JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Metabolism of Metolachlor by Fungal Cultures

D. SANYAL† AND G. KULSHRESTHA*,‡

Analytical Division, Institute of Pesticide Formulation Technology, Sector 20, Udyog Vihar, Gurgaon 122016, India, and Division of Agricultural Chemicals, Indian Agricultural Research Institute, Pusa Campus, New Delhi 110012

Metabolism of metolachlor was studied using a mixed fungal culture isolated from a metolachlor-acclimated field soil. The culture rapidly degraded metolachlor with a half-life of 3.5 days in broth. *Aspergillus flavus* and *A. terricola* purified from the mixed culture also metabolized metolachlor effectively. Five metabolites obtained were identified by co-chromatography on HPLC by comparing with authentic standards and by GC−MS. Hydrolytic dechlorination, N-dealkylation, and amide bond cleavage appeared to be the dominant transformations involved in the metabolism. Metabolites, 6-methyl 2-ethyl acetanilide and 6-methyl 2-ethyl aniline, identified in this study are new metabolites of metolachlor being reported from any mixed or pure microbial cultures. The mixed culture could degrade 99% of metolachlor at a fortification level as high as 100 μg mL^{-1}.

KEYWORDS: Mixed fungal culture; *Aspergillus flavus; Aspergillus terricola;* metabolites; half-life

INTRODUCTION

The biological decontamination of pesticide wastes or spills has become an increasingly important area of research, and it is desirable if a microbial or biological method of degradation is available for the various pesticidal compounds. Metolachlor [2-chloro-*N*-(2-methoxy-1-methylethyl)-2′-ethyl-6′-methyl acetanilide] is a selective preemergence herbicide used extensively for the control of annual grass weeds, broadleaf weeds, and yellow nutsedge common in corn, soybean, sunflower, peanut, and vegetables (*1*). It is more persistent in the soil environment than the other acetanilide herbicides, alachlor and propachlor (*2, 3*). It has the potential to leach to groundwater because of its relatively high water solubility (550 mg L^{-1} at 20 °C) and low sorption to soil particles (*4*). Additionally, the data on bioaccumulation of metolachlor, particularly in edible fish species, have raised concern about its safety to human health (*5, 6*).

Several studies have confirmed that microbial transformations are the essential mechanisms responsible for the degradation of metolachlor (*7*−*10*). Microorganisms capable of metabolizing it have been isolated to elucidate degradation using mixed and pure cultures.

Fungal metabolism of metolachlor, using resting cells of *Chaetomium globosum* was first reported by McGahen and Tiedje (*8*) who found 45% disappearance of metolachlor after 144 h of incubation. Several metolachlor metabolites, which indicated removal of chloro, methoxy, or ethoxy substituents from the *N*-alkyl groups and subsequent hydroxylation at that position, were identified. Metolachlor was completely degraded in 16 days by an actinomycete isolated from metolachlor-treated soil (*7*) in the presence of sucrose and yeast extract, indicating an inability to utilize metolachlor as a sole source of carbon. The degradation involved benzylic hydroxylation of the aryl alkyl side chains and/or demethylation at the *N*-alkyl substituent to form diasteriomeric isomers of 2-chloro-*N*-[2-(1-hydroxymethyl)-6-methylphenyl]-*N*-(2-methoxy-1-methylethyl) acetamide and 2-chloro-*N*-(2-ethyl-6-hydroxy-methyphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide as major metabolites. In a study by Saxena et al. (*11*), *Bacillus circulans, B. megaterium, Fusarium sp., Mucor racemosus,* and an actinomycete were able to degrade metolachlor and formed hydroxylated products similar to those reported by Kruase et al. (*7*). Microorganisms such as *Rhizopus, Actinomyces, Streptomyces,* and bacteria isolated from a soil previously treated with metolachlor for 5 years, were able to extensively degrade the herbicide but were not capable of mineralizing it (*9*).

Various dechlorinated products were also identified from mixed bacterial community (*12*), *Streptomyces* sp., *Phanerochaete chrysosporium, Rhizoctonia praticola,* and *Syncephalastrum racemosum* (*13*). Liu et al. (*14*) and Libra et al. (*15*) reported the degradation of metolachlor by the white-rot fungus *P. chrysosporium*. The predominant metabolites formed were the same as those reported by other workers (*8, 13*). Metolachlor was degraded to 99% within 48 h by the fungus *Cunninghamella elegans* (*16*). Six metabolites were identified comprising 81% of the total metobolized ^{14}C-metolachlor.

In this study, we report the metabolism of metolachlor by a mixed fungal culture isolated from a metolachlor-acclimated field soil.

MATERIALS AND METHODS

Chemicals. Metolachlor (I, 99.0%, analytical grade) and its authentic metabolites [viz., 6-methyl 2-ethyl-acetanilide (II), 2-chloro 6′-methyl

* Corresponding author. Fax: +91 011 576 6420. E-mail: gkagch@iari.ernet.in.
† Institute of Pesticide Formulation Technology.
‡ Indian Agricultural Research Institute.

