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Effects of Two Acetanilide Herbicides on Microbial Populations and Their Cellulolytic Activities

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Organic matter decomposition is one of the most important processes occurring in soil. Organic matter is decomposed in the soil primarily through microbial processes. The importance of the microflora responsible for this degradation is considerable because their activity determines the accumulation of plant debris on the soil surface. One of the side effects of herbicide application is the delay in cellulose decomposition, an important process in the degradation of organic matter in general.

The acetanilide herbicides alachlor, metolachlor and propachlor are registered for control of most annual grasses and certain broadleaf weeds in many crops such as corn (*Zea mays* L.), soybean (*Glycine max* [L.] Merr.), peanut (*Arachis hypogaea* L.), and rice (*Oryza sativa* L.). In Malaysia, alachlor and metolachlor are used for weed control in peanut crops, while propachlor is used for control of grasses in rice fields.

Acetanilide herbicides are degraded quickly by soil microbes; their half-lives are relatively short. Zimdahl and Clark (1982) reported that the half-lives of alachlor and metolachlor at 50% field capacity were 8 and 12 days, respectively. However, the persistence of these herbicides is dependent on many factors (Beestman and Deming 1974). Their effect on cellulose decomposition has never been reported, unlike such effects of other herbicides such as paraquat, aminotriazole and glyphosate (Grossbard and Wingfield 1978) and asulam (Wingfield 1980).

The determination of enzymes involved in stages of litter decomposition is significant for assessing the degradation potential of soils. Recording the enzyme activities is also of interest for assessing the influence of fertilizers and agrochemicals on the metabolic properties of the soil (Schinner et al. 1980; Schinner and Mersi 1990). For instance, a previous report has shown that cellulase activity was reduced in soil treated with herbicide (Cole 1976).

This study was conducted to investigate the effects of two

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acetanilide herbicides viz. alachlor and metolachlor on the population of cellulose-decomposing bacteria and fungi and also on their cellulolytic activities.

MATERIALS AND METHODS

The soil used was taken from the experimental plot of Universiti Kebangsaan Malaysia, Bangi, Selangor. The soil was a clay loam containing 45% sand, 35% silt, 20% clay, 0.37% organic carbon and a pH of 5.9. The soil was sifted through a 3-mm sieve and placed in black polyethylene bags before use. The cellulosic material (100% cotton yarns) used as substrate was obtained from British Textile Technology Group. This substrate was specially made for soil burial tests. Herbicides tested were alachlor as Lasso™ 2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide (Monsanto Malaysia): active ingredient 480 g/L (w/v); and metolachlor as Dual™ 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide (Ciba-Geigy): active ingredient 720 g/L (w/v).

A single layer of the substrate was sprayed on both sides with the herbicide at rates equivalent to 0, 1.12 and 4.48 kg/ha for alachlor, and 1.08 and 4.32 kg/ha for metolachlor at spraying volume 45 mL/m^2. For untreated control, substrate was sprayed with an equal volume of water. The concentrations used were at the recommended and at four times the recommended rate. The substrate was then cut into strips 2 cm x 11 cm and each one was mounted onto a glass slide for burial in soil as described by Greaves et al. (1978). Five substrate-covered slides of each concentration for each herbicide were separately placed sideways in the soil in a box. The box was filled with more moist clay loam soil using, a glass rod to make the soil firm between the slides. This ensured good contact between the soil and the substrate. For another set, similar treatment was given to the substrates, but instead of burying they were placed directly on the soil surface. Ten replicate strips of substrate placed in two boxes of soil were prepared for each treatment. The boxes were then placed in polyethylene bags which were secured with elastic bands and then inflated using a compressed air supply. Each box was weighed and incubated at 27 C for up to 6 wk. The moisture content of the treated and control soils was adjusted to 50% of the field capacity.

