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Effects of trifluralin and metabolites on the decomposition of selected substrates by soil microorganisms

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Summary. The effects of trifluralin and 12 of its soil-formed metabolites on the decomposition of radiolabelled glucose, protein and cellulose were determined, using $^{14}CO_2$ evolution from soil as a measure of decomposition. Trifluralin increased ^{14}C-glucose mineralization rates, but these increases could be eliminated by adding N. Trifluralin had no inhibitory effect on the mineralization of protein or cellulose, but five of the metabolites inhibited glucose mineralization. None of the trifluralin metabolites affected protein mineralization. Seven trifluralin metabolites increased the rate of cellulose mineralization when applied at rates exceeding those that would be expected in soil. After considering the rate of metabolite application and the magnitude of the responses observed these compounds are expected to have no major effects on the microbial decomposition processes in soil.

Key words: Decomposition – Herbicides – Microbial inhibitors – Cotton – Herbicide effects – Trifluralin – $^{14}CO_2$ evolution

As a result of the long-term use of trifluralin [2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)benzenamine] and other dinitroaniline herbicides during a period of decreasing cotton yields (Meredith and Bridge 1984), these chemicals have been suggested as possible factors in the yield decline (Hurst 1977; Frans et al. 1983). Trifluralin can reduce the adventitious root growth of seedlings (Anderson et al. 1967; Oliver and Frans 1968; Christiansen and Hilton 1974), leading to reduced nutrient uptake (Cathey and Sabbe 1972; Buchholtz and Lavy 1979). The effects of trifluralin on cotton yields are inconsistent; yields are generally unaffected (Miller et al. 1975; Hayes et al. 1981), but may be reduced in comparison to yields obtained without herbicide and no weed competition (Gaylor et al. 1983). Damage and yield losses are both accentuated by adverse environmental conditions and high rates of herbicide use, but occasional damage from trifluralin would not result in the cumulative decline that the yield data suggest (Meredith and Bridge 1984). Furthermore, trifluralin and other dinitroaniline herbicides do not appear to accumulate in soils (Parka and Tepe 1969; Savage 1973; Brewer et al. 1982). Koskinen et al. (1984) found that neither trifluralin nor any of 12 principal metabolites decreased cotton yield, even though metabolites were applied at concentrations equivalent to accumulations of 14 to more than 400 years.

Although these findings showed that trifluralin and related compounds were not phytotoxic to cotton, the possibility remains that dinitroaniline herbicides or their metabolites could have deleterious effects on the soil microorganisms responsible for soil fertility, and thus have an indirect effect on cotton yields. The objective of this study was to determine the effects of trifluralin and trifluralin metabolites on the microbial decomposition of organic substrates common in soil. The decomposition process was selected for study because of its importance in soil organic-matter formation and interaction with other nutrient cycles in soil.

Materials and methods

Technical-grade trifluralin from Polysciences, Warrington, Pa, was used without further purification. Twelve metabolites of trifluralin, originally isolated and described by Golab et al. (1979), were synthesized by Lilly Research Laboratories, Greenfield, Indiana, and the USDA-ARS Pesticide Degradation Laboratory, Beltsville,

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Maryland. The metabolites were 2,6-dinitro-*N*-propyl-4-(trifluoromethyl)benzenamine, [coded TR-2]; 2,6-dinitro-4-(trifluoromethyl)-benzenamine, [TR-3]; 3-nitro-5-(trifluoromethyl)-1,2-benzenediamine, [TR-6]; 5-(trifluoromethyl)-1,2,3-benzenetriamine, [TR-9]; 2-ethyl-7-nitro-1-propyl-5-(trifluoromethyl)-1*H*-benzimidazole, [TR-13]; 2-ethyl-4-nitro-6-(trifluoromethyl)-1*H*-benzimidazole, [TR-15]; 7-nitro-1-propyl-5-(trifluoromethyl)-1*H*-benzimidazole, [TR-17]; 4-(dipropylamino)-3,5-dinitrobenzoic acid, [TR-21]; 2,2'-azoxybis-(6-nitro-*N*-propyl-4-(trifluoromethyl)benzenamine), [TR-28]; 2,2'-azobis-(6-nitro-*N*-propyl-4-(trifluoromethyl)benzenamine), [TR-32]; 3-methoxy-2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)-benzenamine, [TR-37M]; *N*-(2,6-dinitro-4-(trifluoromethyl)phenyl)-*N*-propyl-propanamide, [TR-40].