10.1021/jf010850d CCC: $22.00
Published on Web 01/04/2002

2′-ethyl-acetanilide (III), 6-methyl 2-ethyl aniline (IV), 2-hydroxy 6′-methyl 2′-ethyl-*N*-(2-methoxy-1-methylethyl) acetanilide (V), and 6′-methyl (2′-ethyl-*N*-(2-methoxy-1-methylethyl) aniline (VI)] were provided by M/s Gharda Chemicals Ltd. (Dombivilly, India) (shown later in **Figure 5**).

High-performance liquid chromatography (HPLC) grade solvents were purchased from E-Merck (India) Co. All other chemicals were of reagent grade and of highest purity available.

Microorganisms and Growth Condition. A mixed fungal culture was isolated, by enrichment culture technique, from a field soil receiving metolachlor application repeatedly for four times in a span of eight months which included two cropping seasons (*17*). Soil samples were collected randomly from 10 to 12 points at the termination of the field experiment. The culture was isolated from a representative soil sample in a mineral salt medium containing 3.0 g of sodium nitrate, 1.0 g of dipotassium hydrogen phosphate, 0.5 g of magnesium sulfate, 30.0 g of sucrose, and 20 mg of metolachlor dissolved in 1 L of distilled water.

Identification of Microorganisms. The mixed fungal culture was found to be a mixture of two pure cultures which were then purified by spread-plate technique (*18*). They were identified from their morphological details (*19*) as *Aspergillus flavus* and *Aspergillus terricola*. *A. flavus* is a soil-borne fungus abundantly found in soil. The conidial heads of the fungus were yellow and quickly changed to a dark-yellow green shade, and finally changed to deep grape green on aging with the reverse being drab pink. *A. terricola* was at first white in color, later becoming yellow with the formation of conidia, and finally turned old gold to brown shade with the reverse having no color. They had very long conidiophores (1 mm). The chloroform extract of the fungal isolate *A. flavus* did not show any fluorescence on the TLC plates when exposed under UV light, thus confirming the absence of any mycotoxin, namely B_1, B_2, G_1, and G_2 in this isolate.

Degradation Rate Kinetics of Metolachlor. A rate kinetics study was carried out separately using mixed fungal culture and their pure isolates, *Aspergillus flavus* and *A. terricola*, at 20 μg mL^{-1} metolachlor concentration.

Metolachlor (5 mg), in a 0.5-mL acetone solution, was introduced to a sterilized flask (500 mL) containing sterile mineral salt broth (250 mL). Inoculum of conidial suspension (5 mL) from actively growing culture was added aseptically to the flask. The flask was swirled for even distribution of the organisms in the broth. The flasks in triplicate were incubated at stationary condition at 28 ± 1 °C in dark. At regular intervals of time, a 20-mL portion of the growth medium was processed for detecting the presence of the parent compound and its metabolites by using HPLC and GC−MS. The broth samples were centrifuged at 10000*g* for 20 min, and the supernatant was partitioned with an equal volume of hexane/ethyl acetate (4:1, 3 × 20 mL) after saturation with sodium chloride in a 125-mL separatory funnel. The organic phase was passed through anhydrous sodium sulfate and evaporated to dryness on a rotary vacuum evaporator, followed by dissolution in 2 mL of methanol (HPLC grade). The extract was then passed through a PTFE membrane sample filter (30 mm diameter, 0.45 μm pore size) prior to co-chromatography using HPLC. Necessary control samples were also included in the study.

Intermediary Metabolite Formation. To confirm the degradation pathway as postulated for metolachlor by the mixed fungal culture, an experiment was set up in which 10 mL of mineral salt broth was amended with standard solutions (20 μg mL^{-1}) of each of the authentic degradation products (II, III, IV, V, and VI) separately in 50-mL flasks. Spore suspension (0.2 mL) of the actively growing mixed fungal culture was added to each flask and incubated for 7 days at 28 ± 1 °C in the dark. After the conclusion of the incubation period, samples were extracted and analyzed by reversed-phase HPLC to detect the presence of intermediate metabolites formed during the incubation period. Necessary control samples were also included in each case.

Successive Enrichment of the Mixed Fungal Culture. Successive enrichment of the mixed fungal culture was carried out by increasing the herbicide concentration from 20 to 100 μg mL^{-1} of the culture media in six successive increments.

Sterile mineral salt medium (20 mL) was taken in a 50-mL Erlenmeyer flask. Metolachlor (400 μg) in 0.1 mL of acetone solution was introduced to the flask. Actively growing cell suspension (0.2 mL) from the preceding enrichment flask was added as inoculum and swirled for uniform distribution in the medium. The flasks (three replicates) were incubated in dark at 28 ± 1 °C for 20 days. At the conclusion of the incubation period, 0.2 mL of this medium was added as inoculum in the succeeding enrichment flask containing 600 μg of metolachlor in 20 mL of broth. The remaining contents of the flask were extracted and analyzed in manner similar to that mentioned above for the presence of metolachlor residues. Likewise, enrichment up to 100 μg mL^{-1} was achieved in subsequent enrichments. The extraction solvent used in this case was hexane, and 3 μL of the extract was injected into a gas chromatograph (GC).

