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Accelerated biodegradation of atrazine by a microbial consortium is possible in culture and soil

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Abstract

A mixed enrichment culture of microorganisms capable of accelerated mineralization of atrazine was isolated from soil treated with successive applications of the herbicide. Liquid cultures of this consortium, in the presence of simple carbon sources, mineralized 96% of the applied atrazine (0.56 mM) within 7 days. Atrazine mineralization in culture is initiated with the formation of the metabolite hydroxyatrazine. In soil treated with atrazine at a concentration of 0.14 mM (concentration is based on total soil mass), and then inoculated with the microbial consortium, the parent compound was completely transformed in 25 days. After 30 days of incubation, 60% of the applied atrazine was accounted for as $^{14}CO_2$. As was found with the liquid cultures, hydroxyatrazine was the major metabolite. After 145 days, soil extractable hydroxyatrazine declined to zero and 86% of the applied atrazine was accounted for as $^{14}CO_2$. No metabolites, other than hydroxyatrazine, were recovered from either the liquid culture or soil inoculated with the consortium. The use of the mixed microbial culture enhanced mineralization more than 20 fold as compared to uninoculated soil.

Introduction

The herbicide atrazine [2-chloro–4-(ethylamino)–6-(isopropylamino)-*s*-triazine] is used throughout the world. Because of the detection of the herbicide in surface and ground water, its degradation and metabolism are the subjects of much attention. Moreover, conditions exist at many chemical storage and mixing facilities where repeated spills of formulated material have enriched soil to extremely high levels. These sites may serve as a source for stream and ground water contamination. For example, work in Wisconsin and other states has shown mixing and handling locations to be a very significant environmental source of the chemicals (Habecker 1989). Atrazine, alachlor, cyanazine, are the most commonly encountered pesticites in well water samples collected near mixing/loading sites. Atrazine was detected in 37% and 43% of the water samples collected during the Illinois and Wisconsin studies, repectively (Long 1987; Habecker 1989). The atrazine residue levels in the well waters ranged from 0.024 to 220 μg ml^{-1}, the latter value is 7,000 times the current Maximum Contaminant Level (MCL) established by the Office of Water, U.S. EPA.

Although atrazine can undergo chemical transformations into hydroxylated derivatives, total mineralization is a microbial process (Kaufman & Kearney 1970; Giardina et al. 1980, 1985; Bollag 1982; Behki & Kahn 1986). However, this process is very slow and the half-lives of atrazine can exceed one year under some conditions (Armstrong et al. 1967). In most agricultural soils less than 40% of applied atrazine is mineralized after one year. Soil half-lives of atrazine metabolites can exceed that of the parent compound (Winkelmann & Klaine 1991). Several studies investigating atrazine degradation in soil have implicated an important role of soil fungi in the breakdown of the atrazine moiety to its dealkylated metabolites (Armstrong et al. 1967; Russell et al. 1968; Kaufman & Blake 1970).

Reports of isolated microorganisms capable of atrazine degradation are common. However, these isolates degrade atrazine under restricted environmental conditions and may require the presence of a particular metabolite of atrazine. Behki & Kahn (1986) isolated a *Pseudomonas* sp that required the presence of the mono-N-dealkylated product of atrazine before any dehalogenation of the *s*-triazine ring could take place. These *Pseudomonas* produced dehalogenated metabolites of atrazine, but mineralization was not reported. Jessee et al. (1981) isolated a facultative bacterium that required anaerobic conditions and an energy limiting growth medium. Wolf & Martin (1975) reported a consortium of bacteria that when grown in the presence of a carbon source was capable of degrading the triazine herbicide simazine. However, significantly accelerated atrazine mineralization in either soil or culture has not been reported. Accelerating the degradation of the herbicide is a desirable goal if the bioremediation of contaminated environments is to be achieved. We report on a mixed population of microorganisms that are capable of the complete and rapid mineralization of atrazine in both liquid culture and soil.

Materials and methods

Soil

A Drummer soil (silty clay loam, 4% organic matter, pH 6.5) was collected from the upper 6 cm of a conventionally tilled field maintained in continuous corn for over 18 years. The site has been treated with atrazine at application rates recommended for weed control.

