaline conditions promoting hydrolysis of atrazine (Fernandez-Quintanilla et al., 1981; Best and Weber, 1974). The transformation of atrazine to hydroxyatrazine is of environmental significance. The latter compound is not effective as a herbicide (Gysin and Knusli, 1960). Several studies have shown that hydroxyatrazine rapidly becomes unavailable to extraction from soil, either due to biodegradation, bound residue formation, or both effects (Skipper and Volk, 1972; Hance and Chesters, 1969; Goswami and Green, 1971).

The objective of this research was to enrich for microorganisms capable of mineralization of high concentrations of atrazine. Previous studies using atrazine as the sole N-source failed to yield bacterial cultures that effectively mineralize atrazine. In contrast to other studies, we have used citrate as a carbon source and succeeded in the enrichment of stable mixed bacterial cultures that could liberate $^{14}CO_2$ from the s-triazine ring of atrazine. The rates of atrazine degradation observed were significantly faster than those previously reported for atrazine side chain or s-triazine ring metabolism. These experiments are reported in Section 2.

In Section 3, we report the rapid transformation of atrazine to hydroxyatrazine at neutral pH by a soil bacterial mixed culture LFB6. Addition of bacteria to atrazine-containing artificial growth media or soils yielded hydroxyatrazine. The transformation was hydrolytic as demonstrated by [^{18}O]-labeling experiments. The observed rates are extremely fast, which suggests that small populations of soil bacteria may produce significant quantities of hydroxyatrazine.

In Section 4 , we report results of three experiments to test ability of soil bacterial mixed culture LFB6 to degrade atrazine in soils. Finally, in Sections 5,6 and 7, we report the results of our experimental attempts to enrich for alachlor degradation, enhance pesticide degradation in plant rhizospheres, and detoxify atrazine by glutathione conjugation.

2.0 Mineralization of the Atrazine s-Triazine Ring by Stable Bacterial Mixed Cultures

2.1 Materials and Methods

2.1.1 Sampling Sites

Soil samples were collected from three Minnesota sites which were formerly operated as agricultural chemical dealerships and are being considered for bioremediation. Two sites in the city of Little Falls and a third site in Albany, MN are referred to as LFA, LFB and ALB, respectively. The native soils of the LFA site are Hubbard and Nymore sands, and at the LFB site they are loamy sands. The native soils at the ALB site are Cordoba loam and unclassified ponded histosols (Helgesen et al., 1975).

Twelve cores (1.9 cm inner diameter x 30 cm deep) were collected at random locations at each site and stored at 40°C until used. Some grassy weeds were also collected from the boundaries of the LFA site for isolation of potential atrazine degrading microorganisms from the rhizoplane. Physical and chemical data are presented in Table 1.

Table 1

Physical and chemical properties of the spill site soils.

Sampling site	Soil type (USDA)	Particle size distribution (%) sand-silt-clay			pH in water	pH in 0.1 N $CaCl_2$	Total N ($\mu g\ g^{-1}$)	Olsen's P ($\mu g\ g^{-1}$)	Total organic matter (%)	Total organic carbon (%)
LFA	Loamy sand	85	7	8	5.04	4.88	1,240	135	1.5	0.90
LFB	Loamy coarse sand	87	7	6	7.04	7.18	500	95	0.8	0.44
ALB	Loam	44	42	14	5.48	4.88	1,280	500	2.3	1.65

2.1.2 Enrichment Cultures

Selective enrichment cultures were used to obtain mixed bacterial populations that were capable of growth on atrazine as a nitrogen source. Atrazine, metolachlor and alachlor concentrations in soil samples were analyzed according to Koskinen et al. (1991). For samples that contained one or more herbicides, 10 g of soil was suspended three times in 30 ml 0.1 M phosphate buffer (pH 7.3), centrifuged (7000 x g) for 10 min at 40 C, and the supernatant fluids discarded to reduce the quantity of extraneous nitrogen source(s).

To obtain enrichments of weed rhizoplane microorganisms, loose soil particles were removed by hand and the roots were submerged for 10 min in tap water, rinsed 1 min under slow running tap water and air dried. Roots were cut into 5 cm segments and 10 segments were shaken in a 250 ml Erlenmeyer flask with 10 g glass beads (3 mm diameter) and 50 ml 0.1 M phosphate buffer on a reciprocal shaker for 2 h. Enrichment cultures were prepared as described below using 5 ml of the buffer as inoculum. A 5 g wet soil pellet or 5 ml rhizoplane suspension was inoculated into 20 ml atrazine medium containing the following ingredients per L deionized water: K_2HPO_4 1.6 g; KH_2PO_4 0.4 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; NaCl 0.1 g; $CaCl_2$ 0.02 g; sucrose 1 g; sodium citrate 1 g; atrazine stock solution 2.5 ml; salts stock solution 20 ml; vitamins stock solution 20 ml. The salt stock solution consisted of the

follo... in 1 L deionized water: EDTA 2.5 g; $ZnSO_4$ 11.1 g, ...O_4 5.0 g; $MnSO_4 \cdot H_2O$ 1.54 g; $CuSO_4 \cdot 5H_2O$ 0.4 g; $Co(NO_3)2 \cdot 6H_2O$ 0.25 g; $Na_2B4O7 \cdot 10H_2O$ 0.18 g; and 5.0 ml of concentrated H_2SO_4 to retard precipitation of salts. The vitamin stock solution contained per 1 L deionized water: thiamin • HCl 5 mg; biotin 2 mg; folic acid 2 mg; nicotinamide 10 mg; pyridoxine • HCl 10 mg. Atrazine stock solution was prepared in methanol (20 mg/ml) and was vigorously shaken for several hr prior to incorporation into the medium. Salts and vitamin stock solutions were filter sterilized and kept at 40 C. The atrazine stock solution was stored unfiltered at room temperature in the dark. Cycloheximide (50 mg L^{-1}) was added to media for isolation of bacteria and the pH was adjusted to 7.3. Media for isolation of fungi were acidified to pH 5.5 with a concentrated HCl solution, but were not amended with antibiotics. Cultures were incubated without shaking at 30°C in the dark to preclude photolysis reactions. All enrichment cultures were subcultured into a homologous medium in two week intervals. From a two week old culture, 0.5 ml was transferred to 20 ml of freshly prepared atrazine medium. After subculturing four times under conditions of nitrogen limitation, atrazine degradation was quantified by high pressure liquid chromatography (HPLC) as described below. Positive enrichments were harvested by centrifugation, resuspended in buffer and frozen at either -20°C or amended with 20% glycerol (by volume) and stored at -70°C.

Each culture received an identification name based on the site where the initial inoculum was collected, the sample number within the site, and a letter: A = grown in an acidified medium for the first 4 growth cycles; N = grown in a neutral medium throughout the enrichment period; R = isolated from the grassy weed rhizoplane.

2.1.3 Atrazine Mineralization By Growing Cultures

Uniformly ring labeled [^{14}C]atrazine was added to 100 ppm unlabeled atrazine to yield 1.1x10-3 mCi/ml. Twenty ml of medium (pH = 7.3) were aseptically transferred to 250 ml biometric Erlenmeyer flasks. The flasks were inoculated and sealed after being flushed for a few seconds under a stream of oxygen (99.6 % pure) to ensure aerobic conditions and to reduce the amount of N2 in the flask atmosphere. At periodic intervals, the flasks were opened and the amount of $^{14}CO_2$ evolved and trapped in 2 N NaOH solution was determined with a scintillation counter. An aliquot of the atrazine medium was also analyzed for residual radioactivity. Prior to resealing and further incubation, the NaOH in the trap was replaced with a fresh solution and the flask reflushed with oxygen as before.

2.1.4 Determination Of Microbial Growth

Cell growth was measured as absorbance at 600 nm using a Beckman DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA). Protein content of the cultures was determined after cell lysis in 0.1 N NaOH at 80°C for 1 h. A bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL) with a bovine serum albumin standard was used.

2.1.5 Assessme... Nitrogen Fixation Activity In The Enrichment Cult...

The acetylene reduction assay was conducted according to Krieg (1981). The presence of nitrogen fixing bacteria was also tested using a nif gene probe and the hybridization procedure of Holden, et al (1989). The probe was obtained from Dr. Michael Sadowsky (Department of Soil Science, University of Minnesota).

2.1.6 Analytical Methods

Analysis of total N in soils was determined following the procedure of Bremner and Mulvaney (1982). Soil phosphorus content was determined following the procedure of Olson et al. (1954). Soil total organic carbon was measured using a TOC analyzer (Leco Corp., St. Joseph, MI), total organic matter was measured as loss of weight from dry soil sample after pyrolysis. Ammonia in the growth media was determined with the Ammonia 31 enzymatic kit (Sigma, St. Louis MO). Radiorespirometry was performed in 250 ml biometric Erlenmeyer flasks containing 20 ml medium supplied with uniform ring-labeled [^{14}C]atrazine. The flasks were equipped with a CO_2 trap containing 2 ml of 2N NaOH. Radioactivity in samples and CO_2 traps was determined with a Beckman LS 6800 scintillation counter (Beckman Instruments, Irvine, CA) with channels set to 0-670 and 250-670 for background and sample readings respectively. To determine the formation of non-volatile atrazine metabolites in the growth medium, equal volumes of ethyl acetate and growth medium were vigorously mixed and radioactivity in the aqueous phase was measured. Typically more than 98% of the atrazine in non inoculated control treatments partitioned into the organic phase. Thin layer chromatography (TLC) was performed on silica gel TLC precoated plates with a 3 cm preadsorbant spotting layer (J.T. Baker Inc., Phillipsburg, NJ), using chloroform: methanol: water: acetic acid (70:25:4:2 v/v). High Pressure Liquid Chromatography (HPLC) measurements were done using a Spectra Physics system (Spectra Physics, San Jose, CA) consisting of an 8800 pumping system with a Rheodyne 7125 valve-and-loop injector (Cotati, CA) fitted with a 20 or 100 ml loop and a UVIS-204 detector (Linear Instruments Inc., Reno, NV). Absorption at 220 nm was recorded. Routine analysis of residual atrazine in enrichment cultures was performed using a Spheri-5 C18 RP column (Alltech Associates Inc., Deerfield, IL) 100 mm in length and 4.6 mm in internal diameter. A reverse phase isocratic HPLC mobile phase was adopted from Wenheng et al. (1991) containing 50:50 (v/v) acetonitrile-aqueous 0.1 M H_3PO_4, and 20 mM n-heptanesulfonic acid. The eluant pH was adjusted to 2.8. The flow was adjusted to 1.0 ml/min, and the chromatography conducted at room temperature. For determination of atrazine metabolites, a C8 column (Phase Separation Inc. Norwalk, CT) 300 mm length and 4.6 mm internal diameter was used. The procedure suggested by Vermeulen et al.(1982) was followed, except that the isocratic mobile phase contained methanol:aqeous 50 mM ammonium acetate (50:50 v/v). The flow rate was 1 ml/min with the column at room temperature. The chromatograms were displayed on a Spectra Physics Chromjet recording integrator (Spectra Physics, San Jose, CA). Authentic standards were chromatographed to aid in the identification of metabolites.

2.1.7 Chemicals

Atrazine (99.6 %) was purchased from Chem Service (West Chester, PA). [^{14}C] uniform ring-labeled atrazine (7.8 mCi/mmol; 99.6 % radiochemical purity) was purchased from Sigma (St. Louis, IL). Authentic samples of simazine, atrazine, desisopropylatrazine, desethylatrazine, hydroxyatrazine, hydroxydesisopropylatrazine, and hydroxydidesalkylatrazine were a gift from Ciba Geigy, Corp. of Greensboro, NC. Individual 100 ppm stock solutions of authentic atrazine and metabolite standards were prepared at the 100 ppm level in methanol:aqueous 0.1 N H_3PO_4 and stored at 40 C. All other chemicals used were of reagent grade, or better.

2.2 Results

2.2.1 Enrichment Of Microorganisms Using Atrazine As The Sole Nitrogen Source

Successful enrichment cultures, at pH 5.5 and 7.3 and using 100 ppm atrazine as the sole nitrogen source, were obtained from all three herbicide spill sites (Table 2). In the first three transfers, atrazine utilization was indicated by significant growth in 31 out of 38 cultures. The 31 cultures were transferred for the fourth time, and two weeks later they were extracted and analyzed for atrazine disappearance by HPLC; 21 cultures had less than 50% atrazine remaining (Table 2). Atrazine disappearance was determined to be the result of microbial metabolism. Control media which were uninoculated did not show significant atrazine disappearance (< 10%). In studies with [^{14}C]atrazine, less than 10% of the radioactivity was associated with the biomass, mitigating against atrazine depletion by binding to cells. Further experiments with [^{14}C]atrazine, described below, confirmed the metabolism of atrazine to non-volatile metabolites and $^{14}CO_2$.

Table 2.

Atrazine content at spill site sampling locations and atrazine degradation in the corresponding enrichment cultures.