Control flasks containing either a microbial culture in mineral salt broth without metolachlor, or metolachlor in mineral salt solution without a microbial culture, were also included in the study.

Chemical Analysis. Metolachlor residues were determined by GLC following the method described by Singh et al. (*20*).

Metabolites were identified by co-chromatography, using authentic degradation products by reversed-phase HPLC as described by Sanyal and Kulshrestha (*21*).

Metolachlor and its metabolites were quantified by comparing peak areas of the compound to the peak areas of the reference standard solution using eq 1:

$$\text{Concentration} = \text{area} \times \text{RF} \quad (1)$$

where concentration is the concentration of metolachlor in the unknown sample, area is the peak area of the unknown, and RF (response factor) is the concentration of standard/area of the standard.

Mass spectra of metolachlor and its metabolites were obtained on a mass selective detector (GC model 5890 series, MSD model 5973 series) using a capillary column (DB-5, 30 m × 0.25 mm i.d., 0.25 μ film thickness) operated in splitless mode. Operating temperatures were as follows: injections port, 250 °C; column, programmed from 100 to 250 °C at a rate of 15 °C/min. The source temperature was maintained at 250 °C. Helium was used as the carrier gas (2 mL min^{-1} flow). The mass spectrometer was operated at an ionization potential of 70 eV under electron impact mode.

Table 1. Degradation of Metolachlor in Uninoculated Mineral Salt Broth

interval (days)	average residues[a] (μg mL^{-1})	degradation (%)
0	20.00	-
2	19.83	0.86
5	19.62	1.89
10	19.33	3.36
15	18.77	6.51
20	18.67	6.65
	$Y = 1.3005 - 0.0016X$	
	$r = 0.75$, half-life = 189.8 days	

[a] Average of three replications.

RESULTS AND DISCUSSION

Degradation Rate Kinetics. *Of Metolachlor.* Metolachlor degradation due to its hydrolysis in mineral salt broth at different intervals of time was observed by including uninoculated control samples in the study, and it was found to be 6.65% in 20 days having a half-life of 189.8 days (**Table 1**).

Mixed Fungal Culture. Investigations on the degradation rate kinetics of metolachlor by the mixed fungal culture were carried out to determine its half-life in mineral salt broth.

The amounts of metolachlor recovered from broth supplemented with the herbicide (20 μg mL^{-1}) at different time intervals are presented in **Table 2**. The initial residues of 19.408 ppm dissipated to 16.46, 10.36, 5.12, 2.05, and 0.31 ppm in 2, 5, 9, 15, and 20 days, respectively. In a span of 9 days as much as 73.61% of the added metolachlor was metabolized. This was

Table 2. Degradation of Metolachlor in Mineral Salt Broth by Mixed Fungal Culture

interval (days)	average residues[a] (μg mL^{-1} ± S. D.)	degradation (%)
0	19.41 ± 0.19	-
2	16.46 ± 0.29	15.21
5	10.36 ± 0.40	46.61 (31.4)
9	5.12 ± 0.75	73.61 (27.0)
15	2.05 ± 0.64	89.44 (15.83)
20	0.31 ± 0.09	98.41 (8.97)
	$Y = 2.402 - 0.0859X$	
	$r = 0.979$, half-life = 3.5 days	

[a] Average of three replications (figures in parentheses represent % degradation over the previous figure).

Table 3. Degradation of Metolachlor in Mineral Salt Broth by *Aspergillus flavus*

interval (days)	average residues[a] (μg mL^{-1} ± S. D.)	degradation (%)
0	19.14 ± 0.09	-
7	5.98 ± 0.83	68.76
14	2.58 ± 0.36	86.55 (17.79)
21	0.29 ± 0.05	98.49 (11.94)
	$Y = 2.357 - 0.0833X$	
	$r = 0.979$, half-life = 3.6 days	

[a] Average of three replications (figures in parentheses represent % degradation over the previous figure).

followed by 15.83% degradation in the next 6 days. At the end of the experimental period of 20 days, only 1.59% of the applied herbicide remained in broth, representing 98.41% degradation of metolachlor. The rate of microbial degradation was very rapid up to 5 days, but then declined gradually with the passage of time.

The rate of degradation was found to follow first-order kinetics with a half-life of 3.5 days.

Pure Cultures. Upon purification, the mixed fungal culture could be separated into two pure cultures which were identified as *Aspergillus flavus* and *Aspergillus terricola;* and studies on degradation kinetics of metolachlor in mineral salt medium were then conducted using these pure isolates. The amount of herbicide recovered from broth at 20 μg mL^{-1} metolachlor level at weekly intervals are presented in **Table 3** and **Table 4**.

