Anaerobic Degradation of Acifluorfen by Different Enrichment Cultures

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A laboratory study on the biodegradation of acifluorfen in anaerobic conditions was conducted. Mixed and pure cultures isolated from activated sludges of a waste water treatment plant and from a soil with a long history of acifluorfen applications could reduce acifluorfen to aminoacifluorfen in a medium with the herbicide as sole source of carbon. Addition of sodium acetate and sodium 2-nitrobenzoate to the medium enhanced and decreased the reduction rate, respectively. A further transformation of aminoacifluorfen was observed with formation of 5-[[2-chloro-4-(trifluoromethyl)phenyl]oxy]-2-aminobenzamide and 5-[[2-chloro-4-(trifluoromethyl)phenyl]oxy]-2-(acetylamino)benzoic acid.

INTRODUCTION

Acifluorfen, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid (CAS Registry No. 50594-66-6), is a diphenyl ether herbicide largely used as sodium salt in pre-emergence control of broad-leaved weeds in soybean fields. Amino derivatives were found to be the main degradation products of several nitrodiphenyl ether herbicides in soil (Oyamada and Kuwatsuka, 1988; Niki and Kuwatsuka, 1976a). Oyamada and Kuwatsuka (1988) reported that the redox state of the soil remarkably affected the reduction of chlornitrofen: lower Eh values were associated with more rapid degradation of the herbicide. Ruzo et al. (1980) reported that photolysis of typical nitrodiphenyl ethers in solution causes reductive dehalogenation, decarboxymethylation, reduction of nitro substituents, and cleavage of the ether linkage. The degradation of diphenyl ether herbicides probably involves the action of microorganisms. Degradation of chlornitrofen was more rapid in unsterilized soils than in sterilized soils (Oyamada and Kuwatsuka, 1989). Schmidt and Braune (1987) isolated mixed bacterial populations able to degrade nitrofen within 4–5 weeks in the presence of acetate as a cosubstrate for growth. Walker et al. (1988) observed more degradation of oxyfluorfen (in water) in the presence of nonsterile sediment than with sterile sediment. In the literature, very few investigations related to the degradation of acifluorfen are reported. Draper and Casida (1983b) studied the metabolism of acifluorfen by rats and found that the predominant reaction involved the reduction of the nitro group. The disappearance of acifluorfen in five soils with different physicochemical characteristics was monitored by us (Gennari and Nègre, 1990). We found that the half-life of acifluorfen varied from 23 days to more than 112 days, depending on the soil type. Perucci and Scarponi (1993) observed a reduction of the half-life of acifluorfen in soil from 40 days to 28 days after amendment with glucose. Pusino and Gessa (1991) studied the photolysis of acifluorfen in aqueous solution and found that only decarboxylation occurred. We reported on the degradation of acifluorfen by a mixed bacterial culture in the presence of sodium 2-nitrobenzoate (Andreoni et al., 1994). The mixed culture allowed the reduction of acifluorfen to aminoacifluorfen under aerobic conditions.

The objective of this work was to determine what microbially mediated reactions acifluorfen may be subjected to under anaerobic conditions.

MATERIALS AND METHODS

Enrichment Cultures. Activated sludges from a waste water treatment plant (20 mL), a soil from a field treated for 3 years with acifluorfen (5 g), or a mixed culture able to degrade acifluorfen in the presence of sodium 2-nitrobenzoate in aerobic conditions (20 mL) were used as inoculum. The inoculum was placed in 250-mL screw-cap bottles to which 20 mL of sterile water containing 2.5 mg of acifluorfen was added. Eighty milliliters of sterile mineral medium (M9) containing 0.1% sodium acetate or 0.25% sodium 2-nitrobenzoate as source of carbon and energy or without alternative carbon source was added, and the suspensions were incubated at 30 °C in the dark in an anaerobic glovebox (Anaerobic System, Forma Scientific) with $CO_2/H_2/N_2$ (10:10:80) atmosphere.

The composition of the mineral medium M9 is reported elsewhere (Gennari et al., 1991). Every 2 weeks, for 3 months, 20 mL of each culture was used to inoculate fresh herbicide medium identical to that of the parent culture. All operations were performed in an anaerobic glovebox.