In another set of experiments, each herbicide was mixed thoroughly with the soil to give final concentrations of 0, 20 and 150 ppm for either alachlor or metolachlor, respectively, on dry weight basis. For control, the soil was sprayed with distilled water. Untreated substrate was then buried in the treated soil either immediately or after the soil had been kept for 4 wk at ambient temperature. Another substrate was then buried and incubation continued for 6 wk longer before weight loss was determined. The moisture content of the soil was checked weekly and maintained at 50% field capacity during the incubation period. After 6 wk's incubation, the slide-mounted substrate was carefully

removed from the soil and soil particles gently removed from the substrate using a small artist's brush. The weight loss was determined and expressed as a percentage of the weight of an identical piece of substrate which had not been buried. Data presented were an average of five replications for each concentration of each herbicide.

After weighing, about 0.3 g of the substrate was transferred to a flask which contained 15 mL sterilized acetate buffer (20 mM, pH 5.0). The flask was shaken at 150 rpm for 15 min. Ten mL of the sample was then centrifuged at 10,000 rpm at 4 C (Jouan MR 14.11) for 15 min. Subsequently, the supernatant was used for measurement of CM-cellulase activities.

Measurement of CM-cellulase activities was based on the Nelson-Somogyi's methods as modified by Wood and McCrae (1977). About 1.0 mL of 1% CM-cellulose and acetate buffer (0.5 mL) were mixed together. The above supernatant (0.5 mL) was thoroughly mixed with the prepared solution and then incubated in a waterbath at 37 C for 1 hr. The reaction was stopped by the addition of 2 mL of the mixture of Somogyi I and II reagent (Wood and McCrae 1977). The solution was boiled in a waterbath for 15 min, then cooled before adding 2 mL Nelson reagent. Then the mixture was shaken for 2 min using vortex mixture. Finally, 4 mL of water was added before centrifuging the mixture at 1500 rpm for 5 min. The absorbance of the solution was read at 520 nm by using a spectrophotometer model Hitachi 2000. A standard curve was obtained by plotting different amounts of glucose against absorbance.

A flat land situated at the experimental plot of the Faculty of Life Sciences, Universiti Kebangsaan Malaysia, was chosen for microbial populations studies. The plot was originally covered with grasses and had no previous history of pesticide use. It was divided into ten 1-m^2 sub-plots, each separated by a 0.5 m buffer zone. Spraying volume was equivalent to 450 L/ha. The experimental design was a randomized complete block with three replications. The herbicides were applied to the soil separately at 1.12 and 4.48 kg/ha for alachlor and 1.08 and 4.32 kg/ha for metolachlor. Control plots were sprayed with an equal volume of water. The first samples were taken before treatment (referred to as day 0), and days 1, 2, 7, 16, 26, and 35. Four composite samples to a depth of 5 cm were collected from each plot and mixed thoroughly. Soil suspensions were prepared by homogenizing 5 g in 50 mL of quarter-strength Ringer solution for 15 min at 300 rpm. A series of ten-fold dilutions between 10^{-3} and 10^{-5} of the suspension was made with sterile Ringer solution. Each dilution was gently agitated throughout the plating procedure for 15 min. A preliminary experiment indicated that 10^{-4} dilution was suitable for the study and therefore, this dilution was used throughout for enumerating cellulolytic bacterial populations. About 0.1 mL of this suspension was transferred to each of five petri dishes containing Skinner's cellulose medium B (Skinner 1960) with the addition of nystatin and cyclohexamide at 50 ug/mL each for suppression of fungi. For cellulolytic fungi, the above dilutions

were transferred onto Mandel's agar medium (Grossbard and Wingfield 1975) with the addition of streptomycin (30 ug/mL) and Rose Bengal (50 ug/mL) to avoid bacterial growth. All dishes were incubated at 25 C, following which the colonies of cellulolytic fungi and bacteria were counted after 3 days' incubation.