Pesticides

Atrazine [2-chloro–4-(ethylamino)–6-(isopropylamino)-*s*-triazine] was used as non-labeled analytical grade, 99% purity (ChemService, West Chester, PA) and as ring uniformly-labeled ^{14}C-atrazine (ring-ul-^{14}C) with specific activity of 4.5 MBq mmol^{-1} (Sigma Chemicals, St. Louis, MO). The ring-labeled atrazine was mixed with non-labeled atrazine to give a mixture with specific activity of 0.17 MBq μg^{-1}.

Enrichment for atrazine degrading microorganisms

Enrichment for atrazine degrading organisms was carried out in minimal basal salt medium (MBS) containing 1% mannitol and supplemented with 0.14 mM atrazine (AMMBS). The MBS medium contained the following materials: 50 mM K_2HPO_4 and KH_2PO_4 Buffer (pH 7.0), 20 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 0.18 mM $CaCl_2$, 0.005 mM $MnCl_2$, 0.001 mM $CuCl_2$, 0.001 mM $FeCl_3$, 0.001 mM $CoCl_2$, 0.001 mM $NiSO_4 \cdot 6H_2O$, 0.001 mM $Al_2(SO_4)_3 \cdot 18\ H_2O$, 0.001 mM H_3BO_3, 0.001 mM $FeSO_4$, and 0.008 mM sodium molybdate.

Five grams of soil was added to culture flasks containing 50 ml AMMBS medium. Flasks were shaken on a rotary shaker at 200 rpm and maintained at 25 °C. Following 10 days of incubation, the entire content of each flask was transferred to a Teflon centrifuge tube and the tubes were centrifuged at 500 × g to sediment the soil. The supernatants were transferred into a second culture flask and incubation was continued. Starting at the end of the second 10-day incubation period, 10 ml of each flask was transferred weekly into fresh (AMMBS) solution. Atrazine and metabolites concentrations in the cultures were monitored periodically by HPLC (see below). Transfers into fresh media were continued for a total of 10 weeks). From the 10 initial flasks, enrichment cultures with the greatest rates of atrazine loss were selected and atrazine concentration in the media was increased by increments of 0.05 mM atrazine each transfer until a final concentration of 0.56 mM atrazine was reached. Atrazine was applied using ethanol as carrier (using a stock solution of 0.1 M atrazine).

In an attempt to isolate pure cultures containing a single organism or a defined number or organisms capable of atrazine mineralization, a total of 20 isolates (with differences in morphological characteristics) were transferred from nutrient agar plates into liquid AMMBS medium. None of the pure isolates or any of the combinations of the isolates, were able to miniralize atrazine. However, rcombining all the pure isolates restored the atrazine degradation ability to the population. Work is continuing in our laboratory to identify the members of this mixed microbial culture and to investigate their specific role in the mineralization process.

Preparation of working cultures

Culture flasks containing 50 ml of MMBS medium, with no atrazine, were inoculated with 1 ml of the atrazine degrading cultures and grown until mid-log phase. These cultures were used as working cultures in the following experiments.

Atrazine degradation in culture

Five replicates of working cultures (50 ml) prepared as described above, were treated with the ^{14}C-atrazine stock solution (0.1 M atrazine in ethanol) for a final concentration of 0.56 mM. Atrazine treated cultures were transferred into a sterile flow-through system designed to trap the evolved $^{14}CO_2$. The flow-through system consists of a closed culture flask with magnetic stir bar providing continuous agitation of culture. The flask is connected to an air inlet tube equipped with a sterile filter and to an outlet tube passing into two consecutive NaOH traps for $^{14}CO_2$ (40 ml of 1 M NaOH in each trap). The terminal CO_2 trap is connected to a vacuum source providing aeration (10 ml min^{-1} of air) by pulling the evolved CO_2 from the culture flask into the NaOH traps.