Biodegradation of Acifluorfen by Mixed Cultures. Aliquots (20 mL each) of the enrichment cultures were placed in screw-cap bottles, and 80 mL of M9 containing acifluorfen (2.5 mg) was added with or without addition of 0.1% sodium acetate or 0.25% sodium 2-nitrobenzoate as an alternative source of carbon. All operations were performed in an anaerobic glovebox. The cultures were incubated at 30 °C in the dark in anaerobic conditions.

Nonbiological degradation of acifluorfen was assessed in sterile incubation medium. All treatments were in duplicate, and the experiment was repeated three times.

Analysis of Acifluorfen and Aminoacifluorfen. Five-milliliter aliquots were removed from each bottle after 0, 1, 4, 7, 14, 28, and 56 days, respectively, diluted 1:5 with acetonitrile, filtered through a 0.2-μm nylon membrane, and analyzed by HPLC. The liquid chromatograph used was a Perkin-Elmer L35 equipped with a Supelcosil LC_{18} column and a diode array detector operating at 295 nm for acifluorfen analysis and at 230 nm for aminoacifluorfen analysis. The column was eluted with a mobile phase that contained 20% (v/v) water acidified to pH 3 with

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Table 1. Acifluorfen Remaining and Formation of Aminoacifluorfen in Aerobic Enrichment Cultures[a]

	alternative carbon sources								
	acetate			2-nitrobenzoate			none		
time (days)	AC	AAC	AC + AAC	AC	AAC	AC + AAC	AC	AAC	AC + AAC
0	100.0	0.0	100.0	100.0	0.0	100.0	100.0	0.0	100.0
1	101.3	8.9	110.2	96.3	1.5	97.8	101.2	7.8	109.0
4	98.4	12.2	110.6	100.7	2.0	102.7	100.0	1.2	101.2
7	94.5	11.7	106.2	101.1	tr	101.1	92.9	5.9	98.8
18	90.4	15.8	106.2	98.9	1.1	100.0	94.2	8.6	102.8
28	68.1	19.7	87.8	97.8	1.7	99.5	92.0	9.0	101.0
56	0.0	94.2	94.2	90.9	tr	90.9	82.5	8.6	91.1

[a] Data are presented as percentage of the initial molar concentration present in the cultural broth. Standard deviation <10% (mean of six replications). AC, acifluorfen; AAC, aminoacifluorfen; tr, traces.

Table 2. Acifluorfen Remaining and Formation of Aminoacifluorfen in Anaerobic Soil Enrichment Cultures[a]

	alternative carbon sources								
	acetate			2-nitrobenzoate			none		
time (days)	AC	AAC	AC + AAC	AC	AAC	AC + AAC	AC	AAC	AC + AAC
0	100.0	0.0	100.0	100.0	0.0	100.0	100.0	0.0	100.0
1	95.2	10.3	105.5	100.7	0.0	100.7	101.6	8.8	110.4
4	0.0	100.1	100.1	102.3	0.0	102.3	48.8	41.1	89.9
7	0.0	97.3	97.3	102.1	0.0	102.1	26.7	64.8	91.5
18	0.0	100.3	100.3	96.9	2.2	99.1	0.0	100.8	100.8
28	0.0	83.7	83.7	79.5	tr	79.5	0.0	82.3	82.3
56	0.0	80.5	80.5	86.9	tr	86.9	0.0	84.2	84.2

[a] Data are presented as percentage of the initial molar concentration present in the cultural broth. Standard deviation <10% (mean of six replications). AC, acifluorfen; AAC, aminoacifluorfen; tr, traces.

orthophosphoric acid and 80% (v/v) acetonitrile. The flow rate was 1 mL/min.

Confirmation. Identity of the compounds was confirmed by comparing the HPLC retention times and the mass spectra with those of authentic samples. Analytical grade acifluorfen and aminoacifluorfen (97% pure) were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

Isolation of Microorganisms. Pure cultures of acifluorfen-degrading microorganisms were isolated from the mixed cultures with the dilution-plate technique. Five fast-growing cultures were selected and purified by streaking on reinforced clostridial medium (RCM, Oxoid) agar. Isolated colonies were subjected to microbiological analysis and prepared for studies of their biodegradation of acifluorfen activity.