All substrates used were uniformly radiolabelled. The ^{14}C-glucose was obtained from Pathfinder Laboratories, Inc., St. Louis, Mo, and had a specific activity of 1.66 mCi/mg. ICN Radiochemicals, Irvine, Calif., supplied the ^{14}C-algal protein which had a specific activity of 5.11 mCi/mg and the ^{14}C-cellulose which had a specific activity of 0.34 μCi/mg. In all experiments ^{14}C-labelled substrates were diluted with unlabelled materials before use.

Commercially available wettable-powder formulations of pentachloronitrobenzene [Terrachlor, 75% a. i. (w/w), Olin Chemical Co.] and ethylene bis-dithiocarbamate ion, also known as mancozeb [Dithane, 62% a. i. (w/w) as anion, Rohm and Haas, Philadelphia, Pa] were used as microbial inhibitors. Streptomycin (Sigma Chemical Co., St. Louis, Mo) was also used as a microbial inhibitor. All other chemicals were American Chemistry Society reagent grade.

All experiments used a Dundee silt loam soil (fine-silty, mixed, thermic, Aeric Ochraqualf) that has been described previously (Koskinen et al. 1984). Air-dried soil was sieved through a 3.35-mm mesh screen before use. Individual chemicals were applied to the equivalent of 200 g oven-dry soil in 2–3 ml solvent. Vials containing the chemicals were rinsed two to three times with 1 ml of the same solvent, which was also pipetted onto the soil. A total of 5 ml solvent was applied in all instances. The treated soil was mixed thoroughly and allowed to stand at room temperature while the solvent evaporated. Control soils were treated only with 5 ml of the same solvent, applied in the same way. Portions (50 g) of soil from each treatment were placed into each of three biometer flasks (Bartha and Pramer 1965).

The treated soils in the biometer flasks were then amended with radiolabelled substrates and sterile water, so that the final soil water potential was −33 kPa. Each flask was stirred thoroughly to ensure uniform distribution of the water and substrate. The flasks were incubated at 25 °C for 30–90 days in the dark. The $^{14}CO_2$ evolved from the soil was trapped in 0.5 *M* NaOH (10 ml) which was changed at selected intervals. Radioactivity in the NaOH was determined by adding 2- to 4-ml aliquots to 10 ml liquid scintillation medium (PCS, Amersham Corp., Arlington Heights, Ill), the counts being made with a Packard Tricarb 3385 liquid scintillation spectrometer (Packard Instruments, Downers Grove, Ill.). Data were corrected for quenching by use of the external standards ratio method, and background radiation.

The antimicrobial agents pentachloronitrobenzene (PCNB), ethylene bis-dithiocarbamate (EBDC) and streptomycin were applied individually to the soil at rates (a. i.) of 100, 100 and 1000 mg/kg soil, respectively, to determine whether these methods would detect chemically induced changes in microbial metabolism. The three chemicals were dissolved in 2 ml water and applied to individual biometer flasks, and the containers rinsed three times with 1 ml water. A fourth treatment consisted of chloroform fumigation for 36 h followed by removal of the chloroform by vacuum according to the method of Jenkinson and Powlson (1976b). All flasks were amended with ^{14}C-glucose, which had a specific activity of 2.25×10^{-3} μCi/mg glucose, at a rate of 1000 mg/kg soil. A solution of NH_4Cl was added at a rate of 154 mg/kg soil. The flasks were incubated for 30 days.

Trifluralin was applied to soil in acetone at rates of 0.5, 1.0, 10 and 100 mg/kg soil, in two experiments. In the first, ^{14}C-glucose, which had a specific activity of 1.62×10^{-3} μCi/mg, was added at 1000 mg/kg soil. In the second experiment all conditions were the same, except that NH_4Cl was added at a rate of 154 mg/kg soil. Both experiments were incubated for 30 days.

Trifluralin and the trifluralin metabolites were dissolved in methanol, except metabolites TR-28 and TR-32 which were dissolved in benzene, and applied individually at a rate of 0.5 mg kg^{-1} soil. Controls were prepared by treating soils with 5 ml methanol or benzene. N (154 mg NH_4Cl/kg soil) and ^{14}C-glucose were added and the flasks were incubated for 30 days. The specific activity of ^{14}C-glucose was 2.14×10^{-3} μCi mg^{-1}.