Soil sample [a]	Atrazine content in soil samples used as inoculum (ppm)	Atrazine left in enrichment medium[b] (ppm) pH=5.5	pH=7.3
LFA1	12.90	ND[d]	ND
LFA2	5.57	44	50
LFA3	0.50	38	ND
LFA4	0.30	7	3
LFA5	0.30	12	8
LFA6	0.27	6	3
LFA7	0.07	7	3
LFB1	0.87	50	20
LFB2	0.71	54	9
LFB3	0.24	12	ND
LFB4	<0.01	55	99
LFB5	<0.01	58	20
LFB6	<0.01	18	4
ALB1	5.8	88	8
ALB2	4.76	65	10
ALB3	1.03	ND	100
ALB4	0.75	ND	ND
ALB5	0.67	70	11
ALB6	0.53	75	10

a LFA=Little Falls, site A; LFB=Little Falls, site B; ALB=Allbany site.
b Measured in the fourth subculture growth medium after 14 days by HPLC. Starting concentration was 100 ppm for each.
c Amended with cycloheximide.
d Not determined for cultures abandoned prior to fourth subculture due to slow or no growth with atrazine as sole N-source. These could be considered as 100 ppm remaining.

Atrazine utilization as a nitrogen source was also indicated by several lines of evidence. First, atrazine consumption was concomitant with growth in nitrogen limited medium. Second, media lacking atrazine failed to support growth. Some cultures were screened for nitrogen-fixing activity using the acetylene reduction assay and were found to be negative. Concomitantly, a nif-gene probe failed to detect nif genes while a positive control, Rhizobium sp., gave a positive hybridization. Lastly, the addition of ammonium nitrate to cultures

supp[illegible]d growth but suppressed atrazine degradation (data not s[illegible]n). Taken together, these data demonstrated the consumption of atrazine as a nitrogen source by cultures from all three soil sampling sites.

Results shown in Table 2 indicate that atrazine degrading organisms were obtainable from multiple locations at each site. Successful enrichments were observed at both pH 5.5 and 7.3. Generally, the degree of degradation appeared to be more related to the site than to the pH of the enrichment. Only at the LFA site did soils with lower atrazine content give rise to bacterial enrichment cultrues with high atrazine degrading activity.

Plating of bacteria on atrazine solid medium indicated the presence of >10 different colony-types present in most of the cultures. Random selection of over 200 strains purified from the mix cultures failed to yield a single organism capable of degrading atrazine in liquid culture. However, inoculation of liquid media with colony mixtures from plates resulted in cell growth (observed as turbidity) and atrazine mineralization. This suggested that the metabolism of atrazine might require the combined metabolic activities of more than one organism. These mixed cultures were stable on repeated transfers and could be frozen and thawed. Further studies were conducted to characterize atrazine degradation by these stable consortia.

2.2.2 Characteristics Of Stable Atrazine Degrading Consortia

Cultures that degraded more than 50 ppm atrazine in the fifth growth cycle were selected for further study. Ring-labeled [^{14}C]atrazine was used to determine the fate of atrazine in the growth medium. Atrazine concentrations were determined by HPLC and the non-volatile metabolites were determined as radioactivity in the aqueous phase after extraction with ethyl acetate. Clear differences among the cultures were detected. Although 100 ppm atrazine was consumed by all cultures within 7 days, the kinetic course of metabolism differed (Fig. 2). First, while cultures LFAR and LFB6A showed a distinct lag period in atrazine degradation, cultures LFA6A and LFB3A degraded atrazine steadily over the first 3 d. These latter cultures showed an immediate increase in non-volatile ^{14}C-metabolites. The metabolites decreased with time and $^{14}CO_2$ was evolved. Following the lag, LFAR also showed the transient formation of non-volatile intermediates. In contrast, LFB6A showed little accumulation of intermediates. In all cases, 70-85% of the initial atrazine could be accounted for, mostly as $^{14}CO_2$ by the end of the experiment.

The effect of temperature and pH on growth and atrazine degradation was examined with culture LFB5A on the tenth growth cycle. Atrazine degradation was negligible at or below 7°C, but significant metabolism occurred at 15°C and above (Fig. 3A). Atrazine degradation (Fig. 3A) occurred concomitantly with an increase in protein content (Fig. 3B) although lower temperatures led to a lower biomass upon reaching stationary phase. At 30°C, atrazine degradation was not markedly altered over the range of pH 5.5-8.5 (data not shown).

2.2.3 Effect Of [illegible]cated Subculturing In Medium With Atrazine As Sole [illegible]rogen Source

Continuous subculturing of the enriched microorganisms on atrazine as the sole source of nitrogen resulted in a continual increase in degradation rates. This is illustrated with the eighth subcultures by following the disappearance of ethyl acetate soluble atrazine from culture media. In the control without cells, 97% of the added [^{14}C] atrazine partitioned into the organic phase, and this remained constant at both 24 and 48 h (Table 3). In the acclimated cultures, significant amounts (up to 35%) of the [^{14}C] atrazine had been transformed to water soluble metabolites by 24 h. The total ^{14}C in the organic plus aqueous phases were similar to the control indicating that insignificant atrazine mineralization had occurred. After 48 h, most of the radioactivity was no longer in the organic or aqueous phases and in an independent experiment was determined to be $^{14}CO_2$.

Table 3

Transient formation of water soluble metabolites during the degradation of 460 nmole/ml atrazine by eighth-cycle cultures[a].

	^{14}C partitioning (nmole/ml culture)			
Culture	24 h after inoculation		48 h after inoculation	
	Organic phase	Aqueous phase	Organic phase	Aqueous phase
CONTROL	445	15	442	14
LFA5A	303	106	8	20
LFA7A	293	162	10	15
LFA6A	290	157	4	17
LFAR	343	103	13	10
LFB3A	327	101	10	10
LFB6A	405	50	10	50
ALB2N	359	60	15	55

[a]A 2 ml culture medium containing [^{14}C] atrazine was extracted with 2 ml ethyl acetate, the aqueous and organic phases were analyzed by scintillation counting.

The data in Figure 4 demonstrated the differences in degradation patterns between the 6th and 12th growth cycles 24 h after inoculation. By the 6th subculture, almost no atrazine degradation occurred by the end of day 1, as indicated by similar partition patterns in the aqueous and organic phases of the non-inoculated control and the inoculated media. However, after another 6 subculturing cycles, a significant amount of degradation occurred in almost all

cultures 24 h after inoculation. Culture LFB5A and LFB6A were particularly active; only 30% and 41%, respectively, were recovered in the organic phase. The rest of the ^{14}C label occurred as water soluble metabolites (up to 30%) or as $^{14}CO_2$. The mass balance of radioactivity indicated a recovery of >90%. Complete mineralization was observed by the end of the second day of the twelfth subculture cycle in all the cultures except for ALB2N (data not shown).

2.2.4 Chromatography Of Non-Volatile Metabolites

The most rapid atrazine-degrading culture was analyzed by isocratic reverse phase HPLC in a attempt to identify non-volatile metabolites of atrazine degradation. A zero time control (Fig. 5A) showed the starting materials, atrazine and simazine (2-chloro-4, 6-diethylamino-1, 3, 5-triazine); the latter is a minor contaminant of the atrazine preparation (less than 0.5%). After 24 h, atrazine and simazine were degraded by 75% and 85%, respectively. Four new peaks were observed and radioactivity from ring-labeled atrazine was associated with all four. Peaks I and II migrated close to the elution volume of the column and may contain multiple polar materials. Peak III, at 4.8 min, had the same retention time as authentic desisopropylatrazine. The larger peak (IV), at 7.5 min, coeluted with authentic hydroxyatrazine. Ultraviolet spectroscopy of material recovered from peak IV showed an absorption maximum at 240 nm identical to hydroxyatrazine. Rechromatography of peak IV by TLC on silica plates gave a similar Rf value as synthetic hydroxyatrazine.

2.3 Discussion

Previous studies had shown atrazine to be biodegraded by soils (Armstrong and Chesters, 1968; Burkhard and Guth, 1981; Frank and Sirons, 1985), mixed cultures and pure cultures of microorganisms (Behiki and Khan, 1986; Jessee et al., 1983; Kaufmann and Blake, 1970). Atrazine ring cleavage is rarely reported and even side chain cleavage is typically very slow. This is reflected in a recent effort to overcome the recalcitrance of atrazine by using sequential ozonolysis and bioremediation (Leeson et al. 1993).

In the present study, a rapid and extensive conversion (> 80%) of the [^{14}C]s-triazine ring carbon atoms to $^{14}CO_2$ was demonstrated. The enrichments differed from previous studies in that atrazine was used as the sole nitrogen source. This method had previously been used successfully to obtain various s-triazine degrading bacteria but yielded no strains capable of growth on atrazine (Cook and Hutter, 1981). The use of citrate and sucrose as mixed carbon sources may have contributed to the success of the enrichments in these studies.

The cultures described here represent the most rapid rates of biological atrazine degradation, as well as degradation at elevated concentrations (Table 4). Soils often show half-lives of atrazine decrease on the order of months. Pure or enriched cultures typically give half-lives on a time scale of weeks. This is even observed with bacteria which use atrazine as the sole carbon source (Behiki and Khan, 1986). The enrichments in the present study yielded half-lives of

4-8 days by the sixth subculture. After 12 subcultures, the half time for degradation of 100 ppm atrazine was 0.5-2 days. Most of the experiments described here used atrazine at 100 ppm, a concentration typical at a spill site but unlike that used in the field. Indeed, we have observed atrazine degradation on solid media at concentrations as high as 1000 ppm (data not shown). This, coupled with the stability of these consortia, highlights the potential for degrading atrazine under herbicide-spill conditions.

Transient metabolites accumulated in most cultures as demonstrated by detecting non-volatile water soluble materials. HPLC analysis of media indicated the presence of multiple non-volatile compounds resolvable by reverse phase chromatography. The presence of hydroxyatrazine as an apparent intermediate was somewhat surprising. Previous studies have indicated side-chain N-dealkylation reactions as typical first steps in s-triazine metabolism and stressed that the presence of both alkyl groups on atrazine may be inhibitory for bacterial dechlorination (Behiki and Khan, 1986; Erickson and Lee, 1989).

Tab.

Half life times of atrazine disappearance under different conditions in this and previous studies.

Treatment (ref.)	pH	Temperature	Half life- time (days)
Soil (5)	4.8-6.5	22	53-113
Soil (11)	5.6-6.6	Field conditions	37-168
Soil/water slurries (1)	4.5	---	65-113
Filtered sterilized water (5)	5-9	25	64-200
Bacterium from industrial wastes (14)[a]	---	---	>7
Pseudomonas spp.(2)	6.8-7.0	28	>35
Aspergilus fumigatus (15)	---	---	24
Enrichment culture (10)	---	---	>42
Early enrichment cultures (this study)	7.3	25	4-8
Late enrichment cultures (this study)	7.3	25	0.5--2

a Only 40% degradation was detected after 7 days of incubation. No further degradation was reported.

3.0 Soil Bacteria Rapidly Hydrolyze Atrazine to Hydroxyatrazine

3.1 Materials And Methods

The isolation of mixed culture LFB6 is described in Section 2. Culture LFB6 was grown in 500 ml Erlenmeyer flasks containing 300 ml atrazine medium (24) for 3 days (O.D600>1) without shaking. The culture was then harvested by centrifugation (6000 g, 20 min) and washed twice with 0.1N sodium phosphate buffer (pH=7.0) to remove excess nutrients and residual atrazine metabolites. The pellet was resuspended in buffer to yield 1.5 mg protein ml^{-1}

Soil inoculation experiments were conducted in 20 ml screw cap glass vials containing 3 g of air-dried soil (1.5% moisture) sieved through a 20-mesh screen. The soil was moistened with 1 ml of deionized water and preincubated for three days at 30 oC in the dark. Atrazine (460mmole/ml) was suspended in methanol and sonicated for 30 sec at 80% output of a Biosonic sonicator (Bronwill, Rochester, NY) to help solubilize the crystalline atrazine and reduce suspended particle size. The short sonication process did not cause any decomposition of the atrazine as determined by High Pressure Liquid Chromatography (HPLC) analysis. The atrazine suspension (6 ml) was thoroughly mixed into the preincubated soil and allowed to equilibrate at 4°C in the dark for an additional period of 3 days. The experiment was initiated by adding 2 ml of culture LFB6 (0.75 mg cell protein ml-1) to the preincubated soil. The slurry was thoroughly stirred with a sterile spatula, the vials were capped and the mixture incubated on a reciprocal shaker (50 strokes min-1) at 30°C. Slurry samples removed before the end of the experiment were centrifuged to remove the soil. The supernatant was passed through a 0.2 mm filter and frozen at -70°C until analysis.