Biodegradation of Acifluorfen by Pure Cultures. Washed cell inocula were suspended on M9 medium containing acifluorfen (2.5 mg) and a supplementary carbon source (0.25% sodium 2-nitrobenzoate or 0.1% sodium acetate) identical to that of the parent mixed culture. The suspensions were incubated under anaerobic conditions in the dark at 30 °C. At intervals from 0 to 56 days, 5-mL aliquots of the cultural broth were removed for the determination of acifluorfen and aminoacifluorfen residual concentration following the techniques described above.

Extraction of Metabolites. At the end of each degradation experiment (after 56 days of incubation) extraction and determination of metabolites were attempted. The content of each bottle was transferred to a 250-mL separatory funnel and extracted two times with 50 mL of dichloromethane and two times with ether. The aqueous phase was successively acidified to pH 2 with 1 N hydrochloric acid and extracted two times with 50 mL of dichloromethane and two times with 50 mL of ether. The organic phases were collected together, dried over anhydrous sodium sulfate, evaporated to dryness, resuspended in 1 mL of acetone, layered onto 0.25-mm C_{18} thin-layer chromatography (TLC) plates, and developed with a methanol/toluene 1:8 (v/v) solvent system. Separate bands of metabolites, detected under UV lamps, were scraped off and eluted from the C_{18} stationary phase and 1 mL of methanol; then they were analyzed by HPLC and mass spectrometry.

Identification of Metabolites. HPLC analyses were performed under the same analytical conditions used for the determination of acifluorfen and aminoacifluorfen.

Mass spectrometric experiments were run on a Finnigan-MAT 95 Q instrument with magnetic, electrostatic, and quadrupole analyzers mounted in series. Desorption chemical ionization (DCI) (Baldwin and McLafferty, 1973; Cotter, 1980; Vincenti et al., 1992) analyses were executed by loading 1 μL of methanol solution of analytes onto the DCI rhenium filament. The filament was subsequently introduced into the ion source through a probe and heated by an electric current at a heating rate of 4000 °C/min. Methane (0.5 mbar) was used as the reagent gas. Both positive and negative ion spectra were recorded yielding, respectively, $[MH]^+$ and $[M]^-$ molecular ions of the analytes. Ion source temperature was maintained low (50 °C); the electron energy was set to 200 eV, the emission current set to 0.2 mA, and the magnetic analyzer scanned from m/z 130 to 750 at 0.8 s/decade. Electron impact mass spectra were recorded using the same probe utilized in DCI. The electron energy was set to 70 eV, the emission current set to 1 mA, and the magnetic analyzer scanned from m/z 35 to 450 at 0.8 s/decade.

RESULTS

Biodegradation of Acifluorfen by Mixed Cultures. The results of the experiments, regarding the disappearance of acifluorfen and the evolution of aminoacifluorfen with time, are reported in Tables 1–3. In all conditions studied, breakdown of the acifluorfen molecule was accompanied by the appearance of aminoacifluorfen. The mixed cultures derived both from soil previously treated with acifluorfen and from activated sludges demonstrated higher capacity to reduce acifluorfen than the mixed culture previously enriched in aerobic conditions. Almost quantitative conversion of acifluorfen to aminoacifluorfen is observed when the medium contains the herbicide as sole source of carbon. In these cultural conditions a limited growth of the microbial populations was observed, although the degradation proceeds via a reductive pathway not yielding carbon or energy. This growth might be initially attributed to the uncharacterized dissolved organic carbon in the inocula and later to the organic carbon deriving from dead cells present in the preculture. The conversion of acifluorfen to aminoacifluorfen occurs within the first 18 and 28 days in the bottles containing mixed cultures from soil and activated sludges, respectively. In the bottles with the aerobic mixed culture only 17.5% of acifluorfen