The sodium hydroxide traps were replaced periodically and 0.5 ml of sample was added into scintillation vial containing 15 ml of scintillation cocktail (CytoScint^{TM}, ICN Biochemicals, Inc., Costa Mesa, CA). The vials were counted on liquid scintillation counter (Tri-Carb 1600 TR, Packard Instruments, Downers Grove, IL).

Atrazine degradation in soils inoculated with the atrazine degrading microorganisms

Five of the atrazine working cultures were transferred to Teflon tubes and centrifuged at 2000 × g. The resulting mixed population cell pellets were resuspended in 20 ml MBS medium. Each cell suspension was added into one of five replicates of 200 g soil. The inoculated soil samples along with five uninoculated controls were treated with the ^{14}C-atrazine stock for a final concentration of 0.14 mM (atrazine concentration was based on total soil mass). The soils were mixed thoroughly and incubated in biometer flasks equipped with NaOH reservoirs (20 ml 1 M NaOH) to trap evolved $^{14}CO_2$. The NaOH in the traps was replaced periodically and 0.5 ml sample was taken from each trap and counted using liquid scintillation. A 2 g soil sample was taken periodically from each flask and transferred into 50 ml Teflon tubes and shaken for 12 h with 10 ml of CH_3OH. After 12 h the tubes were centrifuged for 5 min at 5000 × g and the supernatants were analyzed on HPLC for atrazine and metabolites. The soil pellets were acidified with 2:8 1M HCl:CH_3OH and shaken for an additional 12 h. The tubes were again centrifuged and the supernatants analyzed on HPLC.

Sample oxidation was utilized as a measure of soil-bound residues which is defined as the ^{14}C activity remaining in soil after methanol and acidified methanol extractions. The extracted soil pellets were air dried, finely ground, and a 0.5 g of soil mixed with 0.3 g of cellulose acetate. These samples were combusted in a sample oxidizer (Model 307, Packard Instrument Company, Downers Grove, IL) and ^{14}C-CO_2 was trapped in 8 ml of Carbosorb E, added to a 10 ml of Permafluor E$^+$ (Packard Instruments, Downers Grove, IL), and radiation counted in a scintillation counter as described above.

High performance liquid chromatography (HPLC)

HPLC analyses of culture solution and soil extracts was carried out on a Varian Model 5000 Liquid Chromatograph (Varian Instrument Group, Walnut Creek, CA). The HPLC was equipped with a reverse phase C_{18} column Spherisorp 10 ODS, size 250 × 4.6 mm (Phenonmenex, Torrance, CA) and a Gilson HM Holochrome UV-VIS variable wave length detector set at 220 nm (Gilson Medical Electronics, Inc., Middleton, WI). Analyses was carried out at ambient temperature and with constant flow rate of 1 ml min^{-1} of 40:60, v:v, H_2O:CH_3OH mobile phase. Ammonium acetate was added to the mobile phase at 50 mM and pH was adjusted to 7.4 when needed. Standards of atrazine metabolites were a gift from CIBA-GEIGY (Greensboro, NC) and were injected into the HPLC every time samples were analyzed.

Results and discussions

The atrazine degrading mixed culture of microorganisms

Following a 90-day enrichment process we were able to isolate a mixed microbial culture capable of accelerated atrazine degradation in the presence of a simple carbon source. These organisms were able to grow on a range of carbon sources including: mannitol, glycerin, ethyl alcohol, and sucrose. Nitrogen as NH_4NO_3, was added to the enrichment medium at 2 mM. However, continuous cultures of the microbial population with atrazine supplement required no further nitrogen source (atrazine was added to the continuous cultures when the concentration in the medium reached zero). This observation supports a general assumption that atrazine is being catabolized as a source of nitrogen