Similar experiments were prepared by substituting ^{14}C-protein or ^{14}C-cellulose as substrates in place of the ^{14}C-glucose. The ^{14}C-protein was prepared by mixing the ^{14}C-algal protein with bovine serum albumin (Sigma Chemical Co.) to form a solution having a specific activity of 6.25×10^{-3} μCi/mg protein, which was applied at a rate of 1000 mg protein/kg soil. Unlabelled cellulose (Difco Laboratories, Detroit, Mich). ^{14}C-cellulose and 16 g soil were ground together and mixed thoroughly. Aliquots were oxidized using a Packard 306 oxidizer, and the radioactivity therein was measured to determine the uniformity of the ^{14}C-cellulose in the mixture. The final mixture had a specific activity of 1.49×10^{-3} μCi/mg cellulose and was added at 1000 mg/kg soil. Both the ^{14}C-protein- and ^{14}C-cellulose-amended soils were incubated for 90 days.

Differences in the kinetics of $^{14}CO_2$ evolution were determined by fitting the data to the non-linear model developed by Brunner and Focht (1984),

$$P = S_0(1-e^{-k_1t-(k_2t^2)/2})+k_0t$$

where P is the cumulative $^{14}CO_2$ produced as a percentage of the ^{14}C applied, S_0 is the fraction of the original substrate mineralized, k_1 is a constant describing the metabolism of the substrate by indigenous populations at the time of substrate addition, k_2 is a constant describing the growth of degrader biomass and k_0 is a zero-order rate constant describing $^{14}CO_2$ evolution from humus or other transformation products of glucose. Regression curves and estimates of model parameters were obtained using the NLIN procedure of SAS (SAS 1982). Differences due to treatments were determined by obtaining a significant ($P = 0.05$) reduction in the pooled residual sums of squares for models describing individual treatments from that of a single model fit to all data across all treatments in an experiment (Hinds and Milliken 1987). If treatments were significantly different, then the significant differences between the treatments on individual sampling dates were calculated.

Results and discussion

Effects of antimicrobial agents

The mineralization of ^{14}C-glucose was influenced by each of the chemical agents, EBDC, PCNB and streptomycin, but not by the chloroform fumigation procedure (Table 1). The regression curves described by these models are presented in Fig. 1 to demonstrate the relationship between the $^{14}CO_2$-production curves and the model parameters, and to illustrate the types of changes induced by these agents. EBDC initially inhibited the $^{14}CO_2$ production, with the maximum rate of evolution occurring 24 h after that of the untreated soil. The differences in initial rates of glucose metabo-

lism in the EBDC-treated and untreated soils are shown by a comparison of the k_1 values (first-order mineralization rates by indigenous microorganisms) determined for these treatments. Subsequent growth of microorganisms, as indicated by the non-zero value of k_2, could be due to the proliferation of surviving or resistant strains in the soil. The activity of these populations produced similar amounts of $^{14}CO_2$ in the treated and untreated soils after the initial EBDC inhibition was overcome. In the untreated soil the glucose was readily metabolized in a first-order process by existing populations. Growth did not influence the kinetics of metabolism, resulting in a k_2 value of zero. Previous reports (Doneche et al. 1983) showed that EBDC was a broad-spectrum microbiocide, reducing both bacterial and fungal populations, and thus accounting for the lower value of k_1 observed in this experiment. Similar delays in the mineralization of specific substrates were reported for the fungicide benomyl (Domsch et al. 1973) and phenol (Isbister et al. 1980) in the soil system.