Table 3. Acifluorfen Remaining and Formation of Aminoacifluorfen in Anaerobic Activated Sludge Enrichment Cultures[a]

	alternative carbon sources								
	acetate			2-nitrobenzoate			none		
time (days)	AC	AAC	AC + AAC	AC	AAC	AC + AAC	AC	AAC	AC + AAC
0	100.0	0.0	100.0	100.0	0.0	100.0	100.0	0.0	100.0
1	99.8	11.1	110.9	101.0	0.0	101.0	95.7	7.8	103.5
4	69.1	25.8	94.9	99.1	1.1	100.2	95.2	15.3	110.5
7	49.3	43.6	92.9	73.3	16.3	89.6	72.1	27.5	99.6
18	0.0	101.3	101.3	23.2	57.1	80.3	12.3	90.9	103.2
28	0.0	96.9	96.9	2.0	72.3	74.3	0.0	100.0	100.0
56	0.0	92.2	92.2	0.0	45.6	45.6	0.0	91.5	91.5

[a] Data are presented as percentage of the initial molar concentration present in the cultural broth. Standard deviation <10% (mean of six replications). AC, acifluorfen; AAC, aminoacifluorfen.

was reduced after an incubation period of 56 days. The disappearance of acifluorfen seems to be affected by the addition of alternative carbon sources. In all cases studied, the addition of sodium acetate in the medium enhanced the reduction rate of acifluorfen to aminoacifluorfen, whereas the addition of sodium 2-nitrobenzoate reduced the conversion rate. In the presence of sodium 2-nitrobenzoate, 100% of the acifluorfen was degraded after 56 days of incubation when mixed cultures derived from activated sludges were utilized, whereas the degradation was negligible with the other mixed cultures.

It is worth noting that the increase of aminoacifluorfen concentration is proportional to the decrease of acifluorfen in the first 18-28 days, depending on the experiments. Subsequently, the mass balance based on these two components decreases, indicating the degradation of the first metabolite. The disappearance of aminoacifluorfen was highest in the mixed culture derived from activated sludges containing sodium 2-nitrobenzoate (Table 3). However, no further metabolites were detected by direct HPLC analysis of the cultural broth. To identify the compounds derived from the biotransformation of the aminoacifluorfen primary metabolite, an extraction from whole culture broths after 56 days of incubation was made, as described under Materials and Methods.

No degradation of acifluorfen and aminoacifluorfen was detected in bottles incubated under sterile conditions, confirming that the microorganisms are responsible for degradation of these compounds.

Identity of Metabolites of Aminoacifluorfen. TLC analysis of the extracts revealed the presence of four bands, corresponding to R_f 0.66, 0.77, 0.81, and 0.87 which were removed, extracted with methanol, and analyzed by HPLC. The compounds eluting at R_f 0.66 and 0.87 turned out to be acifluorfen and aminoacifluorfen, respectively. Compounds at R_f 0.77 and 0.81, eluting from the HPLC column after 3.82 and 3.79 min, were named metabolites II and III and were identified by means of mass spectrometric techniques.

Positive and negative DCI mass spectra (not reported here) were exploited to determine unequivocably the molecular weight of metabolites. Positive ion DCI mass spectra exhibited, for each metabolite, the protonated molecular ion $[MH]^+$ as the base peak, together with a fragment $[M-F]^-$. Negative ion DCI mass spectra showed the occurrence of the molecular ion $[M]^-$, together with an abundant fragment $[M - HCl]^-$. From these data it was possible to establish nominal molecular weights of 331, 330, and 373 for aminoacifluorfen, metabolite II, and metabolite III, respectively.

D-EI mass spectra for aminoacifluorfen, metabolite II and metabolite III are reported in Figure 1. The spectrum relative to aminoacifluorfen (Figure 1a) exhibits the molecular ion as the base peak and some meaningful