For the labeling experiment with $H_2{}^{18}O$, 1 ml of 97.3% $H_2{}^{18}O$ (MSD Isotopes, Canada) was combined with 1 ml of the atrazine suspension (prepared as previously described), and the solution was equilibrated at 4°C overnight. One ml of culture LFB6 (1.5 mg cell protein ml-1) was centrifuged and the pellet was dried for 10 min under a slow stream of air to further reduce its water content. The experiment was started by adding the atrazine suspended in $H_2{}^{18}O$ to the air-dried pellet. The test tube was vigorously shaken for 30 sec, then incubated 1 h at 30°C. The reaction mixture was divided into 10 aliquots of 100 ml each, and immediately frozen at -70°C. Each 100 ml aliquot was analyzed by HPLC. The eluting hydroxyatrazine peaks were pooled, the solvent volume reduced using a rotary evaporator (Buchi, Switzerland) operated at 45°C, and the residue was resuspended in 0.1 ml methanol. Direct insertion mass spectrometry was performed in a glycerol matrix with a Kratos mass spectrometer (Kratos, England) operated in the fast atom bombardment mode with xenon.

Crude protein extracts from a 3 day old LFB6 culture were prepared by sonicating a 3 ml cell suspension (O.D600=5.0) in 0.1N sodium phosphate buffer (pH=7.0) for 45 sec on ice at 50% intensity using a Biosonic sonicator (Bronwill Scientific, Rochester, NY). Broken cells were

removed by centrifugation and the supernatant was filtered through a 0.2 mm filter. Protein content of the resulting crude extract was assayed with a bicinchoninic acid assay (Pierce Chemical Co, Rockford, IL) and adjusted to 0.5 mg ml-1 using 0.1N phosphate buffer (pH=7.0). The crude extract was incubated with 30 ppm atrazine at 30 °C. Metabolites were analyzed by HPLC. Soil metabolites were determined as described in Section 2 and crude extract metabolites were analyzed using a 300 x 4.6 mm, 5mm C8 column (Phase Separation Inc. Norwalk, CT). Atrazine (99.6 %) was purchased from Chem Service Chemical Co. (West Chester, PA). [^{14}C]-uniformly ring-labeled atrazine (7.8 mCi/mmol; 99.6 % radiochemical purity) was purchased from Sigma Chemical Co. (St. Louis, MO). Authentic samples of desisopropylatrazine, desethylatrazine, hydroxyatrazine, hydroxydesisopropylatrazine, and hydroxydidesalkylatrazine were a gift from Ciba Geigy Corp. (Greensboro, NC). Individual 100 ppm stock solutions of authentic atrazine and metabolite standards were prepared in methanol:aqueous 0.1 N H_3PO_4 and stored at 4°C.

3.2 Results And Discussion

The purpose of this study was to determine whether a bacterial mixed culture, known to mineralize atrazine in a liquid growth medium (Section 2), could metabolize atrazine to hydroxyatrazine in soil. This was of interest since hydroxyatrazine formation has not previously been attributed to bacterial activity. Moreover, it is widely reported that the formation of hydroxyatrazine in soil is due to abiotic processes (Erickson and Lee, 1989).

In Webster clay loam and silica sand, each spiked with 100 ppm atrazine and inoculated with mixed bacterial culture LFB6, hydroxyatrazine was detected after 1 h (Fig. 6). Hydroxyatrazine was rigorously identified by HPLC retention time, TLC Rf value, ultraviolet spectroscopy and mass spectrometry. After 24 h, more than 80% and 95% of the atrazine in the clay loam soil and the sand samples, respectively, were degraded. Hydroxyatrazine was formed as a transient intermediate compound which was further degraded. Previously, culture LFB6 in liquid media was shown to liberate the atrazine ring carbon atoms as CO_2 (Section 2). Surprisingly, dealkylated metabolites such as desisopropylatrazine or desethylatrazine were not detected (at a detection level of 100 ppb) except for the uninoculated silica sand treatment in which a trace amount of desisopropylatrazine was formed. Previous reports of microbial atrazine degradation indicated dealkylation to be the initial metabolic step (Erickson and Lee, 1989).

Degradation rates of atrazine in soil by culture LFB6 far exceeded those previously reported for native soils or bacterial cultures. Resting cell suspensions of culture LFB6 degraded atrazine at a rate of 0.13 mmole per mg cell protein per h. Similar degradation rates have only been reported for chemical hydrolysis of atrazine at pH values above 13 or below 1 (Armstrong et al., 1967), or under the combined effect of pH=4 and a high concentration of humic acid in a muck soil (Li and Felbeck, 1972). Thus, it was of interest to determine whether high rates of atrazine degradation could be catalyzed by bacterial enzymes at neutral pH. In Fig. 7, a cell-free crude protein extract of culture LFB6, buffered at pH=7.0, rapidly transformed

atrazine to hydroxyatrazine (Fig. 7B). After 24 h, hydroxyatrazine was further degraded to a more polar metabolite with a retention time similar to those recorded for authentic samples of dealkylated hydroxyatrazine (Fig. 7C). Atrazine degradation did not occur in the buffer alone (Fig. 7A). A control of protein alone indicated that atrazine or hydroxyatrazine were not present in the protein preparation (Fig. 7D). Crude extract boiled for 10 min lost its ability to degrade atrazine (data not shown). These experiments demonstrated that hydroxyatrazine formation occured at neutral pH and required a heat-labile component(s) in cell-free protein extracts.

Dealkylated s-triazines such as desethylsimazine were dechlorinated by a Pseudomonas sp (Cook and Hutter, 1984) via a proposed hydrolytic mechanism. Similarly, culture LFB6 could dechlorinate atrazine under both aerobic and oxygen limited conditions. Thus, we hypothesized a hydrolytic mechanism was operative. However, the apparent hydrolytic dechlorination of pentachlorophenol to tetrachloro-p-hydroquinone is now known to be catalyzed by a flavoprotein oxygenase (Xun et al., 1992). In this context, it was important to determine the source of oxygen in biologically-derived hydroxyatrazine.

We have determined that hydroxyatrazine formation by culture LFB6 is hydrolytic. Atrazine exposed for 1 h to non-growing cells of culture LFB6 in $H_2{}^{18}O$ yielded [^{18}O]hydroxyatrazine as demonstrated by fast atom bombardment mass spectroscopy (Fig. 8). The major peak at m/z 200 (199+1) indicated the incorporation of [^{18}O] from $H_2{}^{18}O$ during atrazine dechlorination. A control treatment consisting of authentic hydroxyatrazine solubilized in 97.3% $H_2{}^{18}O$ did not show any spontaneous exchange of [^{18}O] hydroxyl group, even when the hydroxyatrazine was incubated with $H_2{}^{18}O$ for 24 h. The small peak at m/z 198 in Fig. 8B was due to some residual $H_2{}^{16}O$ carried over from bacterial cells grown in $H_2{}^{16}O$ containing medium. Mass spectra of authentic hydroxyatrazine (Fig. 8A) yielded a hydroxyatrazine peak at m/z 198 (197+1). These findings suggest that microbial dechlorination of atrazine may occur in oxygen limited environments such as groundwater and subsoils.

Many authors cite the work of Armstrong et al. (1967) in support of a chemical mechanism for soil hydroxyatrazine formation (Erickson and Lee, 1989; Skipper and Volk, 1972; Muir and Baker, 1978; Adams and Randtke, 1992; Sorenson, 1992; Nearpass, 1972; Li and Felbeck, 1972; Armstrong and Chesters, 1968; Zimdahl et al., 1970). In contrast, our work suggested that microbial degradation of atrazine to hydroxyatrazine may be significant in many groundwaters and soil. In this light, it is important to reevaluate some of the points supporting the conclusion that hydroxyatrazine in the environment is chemically formed. (a) Soil boiled for 15 min and then incubated for over 30 days enhanced the degradation of atrazine by more than 20 fold (Armstrong et al., 1967). It was concluded that the "sterilized" soil enhanced the degradation of atrazine via a chemical pathway. Numerous soil metabolism studies have shown that boiling for 15 min will not sterilize soils but will likely enrich for heat resistant bacteria (Garrett, 1981). (b) No microbial degradation of atrazine was detected following perfusion of "sterilized" soil with medium containing 0.3 g/L ammonium nitrate and 0.1 g/L of

sucrose as a carbon source (Armstrong et al., 1967). In our studies, such high levels of ammonium nitrate strongly inhibited atrazine biodegradation and sucrose could not serve as a carbon source for atrazine-degrading bacteria. (c) In a non-sterile soil, the correlation between high organic matter and hydroxyatrazine formation could have resulted from increased microbial enzymatic activity associated with high levels of organic matter (Gray and Wallace, 1957).

We demonstrated the hydrolytic dechlorination of atrazine by a bacterial culture in soil. The dechlorination is mediated by bacterial enzymes and not via chemical hydrolysis. We have obtained over 30 atrazine-degrading bacterial cultures out of 100 soil samples taken from three seperate atrazine contaminated sampling sites. Many of those cultures produced hydroxyatrazine from atrazine. This suggests that biological transformation of atrazine to hydroxyatrazine may be widespread in soils previously exposed to atrazine.

4.0 Degradation Of Atrazine In Soils By Soil Bacterial Mixed Cultures

Experiments were conducted with soil bacterial mixed culture LFB6 to determine its effectiveness in degrading atrazine in soils. It is well documented that the activity of microorganisms in soils is influenced by environmental parameters such as pH, temperature, and water content. Therefore, studies of the potential use of microorganisms for soil remediation should be conducted under the most favorable conditions for the activity of the inoculated microorganism, but conditions which are as realistic as possible.

4.1 Experiment I

Soil from Madison, MN that contained high concentrations of atrazine due to an approximately 10 year-old spill was treated with mixed culture LFB6, under oxygen limited (water saturated conditions) to evaluate the treatablility of that soil using atrazine degrading bacterial cultures. Ten g of air-dry soil (sterile or non-sterile) was amended with 30 ml growth medium (pH=7.0) containing sodium citrate and sucrose as additional carbon sources, inoculated with growing cells of cultures LFB6 and LFA7, and incubated at 30 C, in the dark for 3 weeks. Atrazine was not degraded in uninoculated control treatments nor in sterilized soil amended with a carbon source, but not inoculated. However, in an uninoculated treatment that was amended with growth medium alone but not sterilized, about 40% of the atrazine was degraded, indicating the presence of atrazine degrading microorganisms in the soil (Fig. 9). Soils inoculated with either culture LFA7 or LFB6 and amended with additional carbon source degraded atrazine and 80-100 % degradation was recorded. Culture LFB6 performed better than LFA7. In general, sterilization prior to inoculation caused only a minor increase in degradation. Apparently, the inoculated organisms could successfully compete with the indigenous microflora.

4.2 Experiment II

An experiment was conducted to test the mineralization of high concentrations of atrazine in a Webster clay loam soil and in sand. Two moisture treatments (approximately 80% field capacity (FC) and water saturated) were included. Fifty g soil in 250 ml Erlenmeyer biometric flasks was inoculated, amended with growth medium and atrazine to yield a concentration of 100 ppm atrazine, and spiked with uniform ring labeled [^{14}C]atrazine to yield approximately 400,000 cpm/flask. Uninoculated controls and a control treatment with only growth medium were also included. Atrazine mineralization was measured as evolution of $^{14}CO_2$. Each treatment was conducted in three replicates. In the Webster soil similar mineralization rates were found for both moisture treatments when the soil was inoculated with culture LFB6. Under uninoculated conditions, amendment of growth medium alone resulted in better mineralization under the saturated conditions; more than 7.5% mineralization was recorded in the saturated soil as compared to only 3% in the 80% FC soil in 7 days. Apparently, the indigenous microflora in Webster soil is capable of atrazine mineralization when amended with a suitable carbon source. Rapid mineralization occured in both Webster soil and silica sand after inoculation with the mixed culture. In the first day following inoculation, 14-17% mineralization occured in the silica sand and up to 7% in the Webster soil. The mineralization rate in all inoculated cultures decreased and finally leveled off at 20 or 25% mineralization for the Webster soil and silica sand, respectively. Depletion of sodium citrate from the medium could not account for the decrease in mineralization, since amendment of sodium citrate during the second day did not result in an increased degradation rate. Extraction of the soil with methanol and analysis of the residual atrazine indicated that more than 90% of the atrazine in the inoculated treatments was depleted, indicating that the low mineralization rate was probably due to sorption of metabolites to soil, or an inefficient CO_2 trapping system. The exact reason for the decrease in mineralization rate is still unclear at this point. Webster soil and silica sand not amended with growth medium (data not shown) did not mineralize atrazine. Also, silica sand amended with nutrients but not inoculated did not mineralize atrazine.

4.3 Experiment III

A greenhouse experiment was conducted with soil from Madison, MN containing approximately 4000 ppm of aged atrazine, in order to evaluate the degradation of aged atrazine in an upscaled system. Two replicates of each of the following treatments were initiated: uninoculated control; control amended with sodium citrate, treatment inoculated with culture LFB6, and treatment inoculated with culture LFB6 and amended with sodium citrate. The experiment was conducted with 10 L air dried, crushed and sieved (2 mm) soil in 5 gallon buckets. The inoculum consisted of 3 day old culture LFB6 grown in atrazine medium , harvested, washed and resuspended in 10 mM phosphate buffer (pH=7.0) to yield an $O.D_{600}$ of 1.0.