Aspergillus flavus. The initial residues of 19.14 ppm degraded to 5.98, 2.56, and 0.29 ppm in 7, 14, and 21 days respectively (**Table 3**). During the initial 7 days, the rate of degradation was as high as 68.76% (**Figure 1**). This was followed by 17.79% degradation in the next 7 days. After 21 days of incubation, 98.49% of the added herbicide was degraded by *A. flavus*. The half-life was found to be 3.6 days, which was comparable with the degradation by the mixed fungal community.

Aspergillus terricola. The initial residues of 19.44 ppm degraded to 10.86, 6.38, 1.89 ppm, and below detectable limit, in 7, 14, 21, and 28 days respectively (**Table 4**). The rate of degradation of metolachlor by *A. terricola* was relatively gradual compared to that of *A. flavus* (**Figure 1**). After 7 days of incubation, 44.12% degradation was recorded, representing 10.86 ppm of metolachlor residue. This was followed by 23.04% in the next 7 days. The degradation reached 90.28% after 21 days of incubation, and residues were below detectable level after 28 days. The half-life was found to be 6.4 days.

Table 4. Degradation of Metolachlor in Mineral Salt Broth by *Aspergillus terricola*

interval (days)	average residues[a] (μg mL^{-1} ± S. D.)	degradation (%)
0	19.44 ± 0.10	-
7	10.86 ± 0.80	44.12
14	6.38 ± 0.12	67.16 (23.04)
21	1.89 ± 0.05	90.28 (23.12)
28	BDL[b]	
	$Y = 2.3417 - 0.0467X$	
	$r = 0.978$, half-life = 6.4 days	

[a] Average of three replications (figures in parentheses represent % degradation over the previous figure). [b] BDL, below detectable limit.

It was observed that this fungal isolate in association with *A. flavus* degraded metolachlor much faster than when the isolate was used alone.

Identification of Metabolites. Proof of microbial degradation is obtained only after ascertaining the presence of degradation products. Metolachlor metabolites were quantitatively and qualitatively estimated in broth inoculated with mixed as well as that inoculated with their pure isolates. The metabolites formed were identified by co-chromatography using HPLC technique by comparing results with the authentic standards and GC−MS results.

Mixed Fungal Culture. The amounts recovered from broth treated at 20 μg mL^{-1} level at different time intervals are presented in **Figure 2** and the mass fragmentation patterns of the metabolites are presented in **Table 5**.

It was found that, with the passage of time, the mixed fungal culture metabolized metolachlor into various degradation products.

The metabolites started forming from the second day (**Figure 2**) of incubation when the dechlorinated product, namely, 2-hydroxy 6′-methyl 2′-ethyl *N*-(2-methoxy 1-methylethyl) acetanilide (V, 0.33 ppm) and the corresponding aniline 6-methyl 2-ethyl aniline (IV, 0.42 ppm) were recovered. By the fifth day, all the four metabolites, dechlorinated product (V, 1.72 ppm), 2-chloro-6′-methyl 2′-ethyl acetanilide (III, 0.51 ppm), 6-methyl 2-ethyl acetanilide (II, 0.02 ppm), and aniline (IV, 0.07 ppm) were recovered from broth. Thereafter, all the four metabolites were constantly present during the entire experimental period at various proportions. The concentration of the dechlorinated product increased at each successive interval up to 15 days and later declined to 0.72 μg mL^{-1} on the 20th day. A similar trend was also observed for 2-chloro 6′-methyl 2′-ethyl acetanilide (III) where its concentration increased in broth from 5 to 15 days and declined thereafter. Concentration of 6-methyl 2-ethyl acetanilide (II) showed an increasing trend from 5 (0.02 ppm) to 9 days (0.64 ppm), a later declined to 0.07 ppm after 15 days of incubation, and remained constant till the end of the experiment. The concentration of aniline also kept declining from 9 days of incubation to 20 days. The declining trend in the concentration of all the four metabolites, II, III, IV, and V, indicated their further transformation to other metabolites. All the four metabolites formed were major in nature.

GC−MS analysis of the sample gave 7 peaks at R_t 4.531, 6.509, 7.277, 8.42, 9.219, 9.854, and 12.80 min. Peaks at R_t 8.42 and 12.80 min were also present in control samples and