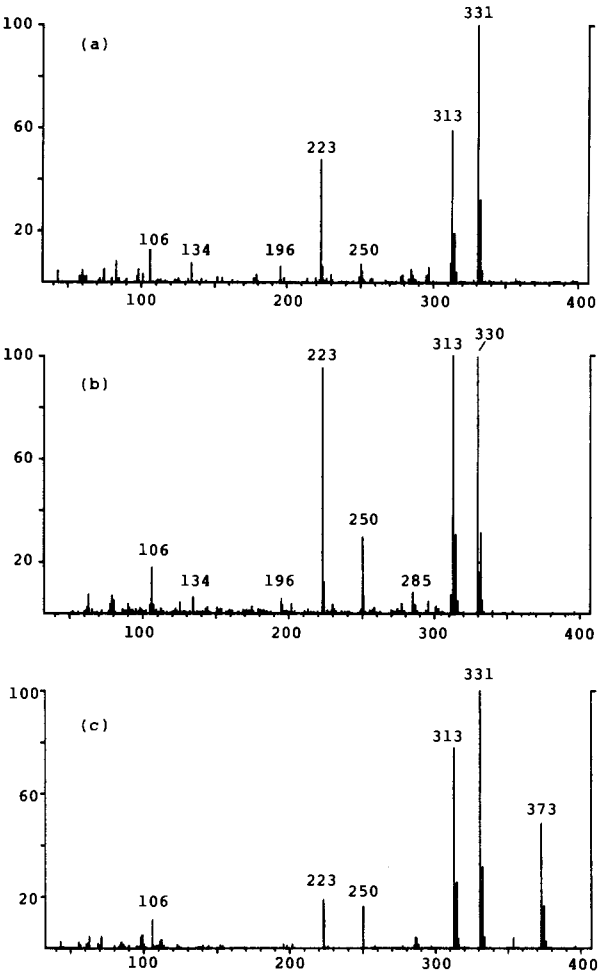

Figure 1. Direct electron impact mass spectra of metabolites of acifluorfen, extracted from a preparative TLC plate: (a) aminoacifluorfen; (b) metabolite II; (c) metabolite III.

fragments, such as m/z 313 $[M - H_2O]^+$ and m/z 250 $[M - HCl - COOH]^+$. m/z 223 might arise from further HCN loss from m/z 250. Lastly, fragment ions at m/z 196 and 179 are relative to the halogenated benzene ring, with and without an OH group, respectively, while fragments at m/z 134 and 106 are relative to the other aromatic ring. This spectrum, obtained from a TLC extract and representing an acifluorfen metabolite, was compared with that obtained from an authentic aminoacifluorfen standard and turned out to be identical.

For the other two metabolites, authentic standards are not available nor are the spectra included in any general and specific mass spectral library. The identification of their structures is based on the interpretation of mass spectra and should be regarded as tentative. The spectrum of metabolite II (Figure 1b) is similar to that of ami-

Table 4. Acifluorfen Remaining and Formation of Aminoacifluorfen in Anaerobic Pure Cultures[a]

	pure cultures											
	C1			C2			C3			C4		
time (days)	AC	AAC	AC + AAC	AC	AAC	AC + AAC	AC	AAC	AC + AAC	AC	AAC	AC + AAC
0	100.0	0.0	100.0	100.0	0.0	100.0	100.0	0.0	100.0	100.0	0.0	100.0
7	11.3	63.5	74.8	101.0	0.0	101.0	99.0	0.0	99.0	100.0	0.0	100.0
14	11.3	46.5	57.8	95.4	0.0	95.4	103.3	0.0	103.3	104.0	0.0	104.0
28	10.1	52.5	62.6	74.4	6.7	81.1	41.1	5.9	47.0	41.6	20.8	62.4
56	10.1	37.8	47.9	71.8	6.9	78.7	41.1	23.4	64.5	40.6	0.0	40.6

[a] Data are presented as percentage of the initial molar concentration present in the cultural broth. Standard deviation <10% (mean of four replications). AC, acifluorfen; AAC, aminoacifluorfen.