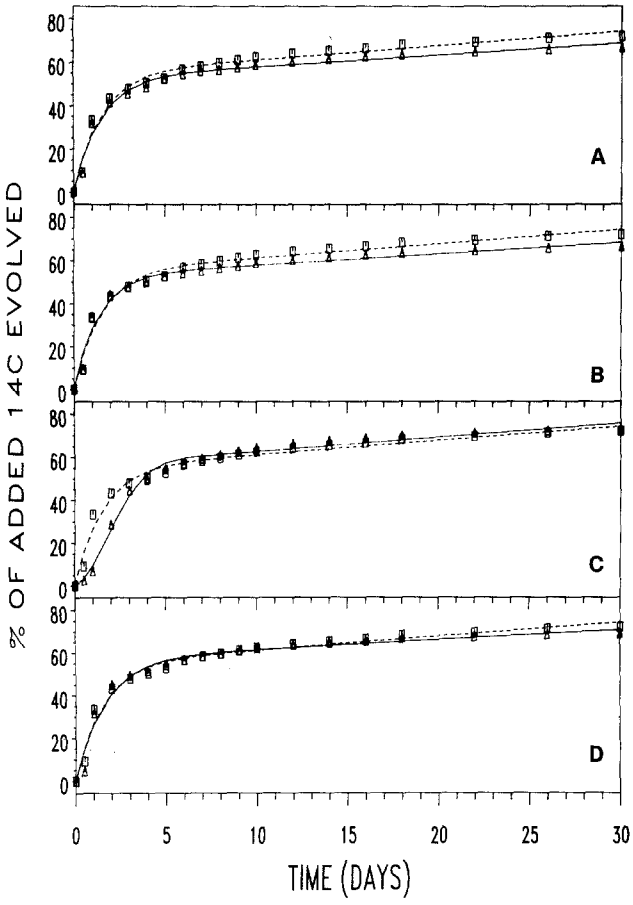

Fig. 1. Comparison of cumulative $^{14}CO_2$ evolution from ^{14}C-glucose-amended soils treated with 1000 mg/kg soil streptomycin (*A*), 100 mg/kg of pentachloronitrobenzene (*B*), 100 mg/kg of ethylene bis-dithiobicarbamate (*C*) and 24-h fumigation with chloroform (*D*). Predicted amounts of $^{14}CO_2$ evolved from untreated (– – –) soil and treated soils (———) are compared to treatment means for treated (△) and untreated (□) soils. See text for details concerning prediction of $^{14}CO_2$ production

PCNB and streptomycin both caused changes in the kinetics of $^{14}CO_2$ evolution, which were different from those caused by EBDC. The effects of these compounds were apparent only after 5 or 6 days of incubation (Table 1, Figure 1), so that the k_0 values for the PCNB- and streptomycin-treated soils were lower than those of the untreated soil. Both compounds affect only a portion of the total microbial populations. PCNB inhibits actinomycetes and some fungi, while streptomycin acts principally against bacteria (Ingham 1985; Ko and Farley 1969). Other studies on the effect of streptomycin on soil microorganisms have shown reductions in glucose mineralization (Song et al. 1986) at higher rates than that used in the present experiments, while Ingham and Coleman (1984) reported no overt effects. Apparently the microorganisms unaffected by PCNB and streptomycin are able to mineralize glucose at rates equal to those in untreated soil (compare k_1 values). However, these compounds apparently reduce the capacity of surviving populations to mineralize the transformed ^{14}C in the later phases of the incubation. While the effects were not

Table 1. Model parameters describing $^{14}CO_2$ evolution from ^{14}C-glucose-amended soil treated with microbial inhibitors or fumigated with chloroform for 36 h. Comparison with control indicates the days during the incubation when cumulative $^{14}CO_2$ evolution from the treated soil was significantly ($P \leq 0.05$) different from that of the untreated soil. Inhibition (I) or stimulation (S) is expressed as percentage of the mineralization in the untreated soil

Treatment	Model parameters				Comparison with control	Maximum inhibition or stimulation (%)
	S_0	k_0	k_1	k_2		
Chloroform fumigation	56.8	0.469	0.610	0	NS	–
EBDC	56.1	0.659	0.045	0.253	0.5–3, 6, 7	79 (I), 3 (S)
PCNB	52.6	0.521	0.748	0	6–30	8 (I)
Streptomycin	52.5	0.545	0.672	0	5–30	7 (I)
None	54.7	0.654	0.657	0	–	–

EBDC, ethylene bis-dithiocarbamate; PCNB, pentachloronitrobenzene

large in magnitude, they persisted throughout the 30-day incubation.

Chloroform fumigation did not affect the mineralization of ^{14}C-glucose, except at the 12-h sampling time when an average of 4.7% of the applied ^{14}C was evolved in the fumigated soil, while 9.1% was evolved in the untreated soil. However, the models describing the mineralization in the fumigated and unfumigated soils were not significantly different. Although the fumigation procedure kills the vast majority of soil microorganisms (Jenkinson and Powlson 1976a), it has no persistent effects. Lynch and Panting (1980) determined that 89% and 99.9% of the bacteria and fungi, respectively, were killed during fumigation of sieved soil. It appears that there are sufficient numbers of surviving microorganisms to metabolize the glucose at rates equal to those of the untreated soil, although the activity of individual microorganisms may be greater in the fumigated soil.

The differences in the kinetics of ^{14}C-glucose mineralization in soil treated with the known inhibitors EBDC, PCNB and streptomycin indicate that these methods could be used to detect metabolite-induced changes in the mineralization of naturally occurring substrates. Other studies have shown that the fungicide benomyl and phenol caused delays in mineralization similar to that observed for EBDC (Domsch et al. 1973; Isbister et al. 1980). The use of radiolabelled materials as substrates has the advantages of precise measurement and the evaluation of specific processes (e. g. cellulose degradation) which may be conducted only by a specific part of the total microbial population. As suggested by Brunner and Focht (1983), the model used in these analyses gave some insight into the action of these inhibitors in soil.