Inoculated treatments were amended with 750 ml culture suspension/ 10 l soil, and stirred until the entire soil was wet. Sodium citrate 30g/L was amended to treatments with nutrient amendment at a rate of 750 ml per bucket. Untreated controls were amended with plain water. Analysis of atrazine in the soil 28 days after inoculation indicated that the unamended control and the inoculated treatment that was not amended with sodium citrate did not degrade atrazine. However, 40 and 60% degradation were observed in treatments with sodium citrate alone or sodium citrate inoculated with culture LFB6 (Fig. 10). These results support previous data that soils previously exposed to atrazine contain indigenous microflora capable of atrazine degradation in the presence of added sodium citrate.

5.0 Alachlor Degradation By Mixed Bacterial Cultures.

Enrichment cultures for the degradation of alachlor were initiated at the same time as those for atrazine degraders. However, a different enrichment strategy was undertaken. Since alachlor is a poor nitrogen source for microorganisms, we used alachlor as the sole carbon source and added an external nitrogen source. Cultures intended to enrich for fungi were acidified to pH=5.5. Cultures for the enrichment of bacteria were at neutral pH, and amended with cycloheximide to prevent the growth of fungi. The remaining medium ingredients were the same as in enrichment media for atrazine degraders (without sucrose and sodium citrate, see Section 2). Soil from the herbicide spill sites was used as inoculum (Corresponding culture identification numbers can be found in Table 5). The cultures were transferred to a fresh growth medium every two weeks. At the end of the fourth transfer, alachlor concentration in the media was measured using HPLC. No alachlor degradation was recorded for most of the acidified growth cultures intended for the isolation of fungal cultures, while up to 30% disappearance of atrazine was recorded for some fungal cultures. However, in media for the enrichment of bacteria many cultures that degraded alachlor were obtained (Fig. 11). Culture 32 that originated from Little Fall Site B (Table 5) and culture 94 that was isolated from a weed rhizosphere were both particularly active. Because of the more dramatic degradation seen for atrazine and the availabilty of ^{14}C-labeled atrazine, we chose not to pursue further research on alachlor. However, it seems that isolation of bacterial cultures capable of alachlor degradation is feasible.

Table 5

Spill site soil sample herbicide concentrations and corresponding isolated microbial culture identification numbers.

Sample Site	Sample Number	Atrazine ppm dw	Alachlor ppm dw	Metolachlor ppm dw	Fungi Atrazine	Fungi Alachlor	Bacteria Atrazine	Bacteria Alachlor
LFA Site								
Near stump N. side loading area 0-15 cm	1A	0.17	42.85	2.14	1	2	3	4
Near stump N. side loading area 0-15 cm	1B	0.84	41.71	0.52				
Near stump N. side loading area 15-30 cm	2A	0.35	22.32	4.18	5	6	7	8
Near stump N. side loading area 15-30 cm	2B	0.26	19.28	4.50				
Near weeds N. side of loading area 0-15 cm	4A	0.29	56.06	9.21	9	10	11	12
Near weeds N. side of loading area 0-15 cm	4B	0.31	68.24	9.55				
Near weeds N. side of loading area 15-30 cm	5A	0.14	31.10	5.21	13	14	15	16
Near weeds N. side of loading area 15-30 cm	5B	0.00	33.67	5.44				
East side of loading area 0-10 cm	6A	15.86	216.98	11.41	17	18	19	20
East side of loading area 0-10 cm	6B	10.85	172.83	10.35				
East side of loading area 10-25 cm	7A	11.86	69.20	4.60				
East side of loading area 10-25 cm	7B	13.95	74.33	5.53	21	22	23	24
Control near railroad 0-10 cm								
LFB Site								
East side of smaller building 0-15 cm	23A	0.00	0.00	0.00	25	26	27	28
East side of smaller building 0-15 cm	23B	0.00	0.00	0.00				
East side of smaller building 15-30 cm	24A	0.00	0.82	0.00	29	30	31	32
East side of smaller building 15-30 cm	24B	0.00	0.53	0.00				
Grass area near mound 0-15 cm	25A	0.89	4.86	0.62	33	34	35	36
Grass area near mound 0-15 cm	25B	0.85	2.30	0.59				
Grass area near mound 15-25 cm	26A	0.58	4.61	0.59	37	38	39	40
Grass area near mound 15-25 cm	26B	0.85	2.30	0.59				
Chlorotic plants near mound 0-10 cm	27A	0.32	3.81	0.71	41	42	43	44
Chlorotic plants near mound 0-10 cm	27B	0.16	0.00	0.00				
Chlorotic plants near mound 10-20 cm	28A	0.00	0.74	0.31	45	46	47	48
Chlorotic plants near mound 10-20 cm	28B	0.00	0.00	0.00				
ALB Site								
25 yards from loading area 0-15 cm	34A	1.04	14.56	4.53	49	50	51	52
25 yards from loading area 0-15 cm	34B	1.03	12.58	3.62				
25 yards from loading area 15-30 cm	35A	4.12	10.74	10.57	53	54	55	56
25 yards from loading area 15-30 cm	35B	5.40	10.35	10.32				
35 yards from loading area 0-15 cm	36A	0.78	34.00	4.00	57	58	59	60
35 yards from loading area 0-15 cm	36B	0.72	23.19	2.76				
35 yards from loading area 15-30 cm	37A	0.79	7.20	0.96	61	62	63	64
35 yards from loading area 15-30 cm	37B	0.55	5.13	0.47				
50 yards from loading area 0-15 cm	38A	0.53	3.56	0.00	65	66.00	67	68
50 yards from loading area 0-15 cm	38B	0.53	2.76	0.00				
50 yards from loading area 15-30 cm	39A	6.77	54.82	29.48	69	70	71	72
50 yards from loading area 15-30 cm	39B	5.00	39.51	22.32				

6.0 Atrazine Conjugation To Glutathione Via Glutathione S Transferase.

Glutathione S-transferase (GST) enzymes are known to play a role in conjugation and detoxification of atrazine in corn and sorghum plants. These enzymes generally catalyze the

reac... of hydrophobic compounds with the -SH group o... ...athione (GSH), thereby neutralizing their electrophilic sites and rendering them more water-soluble. Previous work has shown that the conjugation products of atrazine with glutathione are tightly adsorbed to soil, mitigating against potential leaching of the pesticide into ground water (Clay and Koskinen, 1990). Some soil bacteria and fungi are able to produce glutathione and the enzyme glutathione S-transferase which catalyzes the conjugation of non-polar substrates such as the herbicides atrazine and alachlor to glutathione. To test the possiblility of using this strategy as a remediation technique, studies on the conjugation of atrazine to glutathione in the presence of rat liver crude extract GST were performed.

Rat liver homogenate was prepared in a 10 mM KPi, pH=7.0 buffer containing 0.16 M KCl; 25 uM PMSF; 2 mM EDTA and 2 mM DTT. The crude homogenate was applied to an affinity column (Hexyl-glutathione) at 2 ml/min and chased to baseline with the same buffer. GST was eluted with 50 mM NaPi; pH=7.8, 0.2 M NaCl; 2.5mM s-Hexyl-glutathione. The fraction was dialyzed overnight against 6L of 10 mM Tris; 10 mM NaCl; 25uM PMSF pH 9.4 at 5C.

Atrazine degradation was examined using [^{14}C] Atrazine (0.2 mM) in 100 mM phosphate buffer (pH =7.5). Extraction with ethyl acetate was used to separate atrazine and conjugated atrazine. Extractions were performed after incubation of radiolabeled atrazine in the presence of rat liver GST (9.42 uM) and glutathione (2.6 mM). It was found that after 90 min of incubation, 15% of the atrazine was conjugated to glutathione in the presence of GST, but only 7% was conjugated in the absence of GST (Fig. 12). Incubation for 24 h resulted in increased conjugation; 50% and 60% of the atrazine in the buffered solution was conjugated at pH 7.5 (Fig. 13) and 6.5 (Fig. 14), respectively. Conjugation efficiency varied with substrate and enzyme concentration. Atrazine conjugation to GSH was significantly faster in the presence of GST (Fig. 15). However, since conjugation of atrazine in soils was expected to be slower than conjugation under optimal experimental conditions and since bacterial mixed cultures obtained from soils demonstrated higher atrazine degradation rates than these, further research concentrated on the degradation of atrazine by the mixed bacterial cultures.

7.0 Plant Effects on Degradation.

Another approach to the development of bioremediation technologies for spill site soils was to investigate the potential for enhanced degradation due to plant rhizosphere effects. Although numerous reviews have indicated the importance of root uptake and other potential rhizosphere effects on pesticide fate (Hurle and Walker, 1980; Guth, 1980), very little experimental work has been reported. The "plant activation" effect is one indication of the importance of the rhizosphere (Guth, 1980). As reported in various unpublished reports, four herbicides had significantly shorter half-lives when measured in soils previously planted to corn, cotton and rape. Guth hypothesized this effect was due to increased microbial activity.

A series o... ...ies by Seibert, Cheng and co-workers (Siebert et al., 1... , Siebert et al., 1981; Cheng et al., 1978; Cheng et al., 1975) examined the influence of roots on 2,4-D, atrazine, and MBT degradation in soils. In the case of atrazine, they found no significant increase in atrazine mineralization in the presence of maize plants until after the plant harvest, but they did find a significant (20% in 68 days) uptake of atrazine by the plant. In the rhizosphere soil, 10-20% more atrazine had been converted to hydroxyatrazine compared to the bulk soil (Siebert et al., 1981). In total, up to 25% or more of the atrazine was detoxified (mineralized or converted to metabolites) due solely to the presence of the plants. For 2,4-D there was no significant enhancement of mineralization, but there was a significantly higher buildup of nonextractable residue in the rhizosphere soil. Hsu and Bartha (1979) compared degradation rates of diazinon and parathion in bulk and rhizosphere soils and found 8 and 10% higher rates of mineralization, respectively, in the rhizosphere soil. These results suggest that CO_2 evolution rates are not always affected by the presence of roots, but conversion to detoxified forms and partitioning into more or less mobile phases can be significant.

In addition to the few studies reported here, an extensive body of literature supports the importance of these factors (increases in organic matter and microbial biomass, and changes in pH) for degradation in soils, without specific reference to the role of plant roots.

7.1 Experimental Results

Greenhouse experiments were conducted to study the survival of several plant species in elevated concentrations (25, 50 and 100 ppm) of atrazine and alachlor. Plant species tested included foxtail, Indian grass, switchgrass, big bluestem, Kochia, birdsfoot trefoil, soybean, cicer milkvetch, alfalfa, black medic, sweetclover, Ladino clover, berseem clover, red clover, crimson clover, and sorghum both treated and untreated for atrazine resistance (obtained from Dr. John Grunwald, Dept of Agronomy and Plant Genetics, University of Minnesota).

Elevated levels of alachlor significantly lengthened germination time of all plants, especially those with C_4 metabolism. At 50 ppm, both types of sorghum took twice as long to germinate as the untreated plants, and Indian grass never germinated. Atrazine had little effect on germination. At 50 ppm, only Ladino clover, red clover and sorghum took significantly longer to germinate.

Soybeans were the only legume species to develop true leaves in the contaminated soil. They died in atrazine, but continued to grow at half the control rate in alachlor. Even at 25 ppm, most of the legume plants were dead within 19 days after planting. For all of the grass species except sorghum, development was arrested at a plant height of two inches in all pesticide treatments. The treated sorghum survived at both 25 and 50 ppm in both treatments, but was not healthy. The untreated sorghum also survived, but only barely in the alachlor.

We tested whether transplanting young seedlings that had not been exposed to pesticide might allow them to survive the 25 ppm atrazine and alachlor treatments. All the transplanted species died except for Kochia, berseem, crimson clover, Indian grass, and switchgrass in the alachlor treatment.

Sorghum plants were further used as a plant model to study the effect on degradation of atrazine in a growth chamber experiment. Sorghum was seeded in atrazine contaminated soil (less than 50 ppm) and the disappearance of atrazine from the emerging plants' rhizosphere was monitored 1 and 2 after from seeding. There was no significant difference in the disappearance of atrazine from the seeded compared to the non-seeded control soil. The sorghum plants grew poorly in soil amended with atrazine levels above 25 ppm, and shoot and root biomass were significantly smaller than untreated control plants.

It appears that a more successful avenue of research for attempting to use plants to remediate spill site soils would be to select plants known to have resistance to specific herbicides. These plants may be selected through traditional breeding programs, tissue culture, genetic transformation, or wild type mutants. However, at spill sites, where multiple herbicides and many other chemicals, including petroleum products, may occur in high concentrations it seems unlikely that plants will be found that can tolerate these multiple stresses unless concentrations are very much diluted (as in landfarming).