RESULTS AND DISCUSSION

The rate of cellulose decomposition and the interactions between cellulose, microflora and herbicide varied according to whether the substrate was buried in the soil or incubated on the soil surface. A significant reduction in cellulose decomposition in the soil was observed when the substrate had been treated with either alachlor or metolachlor before burial (Table 1). These results showed that both herbicides exerted an inhibitory effect on cellulose decomposition, especially at the higher rates. At four times the recommended rate (4.48 kg/ha), alachlor reduced cellulose decomposition by 33% of control. The reduction was slightly higher (40% of control) with metolachlor at 4.43 kg/ha. However, neither herbicide affected the decomposition significantly when substrates were placed on the soil surface. At the soil surface, there is less contact between substrate and soil, thus reducing colonization of the substrate by soil microorganisms. This observation was similar to that reported by Grossbard and Wingfield (1978), who also found that cellulose was less degraded when placed on the soil surface.

The effects of alachlor and metolachlor on the decomposition of cellulosic materials buried immediately in treated soil are shown in Table 2. Substrate buried in treated soil with either alachlor or metolachlor had reduced decomposition of cellulosic materials. Metolachlor was found to cause greater inhibition of cellulose degradation than alachlor. The results indicate that with increasing concentrations, both herbicides increasingly reduced the decomposition of the substrate in the treated soil. Treatment of soil with 150 ppm of either alachlor or metolachlor caused a reduction in the weight loss by 63 and 53% of control, respectively.

Incubation of the soil for 4 wk before burial of the substrate partially alleviated inhibitory effects of the herbicide on decomposition. The decomposition rate was 61% of the control at 150 ppm of either herbicide. Factors such as breakdown or adsorption of herbicide during the 4 wk's incubation period prior to the addition of the substrate may account for the decreased effects of the herbicides. When alachlor was used at 150 ppm, the rate of decomposition of cellulosic material doubled in preincubated soil as compared to those buried in soil immediately after treatment. Similarly, decomposition rate in metolachlor-treated soil increased slightly as compared to non-preincubated soil.

The results of this study showed that metolachlor had deleterious effects on cellulose decomposition when applied either directly

Table 1. Effects of alachlor and metolachlor on the weight loss of cellulose materials incubated on the soil surface or buried.

Herbicide (kg/ha)	Control 0	Alachlor 1.12	Alachlor 4.48	Metolachlor 1.08	Metolachlor 4.32
Buried	33±1.3	23±3.0	22±1.6	27±2.3	20±1.6
Surface	33±0.8	33±1.3	32±1.7	30±2.2	32±3.3

(% weight loss, ±S.E).

Table 2. Effects of alachlor and metolachlor on decomposition of cellulosic material which were buried in soil immediately after treatment of soil or preincubated for 4 wk.

Herbicides (ppm)	Control 0	Alachlor 20	Alachlor 150	Metolachlor 20	Metolachlor 150
No incubation	19±2.2	14±1.6	7±2.4	9±1.3	9±0.7
Incubated	23±1.6	21±1.4	14±1.7	22±1.6	14±1.9

The values represent weight loss as % of initial dry weight (±S.E.).

to the substrate or to the soil. Metolachlor showed a marked effect at concentrations higher than the recommended rate. The results clearly show that the differing characteristics of the two herbicides have a marked effect on cellulose decomposition. The known antifungal action of metolachlor probably explains, in part, the inhibitory effect on the degradation of cellulose. In soil, metolachlor was more persistent than alachlor (Zimdahl and Clark 1982), and was also slightly but significantly more toxic to microbes and plants (barnyardgrass) than alachlor (Weber and Peter 1982). On the other hand, alachlor is more readily degraded by soil microorganisms, which reduces its residual activity (Smith and Phillips 1975). It is also readily adsorbed on soils with higher clay and organic matter content, which may reduce its effect on soil microorganisms (Rahman et al. 1978). Therefore, it was less toxic to soil microbes than metolachlor.

