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Degradation of acetochlor by four microbial communities

Jun Xu [a,b], Min Yang [c], Jiayin Dai [a], Hong Cao [a], Canping Pan [b], Xinghui Qiu [a,*], Muqi Xu [a,*]

[a] Institute of Zoology, Chinese Academy of Sciences, Beijing, PR China
[b] College of Science, China Agricultural University, Beijing, PR China
[c] Research Centre of Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, PR China

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Abstract

Four microbial communities capable of degrading acetochlor, designated A, D, E, and J, were obtained from acetochlor-contaminated soil and sludge. Acetochlor at an initial concentration of 55 mg/L was completely degraded by the four mixed cultures after 4 days. At 80 mg/L acetochlor, more than 99% degradation was observed with D, 84% with A and E, and 88% with J after 9 days. There are primary eight strains of bacteria in community A, three in community D, E, and J, respectively. No single isolate was able to degrade acetochlor efficiently. The acetochlor biodegradation products were identified by gas chromatography–mass spectrometry. The probable degradative pathways of acetochlor involved dechlorination, hydroxylation, deethoxymethylation, cyclization, carboxylation, and decarboxylation. Propachlor, alachlor, and metolachlor, which are also the main components of the chloroacetanilide herbicide, could be degraded by the four mixed cultures to some degree. Given the high degradation rates observed here, the four mixed cultures obtained may be useful in the degradation processes of acetochlor.

Keywords: Acetochlor; Degradation; Microbial communities; Bacteria

1. Introduction

The chloroacetamide herbicide acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)-acetamide) is used for preemergence control of annual grasses and small seeded broadleaf weeds in corn and soybean. Acetochlor is a common herbicide used worldwide. Due to low adsorption coefficients, acetochlor is a rather mobile pollutant of the soil, posing a potential danger to the aquatic environment (Lengyel and Földényi, 2003).

In general, chloroacetamide herbicide residues and metabolites are relatively common in watershed and groundwater aquifers in agricultural areas that have a history of herbicide application (Stamper and Tuovinen, 1998). Recent studies proved the presence of acetochlor in groundwater as well as in surface water (Kolpin et al., 1996; Visi et al., 1998). The prevalence of these compounds in the environment has stimulated investigations into the degradation of hazardous substances in water and contaminated soil. The biological decontamination of pesticide wastes or spills has become an increasingly important area of research, and the availability of a microbial or biological method of degradation is generally desirable. Several researchers have emphasized the importance of interacting microbial communities in the degradation of xenobiotic compounds (Alexander, 1981). Synergistically acting organisms were found to be responsible for more rapid mineralization of two organophosphate insecticides (Hsu and Bartha, 1979). However, to date no mixed cultures able to extensively degrade acetochlor have been described in detail. Therefore, the objectives of this study were to (1) isolate and identify the microbial communities able to transform acetochlor, (2) qualify and quantify the biodegradation products of acetochlor, and (3) investigate the degradation of other chloroacetanilide herbicides by the microbial communities.

* Corresponding authors. Fax: +86 10 64807099.
E-mail addresses: qiuxh@ioz.ac.cn (X. Qiu), xumq@ioz.ac.cn (M. Xu).

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2. Methods

2.1. Chemicals

Acetochlor (98.6%) was purchased from Shengyang Kaifa New Technology Co. Ltd. Propachlor (2-chloro-*N*-isopropylacetanilide; 99.5%), metolachlor (2-chloro-2′-ethyl-6′-methyl-*N*-(1-methyl-2-methoxyethyl)-acetanilide; 98.4%), alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide; 99.9%), and 2-ethyl-6-methylaniline (99.5%) were purchased from RdH Laborchemikalien GmbH & Co. KG and Sigma–Aldrich Laborchemikalien GmbH. 2-Chloro-*N*-(2-ethyl-6-methylphenyl) acetamide (CMEPA; 99.0%) was kindly provided by Dr. Randy L. Rose from North Carolina State University.

2.2. Source of samples

Four mixed cultures used in this study were isolated from four different soil and sludge samples (A, D, E, J) contaminated with acetochlor after an enrichment culture of about 3 months. Sample A was collected from the landfill site soil of a pesticide factory that had produced acetochlor for several years. Sample E was from the soil of that factory. Sample D was from the soil in the riverbed near same factory that had been polluted by acetochlor. Sample J was the aerobic sludge from a sewage treatment plant that treats domestic wastewater and industrial wastewater, some of which was discharged from pesticide factories that produce acetochlor. The properties and ambient levels of acetochlor in the four samples are listed in Table 1. Acetochlor was extracted from soil and sludge samples followed by the method of Ye et al. (2002).