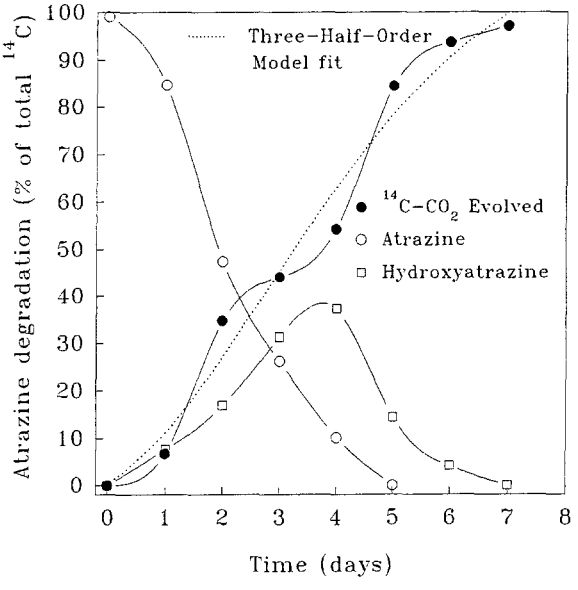

Fig. 1. Dissipation of atrazine and formation of hydroxyatrazine or carbon dioxide by a mixed microbial culture. A three-half-order model fit to atrazine mineralization rate (measured as evolved ^{14}C-CO$_2$) is shown. Atrazine was applied to an initial concentration of 0.56 mM in liquid MMBS medium.

and accounts for our observation of nearly complete carbon mineralization from the ring structure. Nevertheless, the addition of nitrogen to these cultures did not retard atrazine degradation.

Atrazine degradation in culture

Liquid cultures (mid-log) grown on MMBS medium mid-log and treated with 0.56 mM ^{14}C-atrazine degraded all the atrazine within 5 days of treatment (Fig. 1). Atrazine degradation was coupled with the formation of hydroxyatrazine. Concentrations of hydroxyatrazine increased steadily to a high point at 38% of applied atrazine by day 4. No other metabolites were detected in the cultures. Between days 4 and 7, hydroxyatrazine concentration declined to zero. The evolution of ^{14}C-CO$_2$ increased over the incubation period and accounted for 95.7% of the applied radioactivity after 7 days. At all sampling times we could account for 98.6% $\pm$ 1 of the applied atrazine. The changes in the culture concentrations of atrazine and hydroxyatrazine over time are shown in Table 1.

The formation of hydroxyatrazine in media such as soil has been attributed mainly to physiochemical processes. However, studies by Schochen & Speedie (1984) on the degradation of atrazine by the fungus *Periconia prolifica* suggested the formation of hydroxyatrazine is a main pathway in biological degradation. The work of Cook & Hütter (1984) showed that the degradation of deethylsimazine and other *s*-triazines by *Rhodococcus corallinus* appeared to occur in two steps, first a dechlorination to the hydroxy derivative, followed by deamination. Klages et al. (1981) reported a similar dechlorination step was needed for the degradation of other chlorinated aromatics. In our study, the formation of hydroxyatrazine appears to serve as the first step in the mineralization of atrazine. Hydrolysis of chloro-*s*-triazine may also be required for cleavage of the *s*-triazine ring. Skipper & Volk (1972) showed that in several soils $^{14}CO_2$ evolution from *s*-triazine ring was 25 to 40 folds greater from hydroxyatrazine than from atrazine.

Accelerated atrazine degradation in soil

Soil treated with atrazine and inoculated with the mixed microbial culture showed an accelerated atrazine mineralization as compared to uninoculated soil. In the inoculated soil (Fig. 2), 86 and 100% of the parent compound was dissipated by day 10 and 30 days, respectively. In these samples 42 and 62% of the applied atrazine was mineralized to ^{14}C-CO$_2$ by days 10 and 30, respectively. Following 145 days, 86% of applied ^{14}C-atrazine was accounted for as ^{14}C-CO$_2$.

Acidic methanol extraction of soil indicated that after 10 days, 26.5% of the applied atrazine was recovered as hydroxyatrazine. However, hydroxyatrazine concentration declined to zero by day 145. Soil bound ^{14}C-materials residues were highest at day 70 and accounted for 13% of the applied atrazine. At the end of 150 days of incubation, the soil-bound residues comprised less than 8% of the applied atrazine which also represents the only portion not accounted for as evolved ^{14}C-CO$_2$ (Fig. 2).