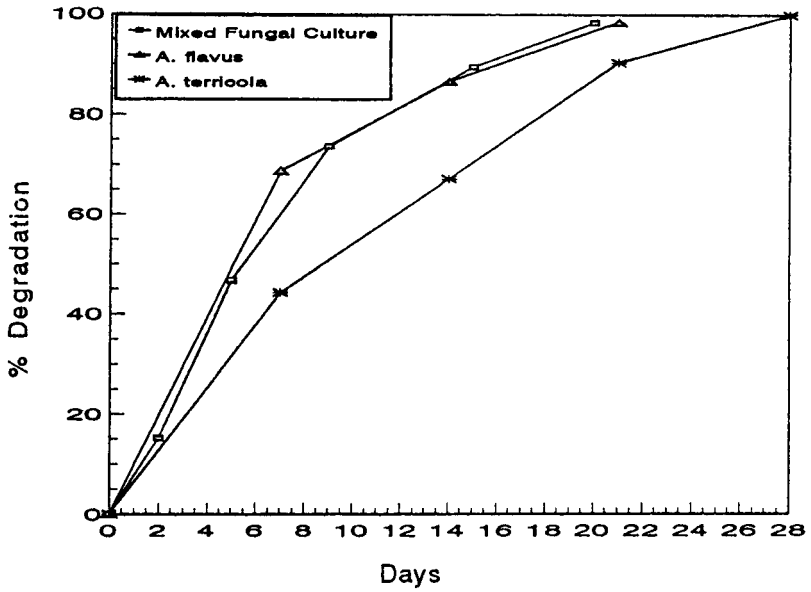


Figure 1. Metolachlor degradation by the fungal culture isolated from soil.

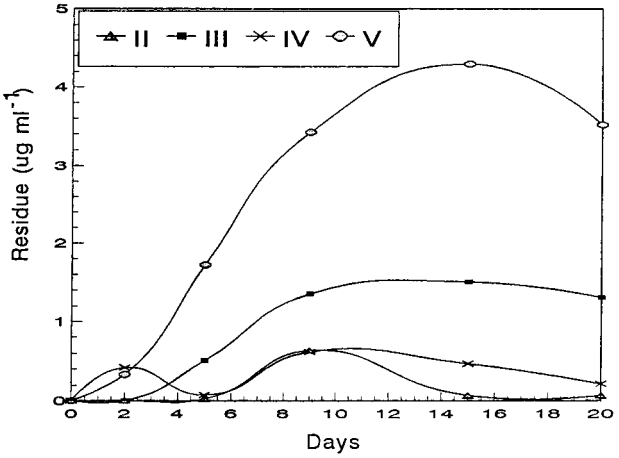


Figure 2. Metolachlor metabolites formed by mixed fungal culture.

thus excluded from the study. Peaks at 4.531, 6.509, 7.277, and 9.219 min represented metabolites IV, II, III, and metolachlor (I), respectively. Thus the compound eluting at 9.854 min was assigned the number VII. The identity of this compound was tentatively assigned as 2-chloro-*N*-(2-hydroxyl-1-methylethyl) 6′-methyl 2′-ethyl acetanilide (VII) after examining its fragmentation pattern (**Table 5**). Krause et al. (*7*) earlier reported this metabolite from an actinomycete isolated from metolachlor-treated soil.

Pure Cultures. Metabolites formed during the study with the pure isolates of *A. flavus* and *A. terricola* in liquid media treated at 20 μg mL^{-1} metolachlor level at weekly intervals are presented in **Figures 3** and **4**.

Aspergillus flavus. In the first week, three major metabolites formed were identified as 2-hydroxy-6′-methyl 2′-ethyl-*N*-(2-methoxy-1-methylethyl) acetanilide (V), 2-chloro-6′-methyl 2′-ethyl acetanilide (III), and 6-methyl 2-ethyl aniline (IV) (**Figure 3**). The highest concentration was that of dechlorinated metabolite (V, 3.29 ppm) followed by chloro-acetanilide product (III, 1.324 ppm). In the following two weeks, the presence of the fourth major metabolite, 6-methyl 2-ethyl acetanilide (II), was also detected in addition to the three metabolites mentioned above.

Aspergillus terricola. In the first week, three major metabolites, viz., III, IV, and V, were formed. The concentration of dechlorinated product (V) was found to be highest (0.649 ppm), followed by 2-chloro-6′-methyl 2′-ethyl acetanilide (III, 0.216 ppm), and 6-methyl 2-ethyl aniline (IV, 0.11 ppm) as was found in case of *A. flavus*. In the following weeks also, all the three metabolites were detected.

The concentration of dechlorinated product (V) showed a declining pattern from the second week (**Figure 4**). Similarly, metabolite III also showed a declining trend from the third week. These observations indicated further degradation of metabolites III and V, which was substantiated by evidence of increasing concentration of aniline metabolite (IV).

The metabolite 6-methyl 2-ethyl acetanilide was not detected in the broth during the entire experimental period.

Intermediary Metabolite Formation. The degradation pathway of metolachlor by mixed fungal culture was postulated by observing the formation of intermediary metabolites in each culture flask containing standard authentic metabolites. The intermediary metabolites formed during the experiment are presented in **Table 6**.

In all cases, the parent molecule was detected in its highest amount in the respective culture flasks, followed by those containing their degradation products. 6-Methyl 2-ethyl aniline (IV) was detected as one of the degradation products in each sample.