2.3. Microorganisms and culture conditions

The four isolated microbial communities were named mixed A, mixed D, mixed E, and mixed J. The cultures were grown in 300-mL Erlenmeyer flasks containing 100 mL liquid mineral salts medium (MSM) with acetochlor. The MSM contained 10 mM phosphate buffer (pH 7.0), 0.25 mM $MgSO_4$, 10 mM NH_4Cl, 5 μM $FeCl_3$, 23 μM disodium EDTA, 25 μM sodium citrate and 0.02% yeast extract (Villarreal et al., 1991). In addition, the MSM contained 1 mL of trace element solution (39.9 mg/L $MnSO_4 \cdot H_2O$, 42.8 mg/L $ZnSO_4 \cdot H_2O$, 34.7 mg/L $(NH_4)_6MO_7O_{24} \cdot 4H_2O$) (Jun et al., 1996). Flasks were incubated in a shaking air bath at 30 °C and 150 rpm in the dark. Transfers consisted of a 1:4 dilution into fresh medium after more than 95% of the acetochlor had been degraded.

Table 1
The properties and the ambient levels of acetochlor in the four samples

	A	D	E		J
pH	7.2	7.1	6.5		7.5
Total N (%)[a]	0.13	0.08	0.09	SV30[c]	35
Org C (%)[b]	2.60	0.99	0.86	SI[d]	95
Acetochlor mg/kg soil	0.94	0.08	17.92		0.93

[a] Total nitrogen content.
[b] Organic carbon content.
[c] Sludge sedimentation volume.
[d] Sludge index.

2.4. Isolation and identification of bacteria

After an enrichment of about three months, mixed cultures were spread on MSM agar plates containing acetochlor (50 mg/L). Plates were incubated at 30 °C for 3–5 days in the dark, at which time individual well-separated colonies were picked out to liquid MSM plus acetochlor (50 mg/L). One milliliter of the growth medium was processed for detecting acetochlor after 5 days; at the same time, cultures were spread on LB agar plates to assess purity. Seventeen strains of bacteria were isolated and purified from the four microbial communities according to colonial morphology and percentage degradation.

The bacteria were identified by sequencing the partial 16S rDNA (Weissburg et al., 1991). A universal primer set for bacteria was used for polymerase chain reaction (PCR) amplification: forward primer (8f: 5′-AGA GTT TGA TCC TGG CTC AG-′3) and reverse primer (1492r: 5′-TAC CTT GTT ACG ACT T-′3). Alignment of the partial 16S rDNA sequences was performed with sequences deposited in the GenBank database.

2.5. Biodegradation assays

Experiments were performed in 300-mL Erlenmeyer flasks containing 100 mL liquid MSM supplemented with acetochlor at a concentration of 55 mg/L or 80 mg/L. The flasks were incubated in a shaking air bath at 30 °C and 150 rpm in the dark. Each experiment was carried out in triplicate, and necessary control samples were included. At regular time intervals, 5 mL of growth medium was processed for detecting the presence of the parent compound and its metabolites by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS).

2.6. Analytical methods

One milliliter of the growth medium was extracted with an equal volume of hexane. This extract was used for quantitative analysis of acetochlor by GC. One milliliter of the growth medium was extracted with an equal volume of ethyl acetate. This extract was used for quantitative analysis by GC–MS of 2-ethyl-6-methylaniline and CMEPA, two metabolites of acetochlor (Istvan, 2000; Ye et al., 2002). Half a milliliter of growth medium was used to monitor growth (OD_{600}). All other 2.5 mL portions were combined with the residual medium at the end of experiment. The combined medium was centrifuged at 1855*g* for 5 min. Half of the supernatant was partitioned with an

equal volume of hexane/ethyl acetate (1:1) or dichloromethane three times after acidification with H_2SO_4 (pH 2.0) and saturation with NaCl (2%) in a 250-mL separatory funnel. The organic phase was passed through anhydrous Na_2SO_4 and evaporated to dryness on a rotary vacuum evaporator, followed by dissolution in 1 mL of hexane/ethyl acetate (1:1) or dichloromethane. This extract was analyzed by GC–MS for detecting the biodegradation products of acetochlor.