8.0 Summary

The purpose of this research was to test innovative approaches using plants and microbes to enhance biodegradation and removal of pesticides from spill sites. The pesticides selected for study were atrazine and alachlor, because they are often detected in groundwater and are commonly involved in pesticide spills in Minnesota. Three major approaches were attempted in seeking remediation technologies: 1) enrichment cultures for microorganisms obtained from soils exposed to repeated spills, 2) conjugation of atrazine via glutathione-S-transferase, and 3) plant rhizosphere enhanced degradation. The most successful approach was enrichment for atrazine degradation utilizing 100 ppm (0.46 mM) atrazine as a sole nitrogen source. Bacterial growth occurred concomitantly with formation of metabolites from atrazine and subsequent biosynthesis of protein. With ring labeled [^{14}C]atrazine, > 80% of the s-triazine ring carbon atoms were liberated as $^{14}CO_2$. Over 200 pure cultures isolated from the enrichment cultures failed to utilize atrazine as a nitrogen source. Mixing pure cultures restored atrazine mineralizing activity. Repeated transfer of the mixed cultures led to increasing rates of atrazine metabolism. Degradation half lives for 100 ppm atrazine ranged from 0.5 to 2 days in liquid culture, which far exceeds the rates previously reported in soils, waters, mixed and pure cultures of bacteria. Hydroxyatrazine was found to be an intermediate in the atrazine mineralization pathway. Bacterial enrichment cultures in two soils and cell free protein extract from the bacteria produced hydroxyatrazine from atrazine, which was then further metabolized. Bacterial atrazine dechlorination was hydrolytic as demonstrated by ^{18}O incorporation from $H_2{}^{18}O$ into hydroxyatrazine. Preliminary experiments testing inoculation of soil bacterial mixed culture on spill site soils indicate significant (60%) degradation of atrazine after 28 days in the presence of an additional carbon source.

9.0 References

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Publications/Presentations Resulting From This Project -

1. Allan, D.L. and R. Mandelbaum. 1991. Degradation and Bioremediation at Pesticide Spill Sites. Workshop on Pesticide-Soil Interaction Research. United States Department of Agriculture National Tilth Laboratory, Ames, IA. November, 1991.

2. Mandelbaum, R., L.P. Wackett and D.L. Allan. 1992. Hydrolytic Dechlorination is the First Step in the Degradation of Atrazine by Some Bacterial Consortia. American Society For Microbiology Conference on Anaerobic Dehalogenation. Athens, GA. September, 1992.

3. Mandelbaum, R., L.P. Wackett and D.L. Allan. 1993. Microbial Degradation of Atrazine. Environmental Science Workshop. The Gray Freshwater Institute. Navarre, MN. February, 1993.

4. Mandelbaum, R., L.P. Wackett, and D.L. Allan. 1993. Bacterial Degradation of Atrazine - A New Mineralization Pathway. American Society for Microbiology Annual Meeting. May, 1993. Atlanta, GA.

5. Mandelbaum, R., L.P. Wackett, and D.L. Allan. 1993. Atrazine Mineralization by Microorganisms Isolated From Atrazine Spill Site. Conference on Bioremediation. Research Triangle, NC. April, 1993.

6. Mandelbaum, R., L.P. Wackett and L.D. Allan. 1993. Mineralization of High Concentrations of Atrazine by Stable Microbial Communities. Appl. Environ. Microbiol. 59: 1695-1701.

7. Mandelbaum, R., L.P. Wackett and L.D. Allan. 1993. Soil Bacteria Rapidly Hydrolyze Atrazine to Hydroxyatrazine. Environ. Sci. Technol. In Press.

C6. **Benefits:** This objective will evaluate the potential for treatment of contaminated soil at pesticide incident sites. Microbes isolated from spill sites will be evaluated for their ability to promote biodegradation or transformation of atrazine.

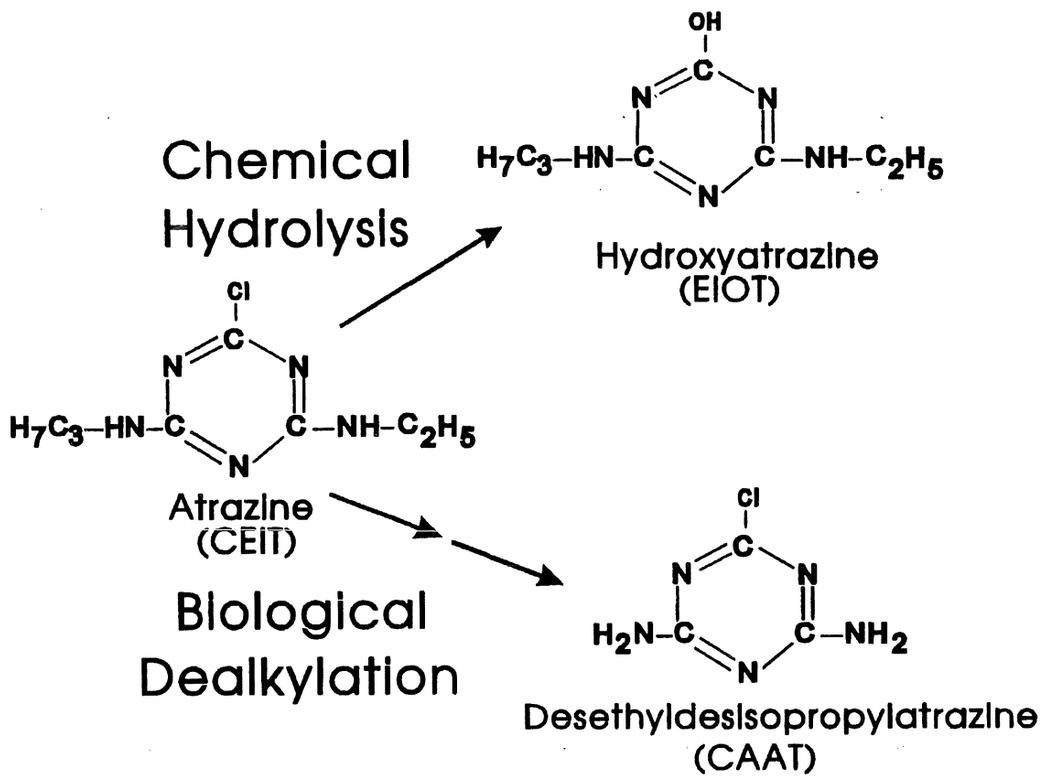

Figure 1. Atrazine degradation pathways in soil. The microbial dealkylation of atrazine to form desethyldesisopropylatrazine may involve more than one microorganism.

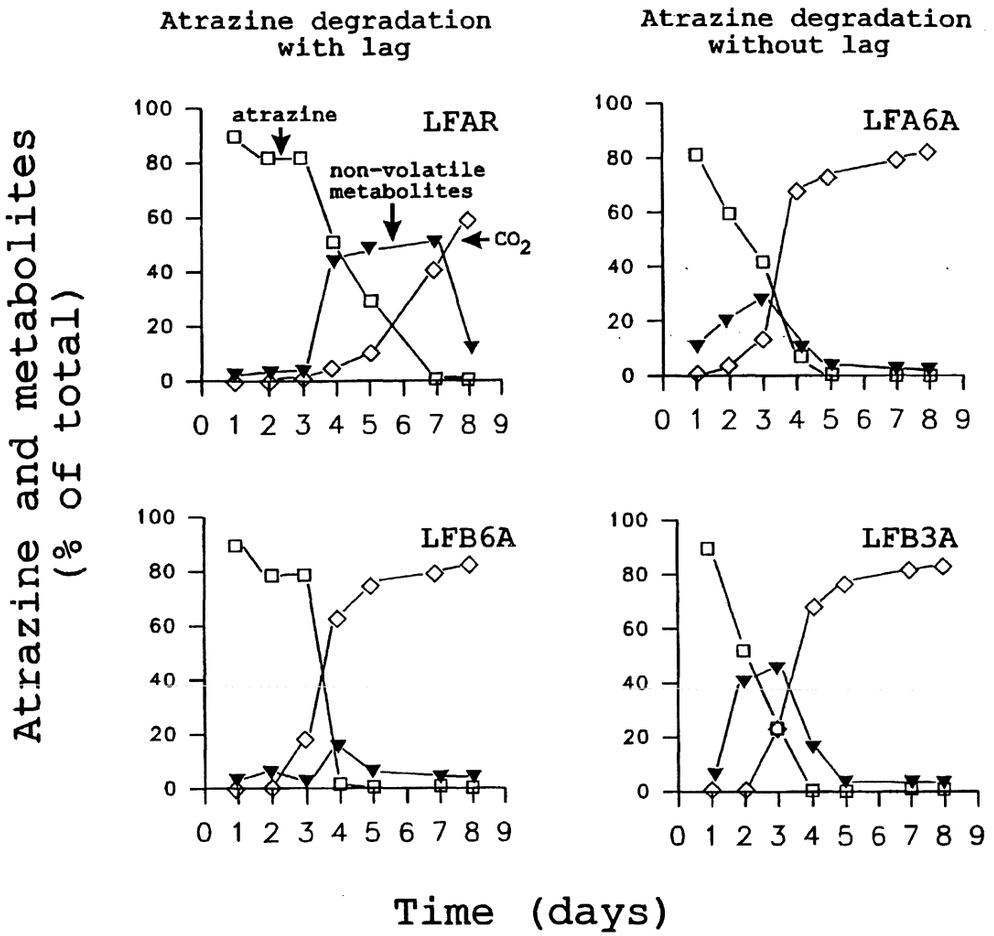

Figure 2. Degradation of 100 ppm [14C] atrazine in the growth medium during the sixth growth cycle. Symbols: □ - atrazine measured by HPLC; ▼ - non-volatile [14C] metabolites minus atrazine as measured by HPLC; ✧ - 14CO2 evolved. The letters in the upper right of each group refer to the culture designation which is explained in the Materials and Methods section.

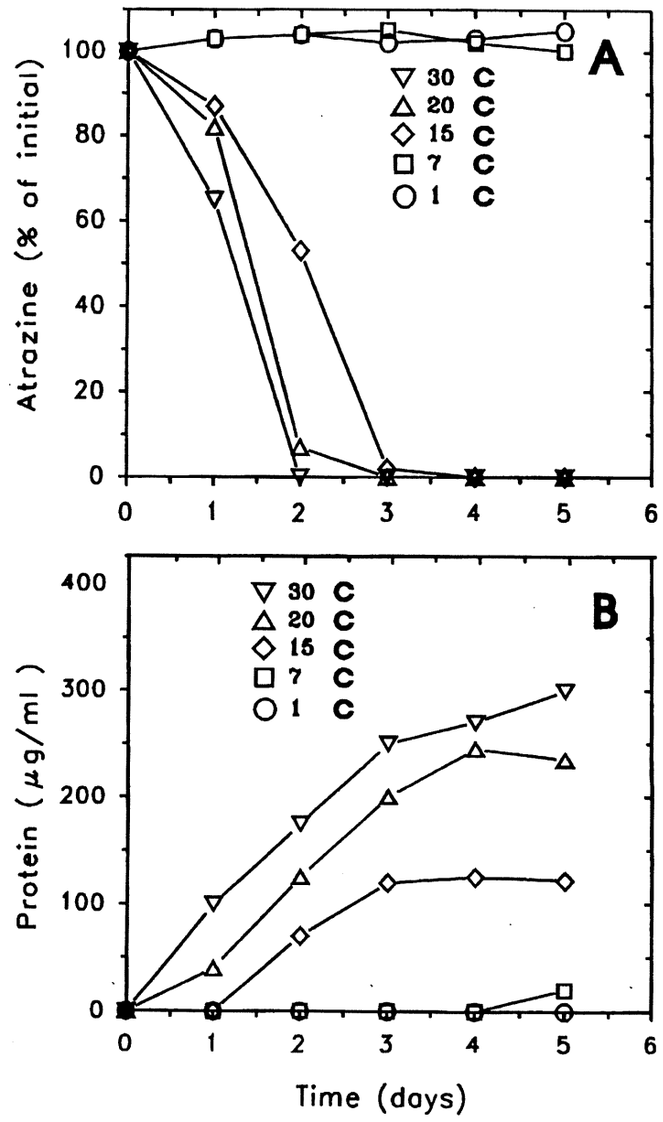

Figure 3. The effect of temperature on atrazine degradation by culture LFB5A during the tenth growth cycle.