Patterns of atrazine degradation in uninoculated soil (Fig. 3) show striking differences when compared to the inoculated soil. In uninoculated soil, atrazine became undetectable after 120 days. This is in contrast to the inoculated soil where dissipation of the applied material took 30 days. A fundamental difference between the two systems is apparent in the mineralization rates and the amount of soil-bound residue formed. In uninoculated soil, 7% and 25% of applied atrazine was mineralized after 30 and 145 days, respectively, compared to 63% and 87% over the same times in inoculated soils. At day 145, soil-bound residues accounted for only 8% of applied atrazine in inoculated

Table 1. Degradation of atrazine and the formation of metabolites in culture.

Time (days)	Atrazine (% of applied)	hydroxyatrazine (% of applied atrazine)	% of applied atrazine evolved as ^{14}C-CO_2
0	99.2	0.0	0.0
1	84.8	7.6	6.8
2	47.5	17.0	34.8
3	26.3	31.4	44.1
4	10.2	37.3	54.2
5	0.0	14.4	84.4
6	0.0	4.2	93.8
7	0.0	0.0	97.2

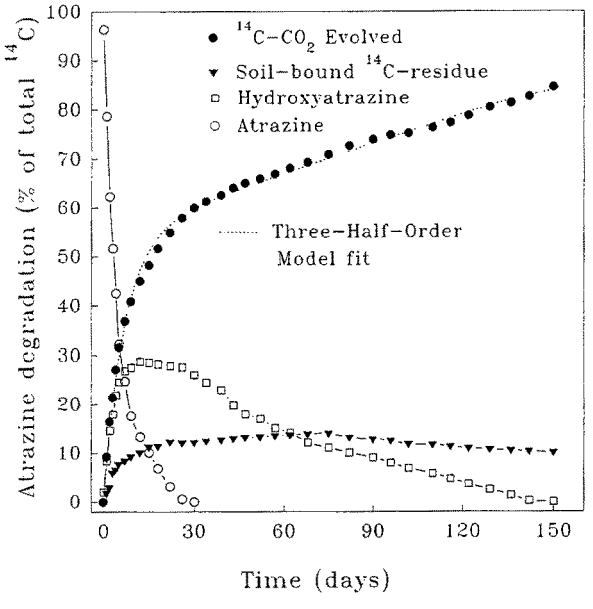

Fig. 2. Dissipation of atrazine and formation of hydroxyatrazine, carbon dioxide and soil-bound residues by a mixed microbial population. A three-half-order model fit to atrazine mineralization rate (measured as evolved ^{14}C-CO_2) is shown. Atrazine was applied to an initial concentration of 0.14 mM in soil. Soil was inoculated with the mixed microbial culture grown on MMBS.

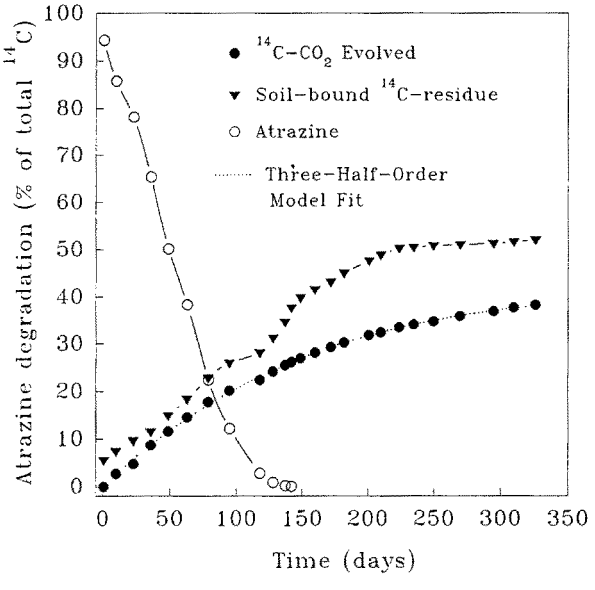

Fig. 3. Disappearance of atrazine and formation of carbon dioxide and soil-bound residues in native uninoculated soil. A three-half-order model fit to atrazine mineralization rate (measured as evolved ^{14}C-CO_2) is shown. Atrazine was applied to an initial concentration of 0.14 mM.

soil. In contrast, the uninoculated soil-bound residues accounted for 40% of the applied label by day 145. The amount of soil-bound residues in the uninoculated soil reached 54% of applied atrazine by day 320.