A Hewlett–Packard 5890 GC coupled with an ECD detector and DB-1 capillary column (25 m × 0.32 mm i.d., 0.25 μm film thickness). Isotherm elution, injector, detector, and column temperatures were 300 °C, 300 °C, 240 °C, respectively. Samples were injected in split mode with a 5.4:1 split ratio.

An Agilent 6890 GC coupled with an Agilent 5973 MSD, operating in selected ion monitoring (SIM) mode, was used. GC was operated in splitless mode and equipped with a DB-5 MS capillary column (60 m×0.25 mm i.d., 0.25 μm film thickness). The temperature program was 100 °C for 7 min, 20 °C/min to 280 °C 5 min; detector temperature was 280 °C. Helium was used as the carrier gas (1 mL/min). The mass spectrometer was operated at an ionization potential of 70 eV under electron impact mode. Masses of the ions selected for qualification were 77, 91, 120, and 135 for 2-ethyl-6-methylaniline, 91, 120, 162, and 211 for CMEPA and 146, 162, 223, and 269 for acetochlor. For the detection of metabolites, the MS was operated in full-scan mode, scanning from 30 to 350 *m*/*z*. The temperature program was 30 °C 7 min, 5 °C/min to 280 °C 5 min.

2.7. Metabolic versatility

The mixed A, D, E and J communities were tested for their ability to degrade 2-ethyl-6-methylaniline, CMEPA, alachlor, propachlor and metolachlor. The biodegradation assays and analytical methods were similar to those for acetochlor.

3. Results

3.1. Composition of the mixed cultures

Acetochlor was detected in all four soil and sludge samples. After an enrichment of about three months, four mixed cultures were obtained, which were primarily composed of 17 strains of bacteria. There are eight strains in mixed A, three strains in mixed D, E, and J, respectively. The partial 16S rDNA sequences of the 17 strains have been determined. Upon comparison of the partial 16S rDNA sequences obtained from the 17 strains with sequences from the GenBank Database, the highest degrees of similarity were obtained (Table 2). The alignment results showed that the strains primarily belong to *Pseudomonas* and *Bacillus*.

3.2. Biodegradation of acetochlor by the mixed cultures

At an initial concentration of 55 mg/L, acetochlor was completely degraded by the four mixed cultures after 4 days (Fig. 1). No single isolate was able to degrade acetochlor efficiently (data not shown).

Degradation of the higher concentration of 80 mg/L acetochlor after 9 days of incubation is shown in Table 3. More than 99% degradation was observed with mixed D, 84% with mixed A and E, and 88% with mixed J. Transformation of acetochlor was accompanied by formation of metabolites. 2-ethyl-6-methylaniline and CMEPA have been reported as two metabolites of acetochlor (Istvan, 2000; Ye et al., 2002); both compounds were recovered from mixed cultures in MSM treated at 80 mg/L acetochlor (Figs. 2 and 3). 2-ethyl-6-methylaniline was detected more frequently in medium incubated with mixed E than in that

Table 2
The composition of four mixed cultures

Mixed cultures	Pure culture	Sequence length (bp)	GenBank Accession Numbers	BLAST results
A	A6	1317	EU372958	*Bacillus luciferensis* LMG18422 (1306/1316=99%)
	A11	1467	EU372959	*Stenotrophomonas acidaminiphila* (1464/1468=99%)
	A13	1449	EU372960	*Kocuria erythromyxa* (1443/1449 = 99%)
	A14	1472	EU372961	Uncultured soil bacterium clone 7 (1463/1474 = 99%)
	A16	1473	EU372962	Uncultured soil bacterium clone 7 (1468/1474 = 99%)
	A18	1473	EU372963	*Bacillus luciferensis* LMG18422 (1457/1473 = 98%)
	A19	1417	EU372964	*Pseudomonas* sp. ML12 (1416/1417 = 99%)
	A21	1472	EU372965	*Bacillus luciferensis* LMG18422 (1462/1472 = 99%)
D	D3	1472	EU372966	*Bacillus luciferensis* LMG18422 (1457/1472 = 98%)
	D6	1460	EU372967	*Pseudomonas putida* KT2440 (1458/1460 = 99%)
	D8	1222	EU372968	*Bacillus luciferensis* LMG18422 (1220/1222 = 99%)
E	E1	1475	EU372969	*Bacillus* sp. 9B_1 (1469/1473 = 99%)
	E6	1473	EU372970	*Bacillus* sp. 9B_1 (1462/1465 = 99%)
	E7	1448	EU372971	*Kocuria erythromyxa* (1448/1448 = 100%)
J	J1	1424	EU372972	*Stenotrophomonas acidaminiphila* (1421/1424 = 99%)
	J4	1418	EU372973	*Pseudomonas putida* (1416/1418 = 99%)
	J5	1431	EU372974	*Bacillus luciferensis* LMG18422 (1405/1414 = 99%)