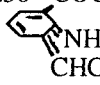
Aniline (IV) metabolized further to some unknown metabolite which was major in nature. Due to the unavailability of an authentic standard and lack of sensitivity of the compound in GC−MS, the identity of this metabolite could not be established either by HPLC or GC−MS.

Substituted aniline (VI) was also further degraded, other than aniline (IV), to an unknown metabolite eluting in HPLC at the same retention time as that formed during aniline degradation.

Therefore, by combining the findings of this experiment with the metabolites formed during the metabolism study of metolachlor using a mixed fungal culture, a possible degradation pathway of metolachlor was deduced, which is presented in **Figure 5**.

Comparison of degradation patterns of metolachlor by the mixed and pure cultures revealed that there was no antagonistic effect of one fungal isolate over the other when present in the mixed form. Though the difference in the rate of degradation by the mixed fungal culture and *A. flavus* was almost negligible,

Table 5. GC Retention Time and Mass Fragmentation Pattern of Various Microbial Metabolites of Metolachlor

Compound	R_t (min)	Mass spectra m/z (ion fragment, RI*)
I	9.219	**238** (M^+ - CH_2OCH_3, 38); **211** (M^+ - CH_3–CH=CH-OCH_3, 7); **162** (238 - $COCH_2Cl$, 100); **142**(162 -CH_3, 18); **134** ( , 6); **91** (134 – N, C_2H_5, 18); **77** (^+CO-CH_2Cl, 20); **73**(-$CHCH_2OCH_3$, 9); **45** (-CH_2OCH_3, 58)
II	6.509	**177** (M^+, 24), **162** (M^+-CH_3, 6); **134** (162 – CO, 49); **120** (134-N, 100); **91** (120 – C_2H_5, 29)
III	7.277	**211** (M^+, 11); **162** (M^+ - CH_2Cl, 100); **134** (162 – CO, 16); **91** (134 – N, C_2H_5, 28); **77** (-$^+COCH_2Cl$, 33)
IV	4.531	**134** (M^+, 27); **120** (M^+ - N, 100); **91** (120 – C_2H_5, 21); **77** (120 – CH_3, 19)
VII	9.854	**269** (M^+, 2); **238** (M^+ - CH_2OH, 42); **211** (M^+ -CH_3–CH=CH-OH, 5); **162** (238 – $COCH_2Cl$, 100); **134** ( , 8); **91** (134 – N, C_2H_5, 36); **77** (-CO-CH_2Cl, 41); **59** (-CH_3–CH – CH_2OH, 14)

*RI = Relative intensity

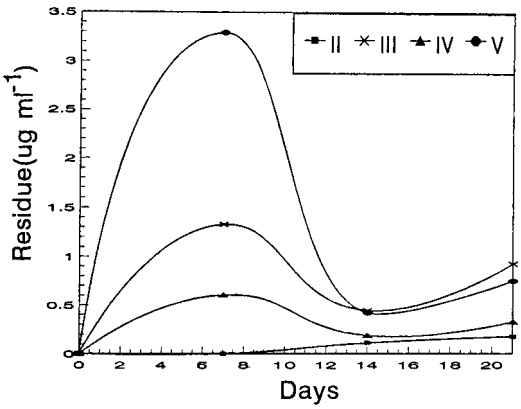


Figure 3. Metolachlor metabolites formed by *Aspergillus flavus*.

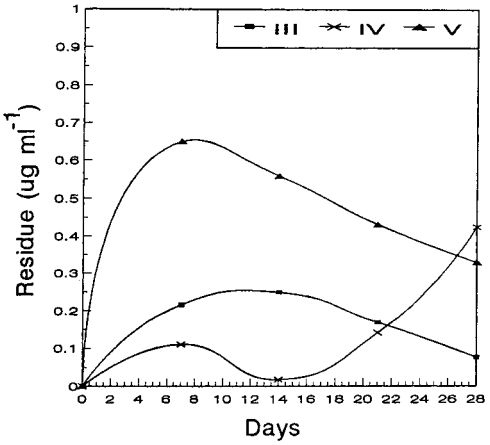


Figure 4. Metolachlor metabolites formed by *Aspergillus terricola*.

the same was not true for *A. terricola*, where degradation was much slower (half-life 6.4 days) as compared to that of the mixed fungal culture (half-life 3.5 days). In other words, it can be concluded that the presence of *A. flavus* in the mixed culture could enhance the rate of degradation of metolachlor.

It was concluded from the study that hydrolytic dechlorination, *N*-dealkylation, and amide bond cleavage appear to be the principal mechanisms involved in microbial degradation of metolachlor.

Metabolite V [2-hydroxy-6′-methyl-2′-ethyl-*N*-(2-methoxy-1-methylethyl) acetanilide] formed during the degradation of metolachlor has been reported in the metabolism study of metolachlor by few fungi and actinomycete (*13*). McGahen and Tiedje (*8*) and Liu et al. (*13*) have reported the degradation of metolachlor into 2-chloro 6′-methyl 2′-ethyl acetanilide (III) by fungi and actinomycete. Metabolite VII has been reported earlier by Krause et al. (*7*), McGahen and Tiedje (*8*), and Pothuluri et al. (*16*).