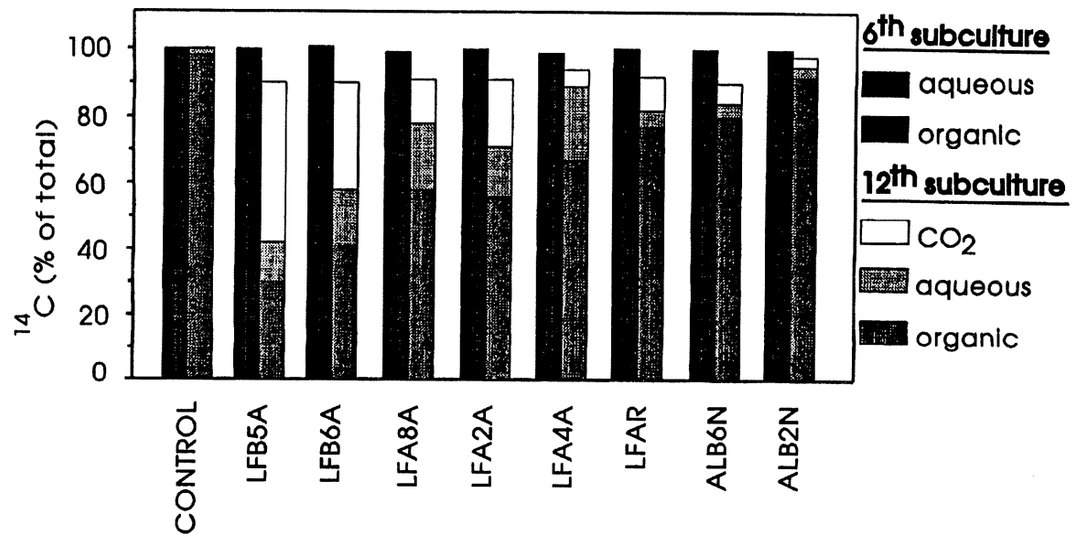

Figure 4. Mass balance of atrazine and its degradation products are compared for the sixth and twelfth growth cycles, each after 24 h incubation. For each pair, the sixth subculture is on the left and the twelfth subculture on the right. Explanations for the cultures names are found in the Material and Methods section.

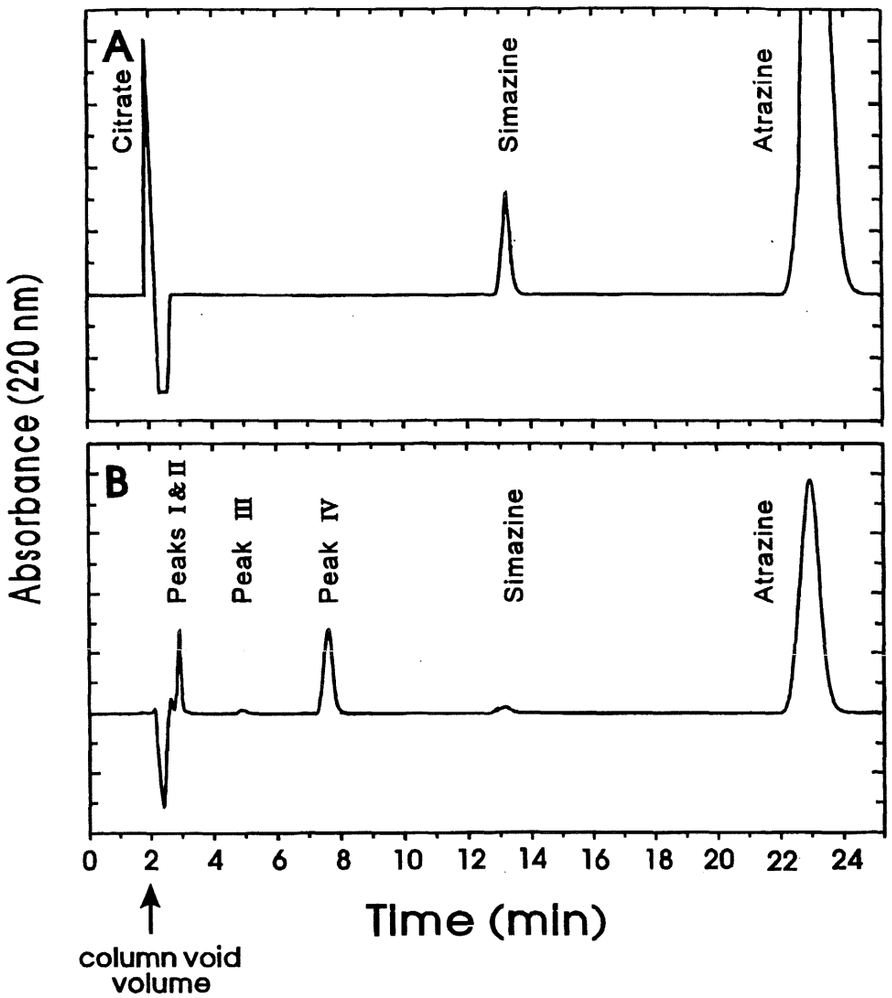

Figure 5. HPLC chromatograms of the culture medium immediately after inoculation with culture LFB5A (1), and 24 h later (B). One ml of culture was centrifuged (15000 g for 3 min), and 100 ml of the supernatant was injected into the HPLC.

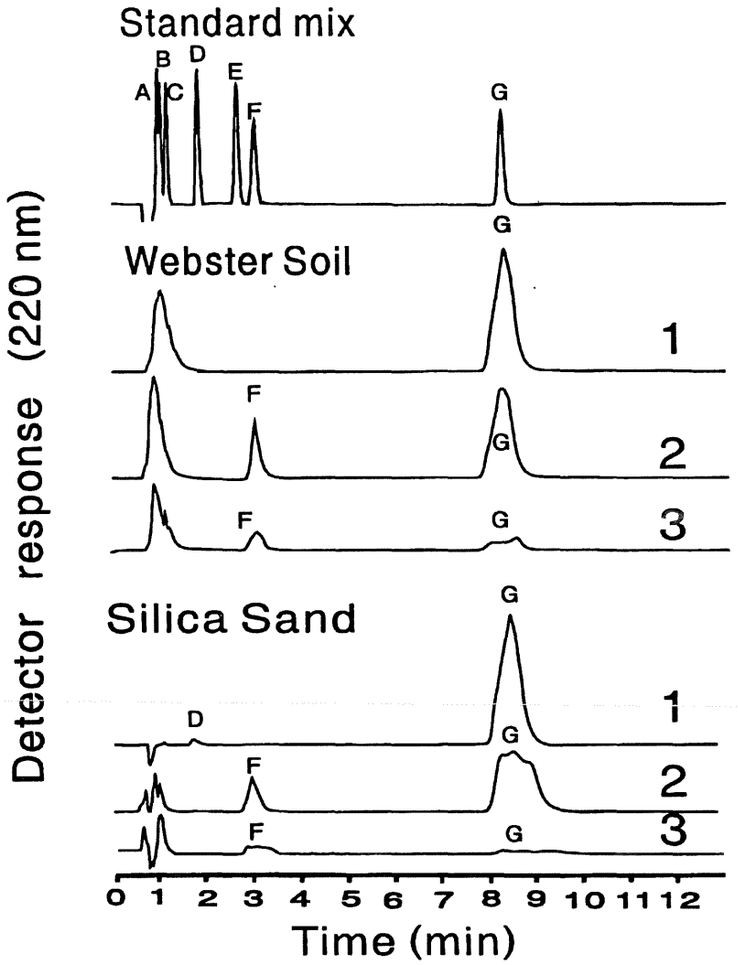

Figure 6. High Pressure Liquid Chromatography (HPLC) analysis of soil inoculated with culture LFB6. A = Desethyldesisopropylhydroxyatrazine; B = Desisopropylhydroxyatrazine; C = Desethylhydroxyatrazine; D = Desisopropylatrazine; E = Desethylatrazine; F = Hydroxyatrazine; G = Atrazine. 1 = extract from uninoculated control; 2 = 1 h after inoculation with culture LFB6; 3 = 24 h after inoculation with culture LFB6.

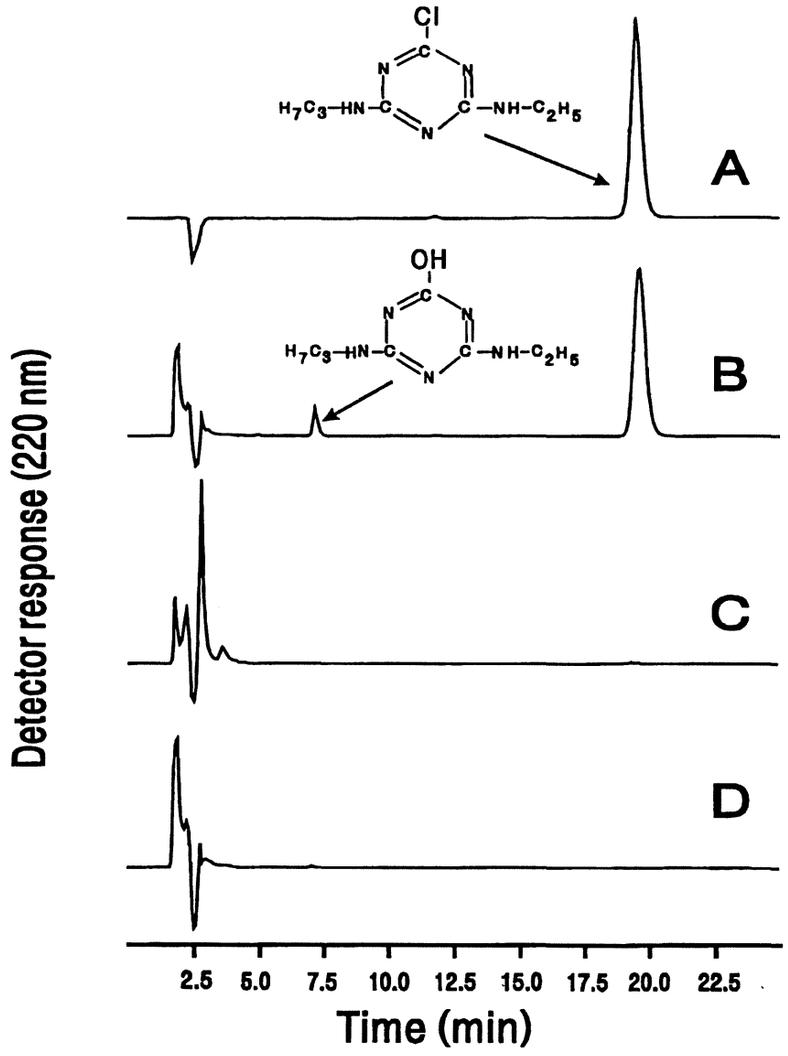

Figure 7. High Pressure Liquid Chromatography (HPLC) analysis of atrazine in crude extract prepared from culture LFB6. A = Control of atrazine in buffer (pH = 7.0) after 24 h; B = Atrazine in crude extract after 1 h; C = Disappearance of atrazine in crude extract after 24 h; D = Crude extract not amended with atrazine.

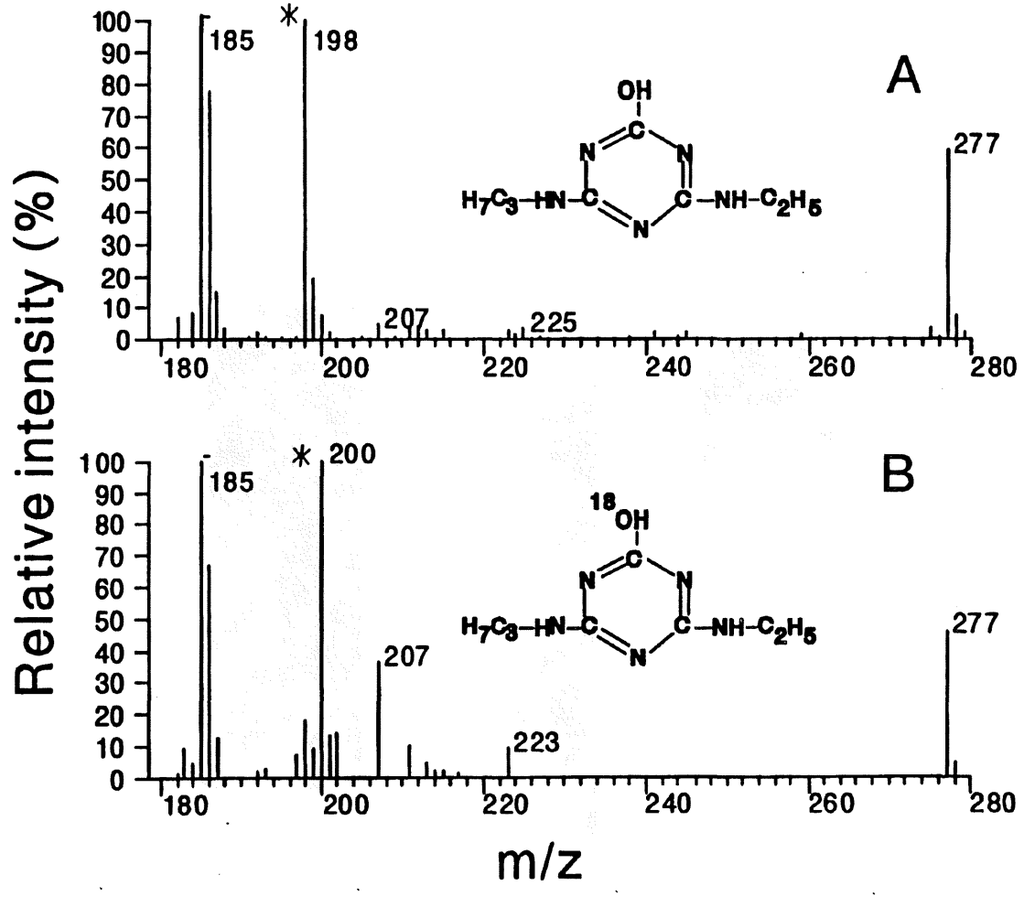

Figure 8. Fast atom bombardment mass spectra of authentic hydroxyatrazine (A), and hydroxyatrazine formed from atrazine by culture LFB6 in H218O (B). The peaks at m/z 185 and 277 are from the glycerol (x2+1 and x3+1, respectively) and m/z 207 represent two molecules of glycerol + sodium. The starred peaks represent the parent ions.