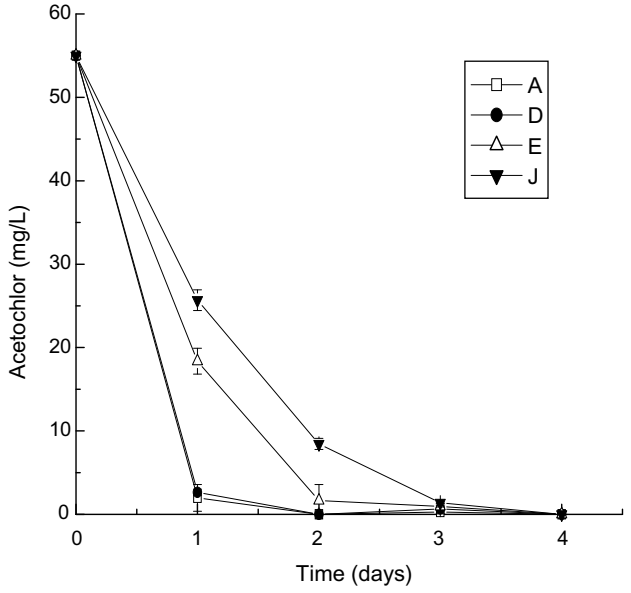

Fig. 1. Degradation of acetochlor (55 mg/L) by four mixed cultures.

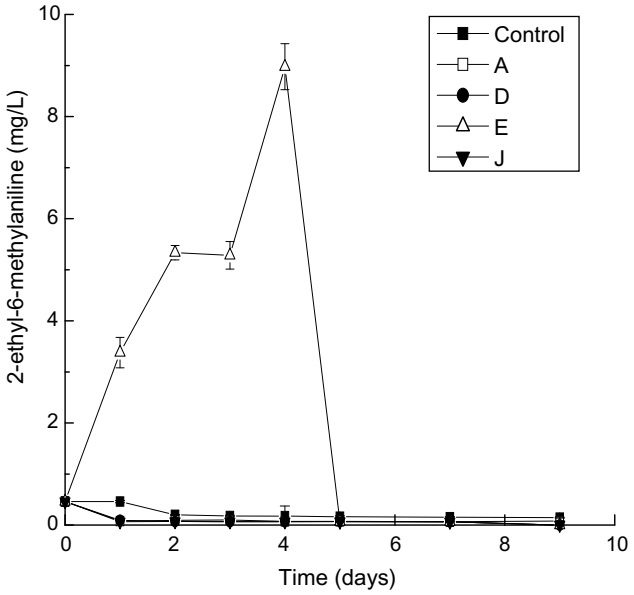

Fig. 2. 2-Ethyl-6-methylaniline detected in acetochlor (80 mg/L) biodegradation by four mixed cultures.

with the other three communities during the biotransformation of acetochlor. In the 4th day of incubation, the concentration of 2-ethyl-6-methylaniline reached 8.98 mg/L and then declined in mixed E. CMEPA was not detected to a great extent in the biotransformation of acetochlor by four mixed cultures. There are may be that CMEPA is produced less or degraded by four mixed cultures further, because it is easily degraded by them (Section 3.4).