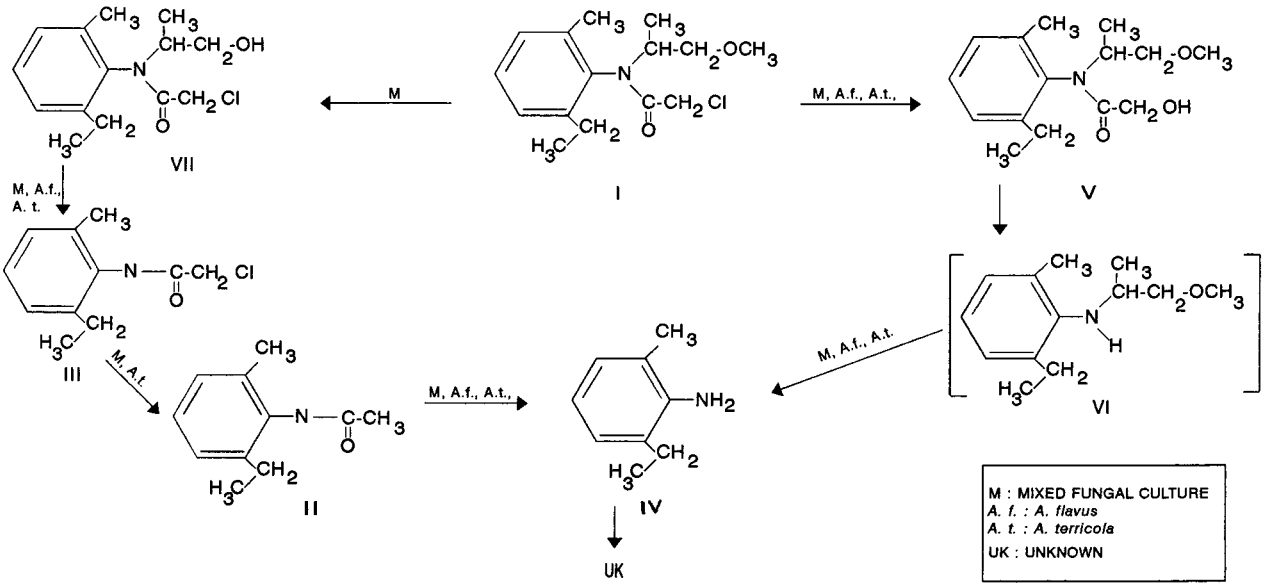


Figure 5. Postulated microbial metabolism of metolachlor by the mixed fungal culture.

Table 6. Intermediary Metabolites Formed by Mixed Fungal Culture in Mineral Salt Medium

amended compound	amount recovered (μg mL^{-1})[a]					
	II	III	IV	V	VI	unknown[b]
II	3.867		0.018			+, major
III	0.229	11.497	0.045			
IV			0.280			+, major
V			0.311	3.169	0.213	+, major
VI			0.043		0.512	+, major

[a] +, Present. [b] Major/minor: Major/minor metabolites with respect to peak area in HPLC.

Table 7. Percent Degradation of Metolachlor in 20 Days in Successive Enrichment by the Mixed Fungal Culture

enrichment	initial concentration (μg mL^{-1})	average residue[a] (μg mL^{-1})	degradation (%)
first	20	0.308	98.41
second	30	0.327	98.91
third	40	1.391	96.52
fourth	60	0.077	99.87
fifth	80	0.021	99.97
sixth	100	0.085	99.92

[a] Average of the three replicates.

Therefore, metabolites II (6-methyl 2-ethyl acetanilide) and IV (6-methyl 2-ethyl aniline) identified in the present investigation are new metabolites being reported from the mixed and pure fungal cultures of *A. flavus*.

Successive Enrichment of Mixed Fungal Culture. The percent degradation of metolachlor in mineral salt broth in six successive enrichments by the mixed fungal culture after 20 days of incubation is presented in **Table 7**.

The percent degradation in all the six successive enrichments was found to be more than 96.0%. The average residue was 0.308 μg mL^{-1} when fortified at 20 μg mL^{-1} metolachlor level and was 0.085 μg mL^{-1} when the medium was amended with 100 μg mL^{-1} metolachlor amounting to 98.41 and 99.9% degradation, respectively.

This clearly indicated the generation of an adapted microbial population with an enhanced ability to degrade metolachlor.

ACKNOWLEDGMENT

The authors are grateful to Dr. D. Sengupta (Director, IPFT, Gurgaon) for providing the GC−MS facility and to Dr. Shashi Lal for fungal identification. We also thank Gharda Chemicals (Dombivilly, Thane) for providing analytical grade herbicide and its metabolites.