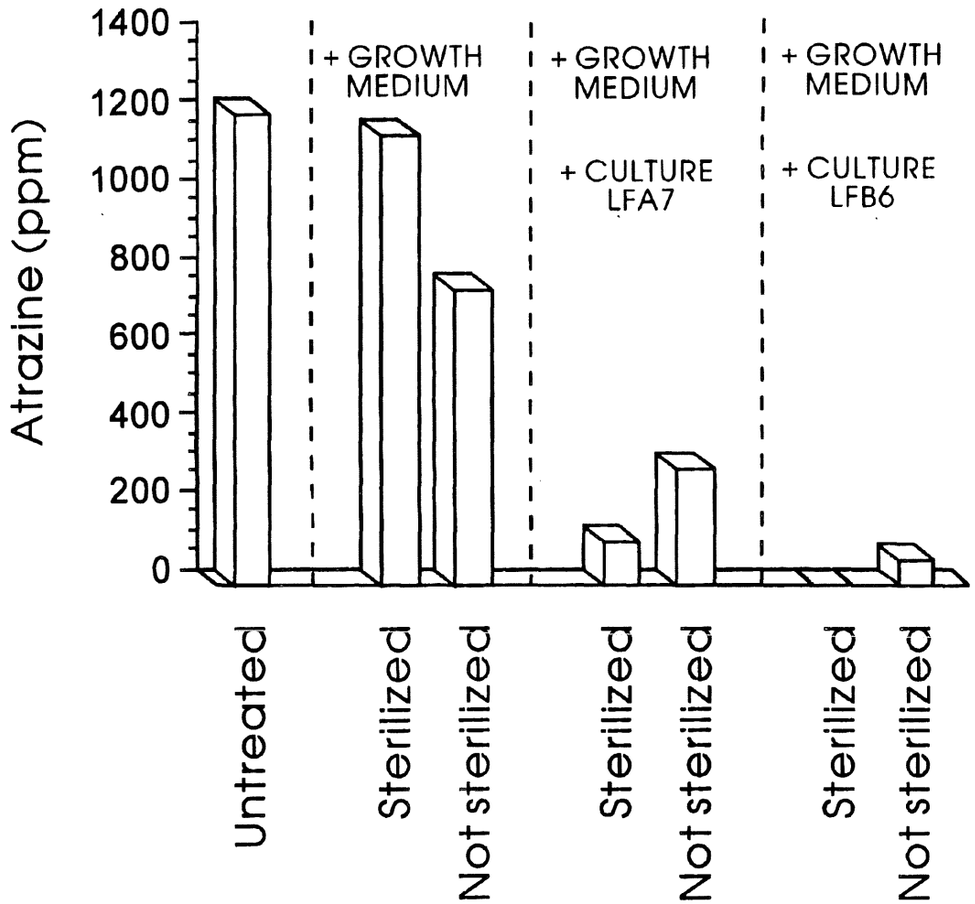

Figure 9. Atrazine content of herbicide-contaminated soils treated with growth medium (nutrients), growth medium plus bacteria obtained from site LFA7, growth medium plus bacteria obtained from site LFB6, and an untreated control. Sterilized soils were autoclaved prior to additions.

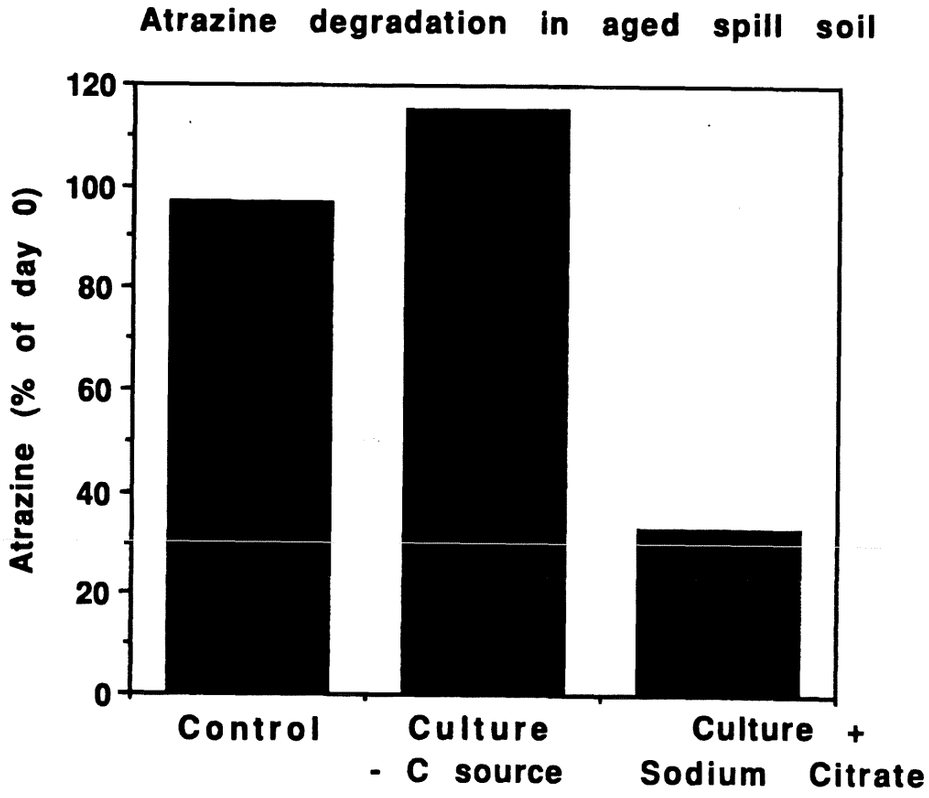

Figure 10. Effect of sodium citrate (C Source) addition on the biodegradation of atrazine in soil.

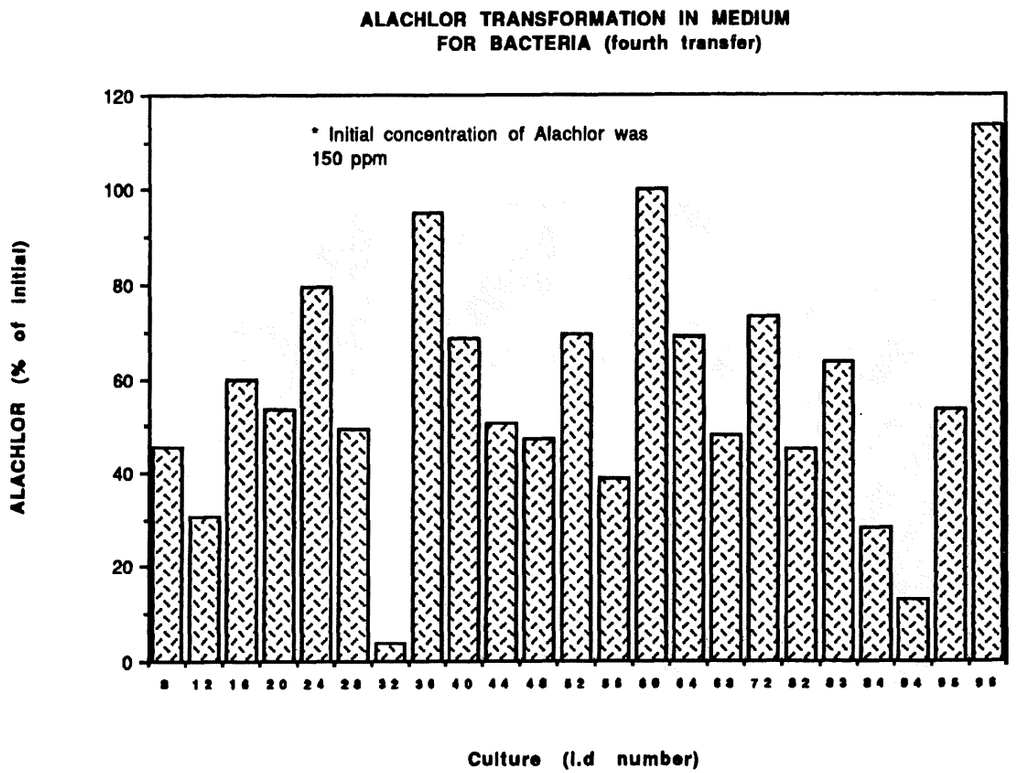

Figure 11. Alachlor biodegradation after a two-week incubation by different bacterial cultures obtained from herbicide spill sites.

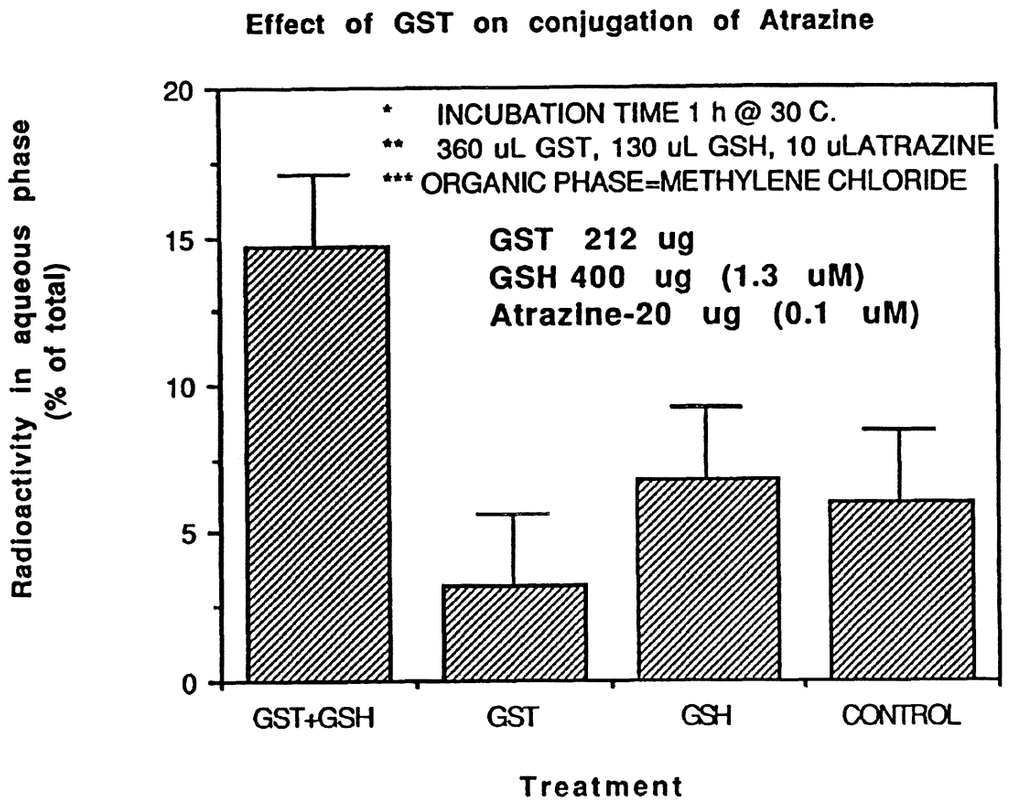

Figure 12. Reaction of glutathione with atrazine after various treatments as measured by determining the amount of radioactive glutathione conjugates found in the aqueous phase. Abbreviations are: GST = glutathione transferase; GSH = glutathione.