As expected, atrazine degradation in culture was faster than that in soil. Because of its sorption to soil surfaces (Clay & Koskinen 1990), hydroxyatrazine may be protected and persist in soil longer than we observed in culture. The detection of hydroxyatrazine in the inoculated soil as the only metabolite parallels our liquid culture work and shows that our mixed microbial culture is functioning when applied to soil. Our liquid culture data shows clearly that the organisms are capable of the rapid mineralization of the metabolites. Other work in our laboratory has shown that atrazine metabolites will build-up in uninoculated soils when atrazine degradation is taking place. As mentioned, it has been shown that in several soils ^{14}C-CO_2 evolution from *s*-triazine ring was 25 to 40 folds

greater from hydroxyatrazine than from atrazine (Skipper & Volk 1972). The mixed microbial culture is functioning in such a manner as to remove the herbicide and its metobolites before soil-bound residue are formed.

The complex nature of the dissipation pattern leads us to fit the atrazine mineralization data from culture and from soil with a three-half-order regression model (Brunner and Focht, 1984). This model as used by Moorman and Harper (1989) estimates k_0, k_1, and k_2 parameters based on cumulative $^{14}CO_2$ evolved. Atrazine mineralization in culture produced estimated k_0, k_1, and k_2 of 1.50, 0.06, and 0.08, respectively. The parameter k_1 represents the initial first order mineralization of the substrate by microorganisms (Brunner and Focht, 1984). We believe that the relatively low k_1 value indicates that very few organisms were able to mineralize the parent compound atrazine. The slightly higher k_2 indicated low microbial adaption to mineralization of atrazine. However, the high value of the k_0 parameter indicates a high mineralization rate of the transformation products of the parent compound, hydroxyatrazine (Brunner and Focht, 1984; Moorman and Harper, 1989). In our experiment the sole transformation product was hydroxyatrazine which accumulates in the culture medium between days 1 and 4 (Fig. 1). The surge in $^{14}CO_2$ evolution after day 1 coincided with the increase in hydroxyatrazine formation. The rate of mineralization reached its peak when hydroxyatrazine in the medium was at its highest levels at day 4. The high rate of $^{14}CO_2$ evolution continued until hydroxyatrazine level declined to zero by day 7 when 96% of the applied ^{14}C-atrazine was accounted for as ^{14}C-CO_2.

For uninoculated soil, the three-half-order model fit to mineralization data provided estimates of k_1 and k_2 that were not significantly different from zero. All parameters were highly correlated indicating that the model was overparameterized for this data (Moorman and Harper, 1989). When values of zero were used for k_1 and k_2, the model reduces to a linear form and gave a value k_0 of 0.048. Therefore, the linear regression form of the three-half-order model shows that a very slow mineralization of metabolites that are formed by either abiotic or biotic processes is occurring in the soil system.

In inoculated soil, however, the estimated parameters of the three-half-order model were 0.27, 0.12, and 0.0003 for k_0, k_1, and k_2, respectively. The k_0 value is 5 times greater and implies degradation is not limited by metabolite formation. The incubation period was 150 days during which 86% of applied atrazine was accounted for as ^{14}C-CO_2. About 62% of the applied atrazine was mineralized during the initial 30 days and is reflected in the relatively high k_1 value. The near zero estimated values for k_2 represents a low initial mineralization of the parent compound atrazine. This was expected because we believe that hydroxyatrazine formation constitutes the first step in the mineralization process. A larger k_0 relative to either k_1 or k_2 indicates mineralization of the transformation product, hydroxyatrazine, which was found at high levels in soil extracts during the first 30 days (Fig. 2). Parallel to what we observed in culture, the surge in $^{14}CO_2$ evolution in the first 30 days coincided with the observed increase in hydroxyatrazine formation. Hydroxyatrazine level was near zero by day 150 when 86% of the applied ^{14}C-atrazine was accounted for as ^{14}C-CO_2. When the three-half-order model was fitted to the mineralization data obtained after the first 30 days, the parameters indicated first order kinetics. Beyond 150 days the evolution of ^{14}C-CO_2 leveled off and the decrease in ^{14}C soil-bound residues equaled the amount of evolved ^{14}C-CO_2.