LITERATURE CITED

(1) Tomlin, C. Metolachlor. In *The Pesticide Manual*, Tenth edition; Worthing, C. R., Ed.; British Crop Protection Council, Crop Protection Publications: Croydon, UK, 1994; pp 693−694.

(2) Zimdahl, R. L.; Clark, S. K. Degradation of three acetanilide herbicides in soil. *Weed Sci.* **1982**, *30*, 545−548.

(3) Sanyal, D.; Yaduraju, N. T.; Kulshrestha, G. Metolachlor persistence in laboratory and field soils under Indian tropical conditions. *J. Environ. Sci. Health* **2000**, *B 35* (5), 571−583.

(4) U.S. EPA. *Pesticide Fact Book*. U.S. Environmental Protection Agency; U.S. Government Printing Office: Washington, DC, 1988; pp 522−529.

(5) Chesters, G.; Simsiman, G. V.; Levy, J.; Alhajjar, B. J.; Fathulla, R. N; Harkin, J. M. Environmental fate of alachlor and metolachlor. *Rev. Environ. Contam. Toxicol.* **1989**, *110*, 1−74.

(6) U.S. EPA. *Metolachlor: Pesticide Registration Standard*; NTIS No. PB81-123820; U.S. Environmental Protection Agency, U. S. Government Printing Office: Washington, DC, 1980; 183 pp.

(7) Krause, A.; Hancock, W. G.; Minard, R. D.; Freyer, A. J.; Honeycutt, R. C.; Lebaran, H. M.; Paulson, D. L.; Liu, S. Y.; J. B. Bollag. Microbial transformation of the herbicide metolachlor by a soil actinomycete. *J. Agric. Food Chem.* **1985**, *33*, 584−589.

(8) McGahen, L. L.; Tiedje, J. M. Metabolism of two new acylanilide herbicides (H-22234) and dual (metolachlor) by soil fungus, *Chaetomium globosum*. *J. Agric. Food Chem.* **1978**, *26*, 414−419.

(9) Liu, S. Y.; Zhang, R.; Bollag, J. M. Biodegradation of metolachlor in a soil perfusion of experiment. *Biol. Fertil. Soils* **1988**, *5*, 276−281.

(10) Miller, J. L.; Wollum, A. G., III; Weber, J. B. Degradation of carbon-14-Atrazine and carbon-14-metolachlor in soil from four depths. *J. Environ. Qual.* **1997**, *26*: 633−638.

(11) Saxena, A.; Zhang, R.; Bollag, J. M. Microorganisms capable to metabolizing the herbicide metolachlor. *Appl. Environ. Microbiol.* **1987**, *53*, 390−396.

(12) Liu, S. Y.; Zheng, Z.; Zhang, R.; Bollag, J. M. Sorption and metabolism of metolachlor by a bacterial community. *Appl. Environ. Microbiol.* **1989**, *55*, 733−740.
(13) Liu, S. Y.; Freyer, A. J.; Bollag, J. M. Microbial dechlorination of the herbicide metolachlor. *J. Agric. Food Chem.* **1991**, *39*, 631−636.
(14) Liu, D.; Maguire, R. J.; Pacepavicius, G. J.; Aoyana, I.; Okamura, H. Microbial transformation of metolachlor. *Environ. Toxicol. Water Qual.* **1995**, *10*, 249−258.
(15) Libra, J. A.; Ro, K. S.; Chung, K. H.; Chung, Y. C. Pesticides and herbicides. *Water Environ. Res.* **1996**, *68* (4), 564−568.
(16) Pothuluri, J. V.; Evans, F. E.; Doerge, D. R.; Churchwell, M. I.; Cerniglia, C. E. Metabolism of metolachlor by the fungus *Chaetomium globosum*. *Arch. Environ. Contam. Toxicol*. **1997**, *32*, 117−125.
(17) Sanyal, D.; Kulshrestha, G. Effects of repeated metolachlor application on its persistence in field soil and degradation kinetics in mixed microbial cultures. *Biol. Fertil. Soils* **1999**, *30*, 124−131.
(18) Cappuccino, J. G.; Sherman, N. *Microbiology − A Laboratory Manual*. Benjamin/Cummings Publishing Company, Inc.: New York, 1992.
(19) Raper, K. B.; Fennel, D. I. *The Genus Aspergillus*. The Williams and Wilkins Company: Baltimore, MD, 1965; pp 361−415.
(20) Singh, S. B.; Yaduraju, N. T.; Kulshrestha, G. Residues of metolachlor herbicide in soil and potato tubers under Indian tropical conditions. *Bull. Environ. Contam. Toxicol*. **1997**, *59*, 216−221.
(21) Sanyal, D.; Kulshrestha, G. A HPLC method for simultaneous analysis of metolachlor and its predicted transformation products in soil and water. *Ind. J. Weed Sci.* **2000**, *32* (1&2), 12−16.

Received for review July 3, 2001. Revised manuscript received November 5, 2001. Accepted November 5, 2001.

JF010850D