Effects of trifluralin and metabolites

Trifluralin increased the mineralization of ^{14}C-glucose in soil when no supplemental N was applied (Table 2). Rates of trifluralin over 1.0 mg/kg soil resulted in greater values of S_0 than in the untreated soil. The parameter S_0 estimates the amount of $^{14}CO_2$ evolved from metabolism of glucose, but excludes $^{14}CO_2$ from transformation products. The maximal (17% – 20%) stimulation of total $^{14}CO_2$ evolution by trifluralin was in the 8- to 12-day period, but the stimulatory effect continued until the end of the experiment. The lower rate of 0.5 mg kg^{-1} produced an intermediate effect. When this experiment was repeated with the addition of enough N to bring the C/N ratio of the amendments to 10, no stimulatory trifluralin effect was found. Model parameters describing the metabolism of glucose under these conditions were: $S_0 = 55.1$, $k_0 = 0.653$, $k_1 = 1.388$ and $k_2 = 0$. The addition of N increased all parameters except k_2. These results suggest that trifluralin may influence microbial N metabolism or uptake. Alternatively, trifluralin may become a source of N under N-limiting conditions.

Trifluralin metabolites both inhibited and stimulated the metabolism of ^{14}C-glucose (Table 3). Stimulatory responses were generally quite small in magnitude, ranging from 2% to 4% of the control. The metabolites which inhibited mineralization to the greatest extent (TR-6 and TR-9) had their greatest effects in the first 2 days after glucose addition to soil.

Table 2. Model parameters describing $^{14}CO_2$ evolution from soil treated with trifluralin and amended with ^{14}C-glucose (– N). Comparison with control indicates the days during the incubation when cumulative $^{14}CO_2$ evolution from the treated soil was significantly different ($P \leq 0.05$) from that of the untreated soil. Stimulation is shown as a percentage of mineralization in untreated soil

Trifluralin rate (mg kg^{-1} soil)	Model parameters				Comparison with control	Maximum stimulation (%)
	S_0	k_0	k_1	k_2		
0	32.2	0.373	0.793	0	–	–
0.5	35.6	0.319	0.542	0	6 – 22	7
1.0	39.8	0.303	0.535	0	3 – 30	19
10	38.8	0.342	0.539	0	4 – 30	17
100	39.3	0.388	0.588	0	3 – 30	20

Table 3. Model parameters describing $^{14}CO_2$ evolution from soil treated with trifluralin or trifluralin metabolites and amended with ^{14}C-glucose (+N). Comparison with control indicates the days during the incubation when cumulative $^{14}CO_2$ evolution from the treated soils was significantly different ($P \leq 0.05$) from that of the untreated soil. Metabolites TR-28 and TR-32 were compared with the benzene control. Inhibition (I) or stimulation (S) is shown as a percentage of the mineralization in untreated soil

Compound (0.5 mg kg^{-1} soil)	Model parameters				Comparison with control	Maximum inhibition or stimulation (%)
	S_0	k_0	k_1	k_2		
Trifluralin	52.8	0.462	1.316	0	1, 4	5 (I)
					22 – 30	4 (S)
TR-2	52.4	0.312	1.250	0	NS	–
TR-3	53.6	0.343	1.279	0	10 – 18	2 (S)
TR-6	51.7	0.379	1.105	0	0.5 – 5	17 (I)
TR-9	50.1	0.352	0.981	0	0.5 – 30	26 (I)
TR-13	53.3	0.362	1.160	0	0.5, 1	10 (I)
					12 – 22	2 (S)
TR-15	52.8	0.354	1.398	0	NS	–
TR-17	53.7	0.343	1.399	0	9 – 18	2 (S)
TR-21	53.9	0.304	1.316	0	NS	–
TR-28	52.8	0.356	1.434	0	NS	–
TR-32	52.4	0.336	1.140	0	0.5, 1	12 (I)
TR-36	51.5	0.427	1.387	0	NS	–
TR-40	51.9	0.386	1.219	0	0.5, 1	10 (I)
Benzene control	52.4	0.389	1.350	0	NS	–
Methanol control	52.8	0.332	1.387	0	–	–