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References

Armstrong DE, Chesters C & Harris RF (1967) Atrazine hydrolysis in soil. Soil Sci. Soc. Am. Proc. 31: 61–66

Behki RM & Kahn SU (1986) Degradation of atrazine by *Pseudomonas*: N-Dealkylation and dehalogenation of atrazine and its metabolites. J. Agric. Food Chem. 34: 746–749

Bollag JM (1982) Microbial metabolism of pesticides. In: Rosazza JP (Ed), Microbial Transformations of Bioactive Compounds (pp 126–168). CRC Press, Inc., Boca Raton

Brunner W & Focht DD (1984) Deterministic three-half-order kinetic model for microbial degradation of added carbon substrates in soil. Appl. Environ. Microbiol. 47: 197–172

Clay SA & WC Koskinen (1990) Adsorption and desorption of atrazine, hydroxyatrazine, and s-glutathione atrazine on two soils. Weed Sci. 38: 262–266

Cook AM & Hütter R (1984) Deethylsimazine: Bacterial dehalogenation, deamination, and complete degradation. J. Agric. Food Chem. 32: 581–585

Giardina MC, Giardi MT & Fialacchioni G (1980) 4-amino–2-chloro–1,3,5-triazine: A new metabolite of atrazine by a soil bacterium. Agric. Biol. Chem. 44: 2067–2072

Giardina MC, Giardi MT & Fialacchioni G (1985) Chemical and biological degradation of primary metabolites of atrazine by a *Nocardia* strain. Agric. Biol. Chem. 44: 1551–1558

Habecker MA (1989) Environmental contamination at Wisconsin pesticide mixing/loading facilities: Case study, investigation and remedial action evaluation. Wisc. Dept. Agriculture, Trade and Consumer Prot., Ag. Res. Mgmt. Rpt. (pp 1–80)

Jessee JA, Valerias C, Benoit RE, Hendricks AC & McNair H (1981) Determination of cyanuric acid by high performance liquid chromatography. J. Chromatogr. 207: 454–456

Kaufman DD & Blake J (1970) Degradation of atrazine by soil fungi. Soil Biol. Biochem. 2: 73–80

Kaufman DD & Kearney PC (1970) Microbial degradation of triazine herbicides. Residue Rev. 32: 235–265

Klages U, Marcus A & Lingens F (1981) Degradation of 4-chlorophenolic acid by a *Pseudomonas* species. J. Bacteriol. 146: 64–68

Long T (1987) Groundwater contamination in the vicinity of agrichemical mixing and loading facilities. Proceedings of the 16th ENR Annual Conference, Illinois Department of Energy and Natural Resources.

Moorman TB & Harper SS (1989) Transformation and mineralization of metribuzin in surface and subsurface horizons of a Mississippi delta soil. J. Environ. Qual. 18: 302–306

Russell JD, Cruz M, White JL, Baily W, Payne WR Jr, Pope JD Jr & Teasley JI (1968) Mode of chemical degradation of s-triazines by montmorillonite. Science 160: 1340–1342

Schocken MJ & Speedie MK (1984) Physiological aspects of atrazine degradation by higher marine fungi. Arch. Environ. Contam. Toxicol. 13: 707–714

Skipper HD & Volk VV (1972) Biological and chemical degradation of atrazine in three Origon soils. Weed Sci. 20: 344–347

Winkelmann DA & Klaine SJ (1991) Degradation and bound residue formation of atrazine. Environ. Toxicol. Chem. 10: 335–345

Wolf DC & Martin JP (1975) Microbial decomposition of ring-^{14}C-atrazine, cyanuric acid, and 2-chloro–4,6-diamino-*s*-triazine. J. Environ. Qual. 4: 134–139