3.3. Identification of the metabolites produced by the mixed cultures from acetochlor

Acetochlor biodegradation products were identified by GC–MS based on mass spectral data and fragmentation patterns. The main biodegradation metabolites identified in our experiment consisted of 2-ethyl-6-methylphenol (compound 1; *m*/*z*, 136, 121, 91, 77), 2-ethyl-6-methylaniline (compound 2; *m*/*z*, 135, 120, 91, 77), chloroacetyl-indoline (compound 3; *m*/*z*, 209, 194, 160, 132), CMEPA (compound 4; *m*/*z*, 211, 162, 120, 91), *N*-(ethoxymethyl)-*N*-(2-ethyl-6-methyl)benzoylamide (compound 5; *m*/*z*, 221, 175, 146, 132), 2-hydroxy-*N*-(2-ethyl-6-methylphenyl)-*N*-(ethoxymethyl)acetamide (compound 6; *m*/*z*, 251, 222, 205, 146), and *N*-ethoxymethyl-2-ethyl-6–methyl oxanilic acid (compound 7; *m*/*z*, 265, 236, 174, 146). Compounds 4, 5, 6 and 7 were acetochlor biodegradation products of mixed A and D. Compounds 1, 2, 3, 5, 6 and 7 were that of mixed E. Compounds 4, 5 and 6 were that of mixed J. Comparison of the metabolites produced by the four mixed cultures revealed extensive similarity.

3.4. Biodegradation of additional compounds by the mixed cultures

Propachlor, alachlor and metolachlor, the analogous compounds of acetochlor, are also the main components of the herbicide chloroacetanilide. The degradation of these three components of chloroacetanilide herbicide by four mixed cultures is shown in Fig. 4. These three components of chloroacetanilide herbicide were degraded by four mixed cultures to different degrees after 21 days of incubation. Of the three compounds, propachlor was more easily degraded by the mixed cultures compared

Table 3
Degradation of acetochlor (80 mg/L) and growth of four mixed cultures (OD_{600})

Time (day)	Concentration of acetochlor (mg/L)					Growth of mixed cultures (OD_{600})			
	Control	A	D	E	J	A	D	E	J
0	80.00	80.00	80.00	80.00	80.00	0.02	0.02	0.04	0.03
1	79.62	70.45	65.25	59.97	63.37	0.10	0.11	0.19	0.17
2	79.60	49.32	52.89	45.62	51.57	0.12	0.19	0.19	0.20
3	79.60	43.76	49.83	41.02	44.45	0.16	0.20	0.21	0.17
4	79.50	34.24	32.49	29.50	30.74	0.17	0.20	0.18	0.17
5	79.02	29.51	19.75	24.59	19.10	0.18	0.18	0.17	0.18
7	76.96	21.08	1.19	16.61	11.01	0.17	0.17	0.17	0.18
9	76.01	11.94	0.37	12.30	9.18	0.16	0.17	0.17	0.18

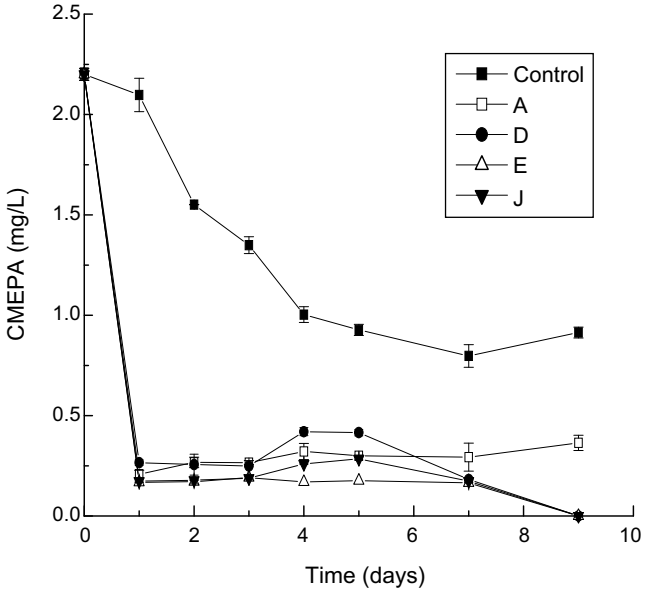

Fig. 3. CMEPA detected in acetochlor (80 mg/L) biodegradation by four mixed cultures.

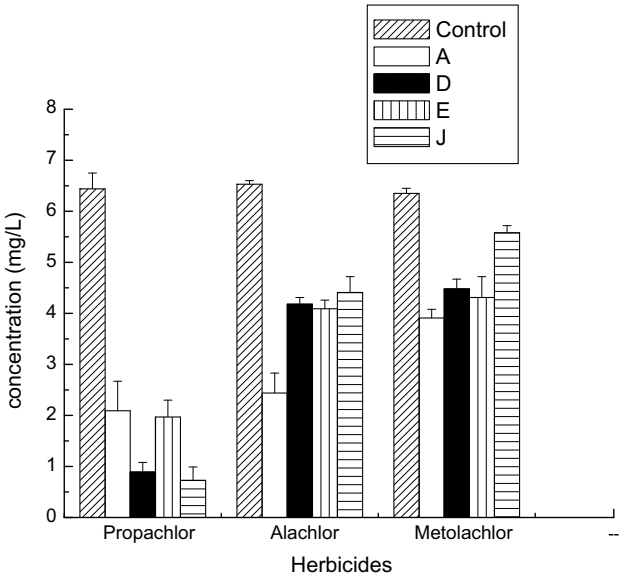

Fig. 4. Degradation of propachlor (7.0 mg/L), alachlor (7.0 mg/L) and metolachlor (6.7 mg/L) by four mixed cultures.