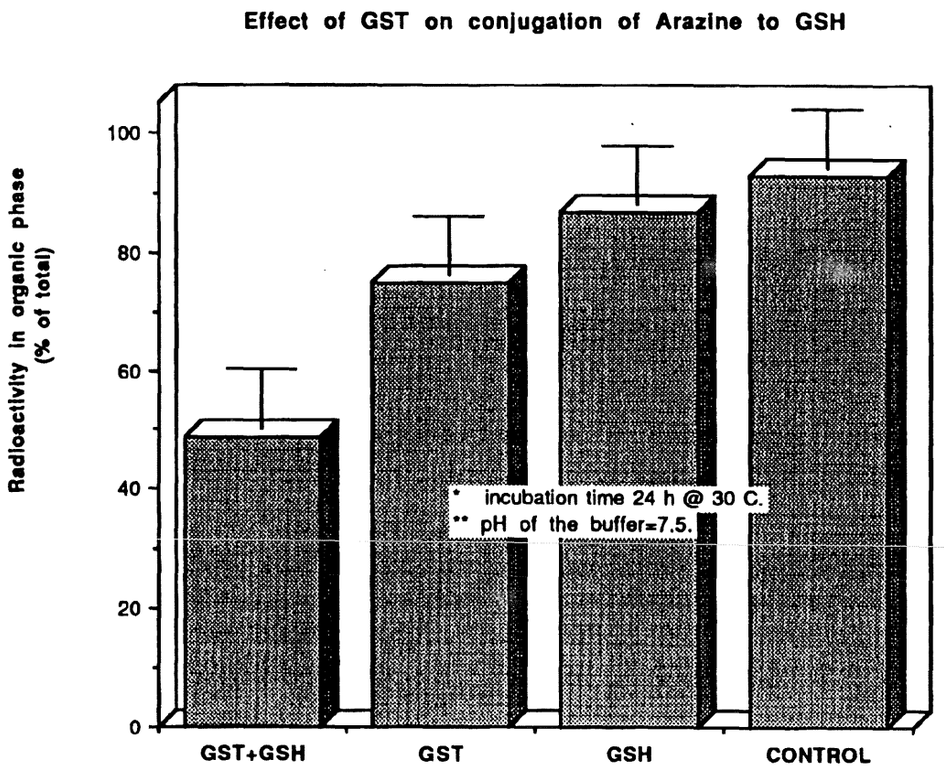

Figure 13. Removal of atrazine found in an organic extract by conjugation of glutathione (GSH) with atrazine catalyted by glutathione S-transferase (GST).

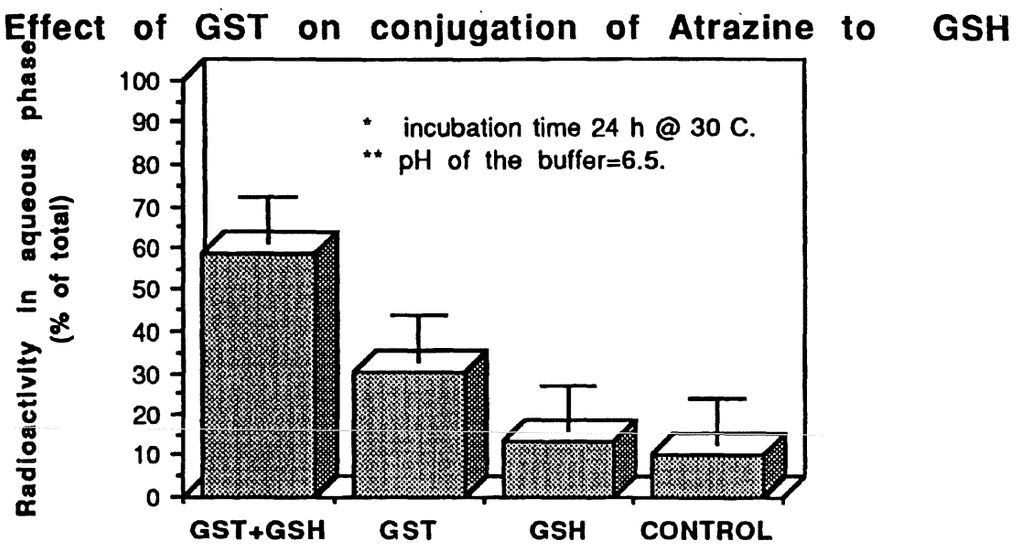

Figure 14. Effect of long incubation time (24 h) on the conjugation of atrazine to glutathione (GSH) catalyted by glutathione S-transferase (GST).

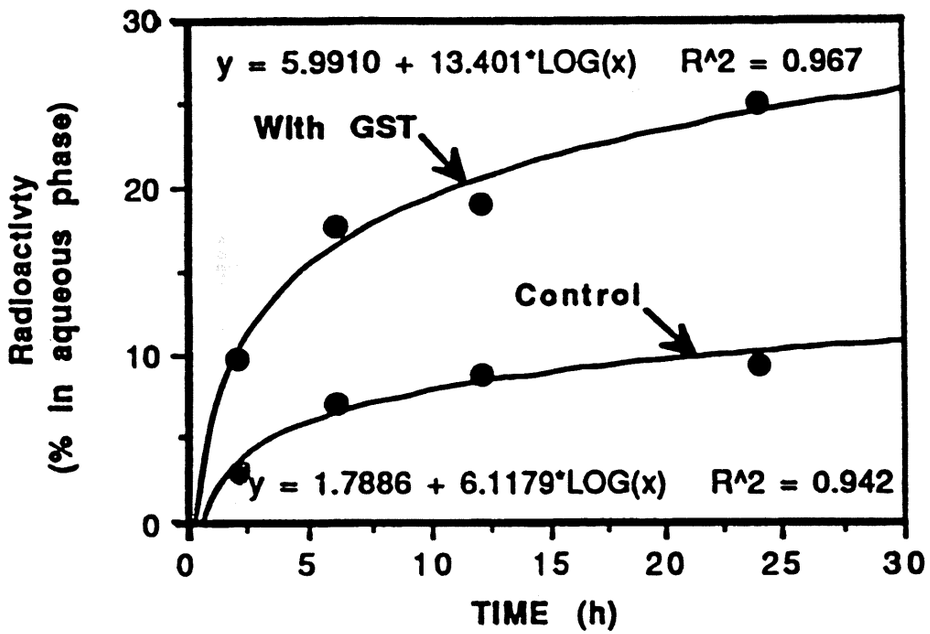

Figure 15. Time course of the reaction of atrazine with glutathione in the presence of glutathione S-transferase (GST) and in its absence (control).

IV. EVALUATION:

For the FY92-93 biennium, this program can be evaluated by the development of new information regarding soils contaminated with elevated levels of pesticides. Each objective can be evaluated as follows:

(1) Objective A can be evaluated by the production of a report describing the existing and applicable technologies.

(2) Objective B can be evaluated by its ability to characterize the mineralization and movement of atrazine and alachlor at elevated levels representative of spill sites.

(3) Objective C can be evaluated by its ability to identify: (1) mixed and/or isolated microorganisms capable of rapid degradation of elevated levels of atrazine; (2) potential mechanisms of atrazine degradation; and (3) whether mixed cultures or isolated organisms can effectively reduce atrazine concentrations in contaminated soils.

The long term success of this project will be evaluated by the incorporation of the information generated into clean-ups and programs to remediate contaminated soils.

V. CONTEXT :

A. Recent studies have identified pesticide mixing, loading and handling sites as potential sources of ground water contamination. Efforts in Minnesota and the Midwest are beginning to attempt remediation of these sites. Very little is known of the fate and transport of elevated levels of pesticides in soil and the remediation of these soils. Virtually all efforts by pesticide registrants, EPA and University researchers to date has focused on fate and transport mechanisms for pesticides used at or near labeled rates in field situations. What little data exists nationwide indicates that elevated pesticide levels may overwhelm mechanisms responsible for degradation and retarding movement of chemicals, thereby increasing the potential for ground water contamination.

The only techniques available for clean-up of pesticide contaminated soils currently available are at out-of-state licensed hazardous material sites, landspreading techniques and on-site encapsulation.

B. Several reports suggest that the presence of live or decomposing plant roots have the potential to increase pesticide degradation or transformation. The enhancement of degradation has been little studied, but confirmed for alachlor on alfalfa, and for atrazine on corn, where up to 25% or more of the atrazine was detoxified (mineralized or converted to metabolites) due solely to the presence of the plants. Most studies have simply looked for disappearance of the parent

compound or evolution of CO_2. The work proposed here will elucidate mechanisms for enhanced degradation, determine application of this bioremediation measure for elevated levels of pesticides, and evaluate plant species appropriate for Minnesota conditions. This project will review existing information and develop new information in a field where little is known.

C. Past LCMR funded projects consisted of efforts to understand the fate and transport mechanisms under normal use. This project will build on existing and presently developing data from the University of Minnesota Center for Agricultural Impacts on Water Quality which has been partially funded through LCMR in the past. This project provides the unique opportunity to access the extensive database being generated at field-use levels to enhance this effort to characterize the mineralization and movement of elevated pesticide levels encountered with spills or mishandling. The equipment is in place to do the laborious and costly sample extraction and analysis.

Based on the results of this program, new projects may be proposed such as correlating laboratory studies to actual field conditions. Potential future studies may involve other bioremediation technologies and fate and transport studies on additional pesticides and under various conditions.

D. Not applicable.

E. Not applicable.

VI. QUALIFICATIONS :

1. Program Manager :

Deborah B. DeLuca
Hydrologist, Incident Response Program
Agronomy Services Division
Minnesota Department of Agriculture
M.S. Land Resources, University of Wisconsin, Madison, 1989.

Ms. DeLuca is a hydrologist in the Incident Response Program at the Minnesota Department of Agriculture. In this role, she provides the technical review for remedial investigations and corrective actions for agricultural chemical spills and incidents. Her areas of interest and expertise are environmental chemistry, site remediation, and regulatory policy on site remediation.

2. Major Cooperators :

A) Dr. Douglas D. Buhler
USDA/ARS and Associate Professor
Department of Agronomy and Plant Genetics
University of Minnesota
Ph.D. Agronomy (Weed Science), University of Nebraska, Lincoln, 1984.
M.S. Agronomy (Weed Science), University of Nebraska, Lincoln, 1982.

Dr. Buhler's expertise and interests are in herbicide mineralization and movement related to agricultural uses. He is the primary contact within the University of Minnesota Center for Agricultural Impacts on Water Quality specializing in field research aspects of the environmental fate of herbicides. Dr. Buhler's role will be to develop the field and laboratory components to derive soil and water samples for analysis.

B) Dr. William C. Koskinen
USDA/ARS and Associate Professor
Department of Soil Science
University of Minnesota

Ph.D. Soil Science (Chemistry), Washington State University, Pullman, 1980.
M.S. Chemistry (Physical Organic), San Diego State University, San Diego, 1974.

Dr. Koskinen's interests and expertise are in the sorption, degradation, and movement of pesticides in the environment. He is the primary contact within the University of Minnesota Center for Agricultural Impacts on Water Quality for pesticide analysis. His specialty is the development and use of analytical techniques to qualify and quantify herbicide sorption and degradation. Dr. Koskinen will have primary responsibility for developing methodologies for, and extraction and assaying of, samples.

C) Dr. Roger L. Becker
Assistant Professor
Department of Agronomy and Plant Genetics
University of Minnesota

Ph.D. Agronomy (Crop Physiology), Iowa State University, Ames, 1982.
M.S. Botany (Plant Physiology), Iowa State University, Ames, 1978.

Dr. Becker's interests and expertise are in weed control and environmental concerns associated with herbicide use. He is the primary contact within the University of Minnesota for Extension Service efforts addressing herbicide contamination of surface and ground water. His major role will be to coordinate Section B, to provide inputs on

...rimental design and focus, and to develop applied utiliz... of research results.

D) Dr. Beverly R. Durgan
Associate Professor
Department of Agronomy and Plant Genetics
University of Minnesota
Ph.D. Agronomy (Weed Science), North Dakota State University, Fargo, 1985.
M.S. Agronomy (Weed Science), North Dakota State University, Fargo, 1983.

Dr. Durgan's interest and expertise are in weed control and environmental impacts of herbicide use. She is the primary contact for extension efforts in weed control on Minnesota's agronomic crops. Dr. Durgan's role will be to provide inputs into the applied aspects of project design and implementation.

E) Dr. Deborah Allan
Assistant Professor
Soil Science Department
University of Minnesota

Ph.D. Soil Science, University of California, Riverside, 1987.
M.S. Agriculture, California Polytechnic State University, 1983.

Dr. Allan's primary interest is in root physiology and soil chemistry of the rhizosphere. She has expertise in greenhouse and growth chamber experiments and analysis of plant roots, their excreted compounds and the rhizosphere soil. Her primary role will be to coordinate and participate in the accomplishment of Objective C.

F) Dr. Lawrence Wackett
Assistant Professor
Biochemistry Department
University of Minnesota

Ph.D. Microbiology, University of Texas, Austin, 1984.
M.S. Microbiology, Louisiana State University, 1979.

Dr. Wackett's major research focus is the use of bacteria to biodegrade hazardous wastes. Expertise has been developed in understanding mechanisms of these processes and the use of that knowledge in bioremediation. He will primarily work on the microbiological aspects of Objective C.

VII. REPORTING REQUIREMENTS :

Semiannual status reports will be submitted not later than January 1, 1992, July 1, 1992, January 1, 1993 and a final status report by June 30, 1993.