Table 4. Model parameters describing $^{14}CO_2$ evolution from ^{14}C-cellulose-amended soil treated with trifluralin or trifluralin metabolites. Comparison with control indicates the days during the incubation when cumulative $^{14}CO_2$ evolution from the treated soils was significantly different ($P \leq 0.05$) from that of the untreated soil. Stimulation of mineralization is shown as a percentage of the mineralization in control soils. Metabolites TR-28 and TR-32 were compared with the benzene control

Compound (0.5 mg kg^{-1} soil)	Model parameters				Comparison with control	Maximum stimulation (%)
	S_0	k_0	k_1	k_2		
Trifluralin	41.1	0.175	0.088	0	NS	–
TR-2	44.6	0.192	0.110	0	26–90	18
TR-3	47.6	0.180	0.102	0	12–90	23
TR-6	53.4	0.188	0.099	0	12–90	36
TR-9	35.4	0.159	0.106	0	NS	–
TR-13	43.4	0.229	0.102	0	26–90	22
TR-15	40.0	0.166	0.092	0	NS	–
TR-17	39.5	0.164	0.100	0	NS	–
TR-21	42.6	0.210	0.108	0	33–90	17
TR-28	37.8	0.197	0.119	0	NS	–
TR-32	56.1	0.179	0.110	0	12–90	47
TR-36	33.8	0.218	0.097	0	NS	–
TR-40	45.1	0.192	0.096	0	26–90	18
Benzene control	36.9	0.156	0.118	0	NS	–
Methanol control	38.6	0.156	0.101	0	–	–

The values of k_1 were reduced by these metabolites in comparison to the value for untreated (methanol only) soil, suggesting that these compounds acted by inhibiting glucose metabolism itself rather than any secondary processes. However, the effects of both compounds decreased afterwards and only TR-6 continued to cause significantly less mineralization throughout the experiment. The inhibitory effects of the other metabolites and of trifluralin were highly transient and of relatively small magnitude.

Neither trifluralin nor any of the metabolites had any inhibitory effects on the mineralization of ^{14}C-cellulose, although seven of the metabolites stimulated the mineralization of cellulose (Table 4). All the metabolites that stimulated ^{14}C-cellulose mineralization still had stimulatory effects at the end of the 90-day incubation. The stimulatory metabolites induced greater S_0 values, indicating that the initial degradation of cellulose was continued to a greater extent than in the control treatments. However, the rates of degradation (k_1) and other model parameters were comparable to those of the respective controls. Several of the metabolites that caused temporary delays in ^{14}C-glucose metabolism stimulated mineralization of ^{14}C-cellulose. Perhaps these metabolites provided some competitive advantage to those organisms able to degrade cellulose by inhibiting the secondary colonists that used glucose produced in the degradation process. None of these compounds had any stimulatory or inhibitory effect on ^{14}C-protein mineralization. The model parameters describing protein mineralization in both treated and untreated soils were: $S_0 = 38.2$, $k_0 = 0.170$, $k_1 = 0.720$ and $k_2 = 0$.

Although no information is available concerning the concentrations of these metabolites in soils receiving applications of dinitroaniline herbicides over many years, the concentrations of metabolites used in the present experiments probably exceed the levels that would be found in field soils. Estimates of metabolite accumulations necessary to equal the rates of application in these experiments were made using the amounts found by Golab et al. (1979), during a 3-year field study. It was estimated that the application rate of 0.5 mg/kg soil represented from 50 to more than 1000 years of metabolite accumulation, assuming no degradation of accumulated metabolite (Koskinen et al. 1984). The total metabolites formed from dinitroaniline herbicides typically account for only a few percent of the applied material 7 months to 1 year after application in field environments (Golab et al. 1975; Kearney et al. 1976).

The substrates used in the present experiments are representative components of more complex organic materials such as crop residues or microbial biomass. The decomposition of these complex materials is an integral part of long-term nutrient cycling in soils. These metabolites probably have a minimal effect on decomposition processes in soil, when the rate of application, or the type and duration of the effects of these compounds are considered. Almost all the effects observed were either stimulatory or reversible. These results do not preclude any potential effects of trifluralin or metabolites on specific microorganisms or groups of microorganisms, such as the reduction of soil fungal populations reported by Breazeale and Camper (1970). However, if specific populations were affected by these compounds they were not highly active in the decomposition process, or other microbial groups may have compensated for the loss of activity. These findings provide evidence that the dinitroaniline herbicides and their breakdown products do not cause significant inhibition of decomposition processes in soil, and suggest that the concurrent transformation of plant nutrients is not impeded.

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