to alachlor and metolachlor. After 21 days of incubation, highest degradation rate of propachlor was 89% by mixed J, that of alachlor was 63%, and that of metolachlor was 39% by mixed A.

2-Ethyl-6-methylaniline (5 mg/L) and CMEPA (7.5 mg/L) were used as substrates to test whether the mixed cultures could transform them. After 5 days of incubation, CMEPA was completely transformed by four mixed cultures, while 2-ethyl-6-methylaniline was completely transformed by mixed A, D, E, and mostly transformed by mixed J (data not shown).

4. Discussion

Acetochlor is a poorly biodegradable organic compound (Qu et al., 1999). In soil treated with 10 mg acetochlor kg^{-1} soil, 66% of the parent herbicide remained without degradation one month after treatment (Istvan, 2000). To date, no mixed cultures able to extensively degrade acetochlor have been definitively described in the literature. In our study, four acetochlor-resistant microbial communities capable of degrading high concentration (80 mg/L) of acetochlor and other chloroacetamide herbicides were obtained, but no single isolate was found to be able to degrade acetochlor efficiently. In our former study, a *Pseudomonas oleovorans* capable of degrading acetochlor was isolated, but it could not transform acetochlor efficiently (Xu et al., 2006). These results may indicate that the metabolites produced in the transformation of acetochlor were toxic to the pure strain and acetochlor could be transformed by the microbial communities quickly. Results of several studies support the hypothesis that a much wider genetic potential for biodegradation might be carried within microbial communities than within a single organism (Sun et al., 1990; Liu et al., 1989).

The primary acetochlor biotransformation products previously reported included 2-ethyl-6-methylaniline, chloroacetyl-indoline, 2-chloro-*N*-(2-ethyl-6-methylphenyl) acetamide, hydroxyacetochlor, oxanilic acid, and ethane sulfonic acid (Istvan, 2000; Ye et al., 2002; Dagnac et al., 2002). The main biodegradation metabolites identified in our experiment consisted of 2-ethyl-6-methylphenol (1), 2-ethyl-6-methylaniline (2), chloroacetyl-indoline (3), CMEPA (4), *N*-(ethoxymethyl)-*N*-(2-ethyl-6 -methyl)benzoylamide (5), hydroxyacetochlor (6), and oxanilic acid (7). Compared with previous results, compounds 1 and 5 have not been mentioned before as acetochlor biodegradation products, and their structures were suggested based on their mass spectra and fragmentation patterns. The hydroxylation of its phenyl ring may indicate that the aromatic ring could be cleaved and acetochlor might be partly mineralized. The remaining products were also detected in previous research. Compounds 2 and 4 were confirmed by comparison with authentic standards. The carcinogenic product 2-ethyl-6-methylaniline was detected in the acetochlor degradation experiments. However, the four mixed cultures also could degrade this compound. Compounds 3 and 6 were identified by comparison with the spectra reported by Istvan (2000). Metabolite 7 was identified according to its mass spectra and fragmentation patterns. Oxanilic acid degradation products of chloroacetanilide herbicides such as metolachlor, alachlor, and acetochlor have been shown to occur in ground water and surface water more frequently (Clark et al., 1999; Kalkhoff et al., 1998). On the basis of the metabolites tentatively identified in this study, the probable metabolic pathways of acetochlor are proposed. The probable degradative pathways of acetochlor involve dechlorination, hydroxylation, deethoxymethylation, cyclization of the remaining *N*-substi-

tute with one of the ethyl groups to produce indole derivatives, carboxylation, and decarboxylation.

In conclusion, the transformation of acetochlor by four microbial communities has been demonstrated, and the main metabolites have been identified in this process. Given the high degradation rates observed here, the four mixed cultures obtained may be useful in the degradation processes of acetochlor.

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