The decrease of cellulose decomposition could be related to the decrease of activity of cellulase, one of the important enzymes produced by cellulolytic microorganisms. CM-cellulase on substrate buried in soil treated with either herbicide is not reported here. Metolachlor reduced CM-cellulase activity significantly on treated substrate with increasing concentrations when buried in soil (Table 3). Alachlor also showed reduced CM-cellulase activity on buried substrate but not as much as the reduction shown with metolachlor. The decreased CM-cellulase activity may reflect the decrease of cellulolytic microorganisms in the presence of herbicides. The activity of CM-cellulase extracted from substrate placed on soil surface was less affected. The presence of herbicide residue on the substrate may reduce colonization by cellulolytic microorganisms.

Table 3. Effect of alachlor and metolachlor on CM-cellulase activity (Ux10^{-3}/mL) of the treated substrate (±S.E.)[a]

Herbicide (kg/ha)	Control 0	Alachlor 1.12	Alachlor 4.48	Metolachlor 1.08	Metolachlor 4.32
Buried	3.5±0.6	3.2±0.3	3.0±0.4	1.8±0.2	1.0±0.2
Soil surface	4.1±0.8	3.4±0.1	2.7±0.6	2.4±0.2	2.7±0.7

[a]1U = lumol/min of glucose equivalent released.

Table 4. Effect of alachlor and metolachlor on the numbers of cellulolytic fungi (x 10^4/g dry soil) and cellulolytic bacteria (x 10^5/g dry soil) in a clay loam soil.

Herbicides (kg/ha)	Days of sampling 0	1	2	7	16	26	35
				Fungi			
Control	12	3	8	9	13	13	37
Alachlor							
1.12		3	3	7	14	13	46
4.48		3	4	10	15	9	30
Metolachlor							
1.08		2	7	8	13	10	24
4.32		2	7	6	10	8	24
S.E.	±1.2	±0.1	±1.3	±1.3	±1.5	±1.4	±2.6
				Bacteria			
Control	10	5	5	6	3	4	5
Alachlor							
1.12		8	12	8	2	5	3
4.48		7	11	4	2	1	1
Metolachlor							
1.08		1	2	5	3	3	5
4.32		1	1	3	2	4	2
S.E.	±1.2	±1.3	±1.3	±1.1	±1.1	±1.2	±1.2

The application of herbicide to soil may change both quantitatively and qualitatively the microbial populations in the soil (Camper et al. 1973). The results of this study show that microbial numbers in soil treated with alachlor decreased during day-1 and day-2 after treatment but increased during the subsequent sampling days (Table 4). It should be noted that the changes in microbial numbers occurred infrequently and randomly during the experiment. Often control data fluctuated widely with time possibly due to environmental factors such as soil temperature and moisture. Metolachlor inhibited more cellulolytic bacteria than cellulolytic fungi.

A significant decrease in the number of cellulolytic bacteria in metolachlor-treated soil was observed at the highest rate of application. In contrast, fungal numbers recovered in

metolachlor-treated soil on day-16, but cellulolytic bacteria did not. The decrease in cellulolytic propagules may have occurred as a result of the death of certain species, leaving resistant strains to dominate the population. These strains could then increase steadily after a few days of treatment. The results obtained in this study agree with the observation of Raju and Rangaswari (1971), who found that herbicides such as atrazine caused a temporary decline in soil microbial numbers. However, this phenomenon depends mainly on the residual activity of the herbicide applied. In this case, metolachlor was found to be more persistent than alachlor (Zimdahl and Clark 1982), and also had a more noticeable and permanent effect on microbial numbers.

The results of this study showed that alachlor and metolachlor affected the number of cellulolytic microorganisms and their ability to colonize the substrate. The reduction of these organisms may affect the production of CM-cellulase, which is important for the decomposition of cellulosic materials or organic matter in general in the soil. However, these herbicides seem only to delay the decomposition process, which will recover when there is no residual activity in the soil. After further incubation for 12 wk, alachlor or metolachlor in soil did not affect significantly the decomposition rate of cellulolysic materials (data not shown). This indicated that the reduction in cellulose degradation is likely to be a temporary effect. Whether the same phenomenon occurs in the field is yet to be verified.

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