Table 5. Morphological and Physiological Characteristics of the Pure Cultures Able To Metabolize Acifluorfen

isolate	Gram reaction	shape and dimension	catalase	optimum growth conditions		sporulation characteristics	parasporal crystals
				anaerobic	aerobic		
C1	positive	rods, single motile	positive	LB agar[a] at 37 °C	Nutr agar (Difco) at 37 °C	spore cylindrical subterminal or central	present
C2	positive	rods, single irregular	negative	PYG agar[b] at 30 °C	PYG agar at 30 °C	not detected	not detected
C3	positive	rods, single or short chains	negative	BHI broth[c] at 37 °C	BHI broth at 37 °C	not detected	not detected
C4	negative	rods, motile very small	negative	RCM (Oxoid) at 30 °C	no growth	not detected	not detected

[a] LB agar: triptone 1%; yeast extract 0.5%; NaCl 0.5%; agar 1.2%; pH 7.5. [b] PYG agar: glucose 2%; yeast extract 0.5%; peptone 0.5%; agar 1%; $CaCO_3$ spread on the surface. [c] BHI suppl. agar = BHI (Difco) 3.7%; yeast extract 0.5%; cysteine-HCl·H_2O 0.05%; pH 7.2; supplemented with emine 0.5 mg/100 mL and K vitamin.

noacifluorfen, except for the molecular ion, which is shifted 1 mass unit downward. This suggests that the carboxylic functional group of aminoacifluorfen has been modified, in metabolite II, to an amidic group, which readily fragments by releasing an NH_3 molecule (instead of a H_2O molecule) to generate the ion m/z 313, identical to that produced from aminoacifluorfen. The rest of the fragment ions arise from m/z 313 by consecutive bond cleavages and are identical in the two spectra, because the parent ions have the same structure.

The mass spectrum of metabolite III is again similar to that of aminoacifluorfen, but the molecular ion is 42 mass units higher. A neutral loss of 42 Da and an abundant m/z 43 ion indicate the probable presence of an acetyl group in the structure. Also in this case, the acetyl group is easily removed as a carbene (CH_2=CO) neutral loss, releasing a hydrogen on the NH group. Thus, a fragment ion identical to the molecular ion of aminoacifluorfen is created, which fragments further by similar pathways.

Biodegradation of Acifluorfen by Isolated Microorganisms. No anaerobic organism was isolated from the cultural broths containing the aerobic mixed culture in any of the three cultural conditions tested, probably because this population does not grow in anaerobic conditions. Negligible turbidity of the cultural broth was observed even when a supplementary carbon source was added to the medium, demonstrating a very low growth of this microbial population in the anaerobic biodegradation experiments. Three different organisms were isolated from the mixed cultures arising from activated sludges (C1, C2, and C3) and two from soil (C4 and C5). When their ability to degrade acifluorfen was tested, disappearance of the herbicide was observed with four of these organisms (Table 4). However, in these experiments conducted with individual species, the degradation of acifluorfen was never accompanied by a proportional formation of aminoacifluorfen. This might indicate that the isolated populations are able to metabolize aminoacifluorfen more quickly than the parent mixed cultures. Also in these cases, no metabolite of aminoacifluorfen was detected by direct HPLC analysis of the cultural broths. Metabolites II and III were again detected upon extraction from the cultural broths.

Morphological and physiological characteristics of the four microorganisms capable of degrading acifluorfen are given in Table 5.

DISCUSSION

Both mixed and pure bacterial cultures were identified which could degrade acifluorfen in anaerobic conditions. The microbial populations carried out the reduction of the nitro group of acifluorfen in the mineral medium with or without an additional carbon source. Reduction was enhanced when sodium acetate was added to the medium, whereas it was inhibited when sodium 2-nitrobenzoate was added. Our previous studies showed that reduction of acifluorfen by microorganisms may also occur in aerobic conditions (Andreoni et al., 1994).

Formation of the amino derivative of nitrodiphenyl ethers has been observed in other studies. Draper and Casida (1983a,b) observed metabolic reduction of several nitrodiphenyl ether herbicides to amino derivatives by rats presumably via nitroso and the highly instable hydroxylamine intermediates. While studying the degradation of chlornitrofen, nitrofen, and chlomethoxynil in soil under flooded and upland conditions, Niki and Kuwatsuka (1976a) observed formation of the amino derivatives in flooded conditions but not under upland conditions. These authors also observed disappearance of the amino derivatives, but they were not able to assess whether the metabolite was degraded or adsorbed on the soil constituents. Oyamada and Kuwatsuka (1989) reported the reduction of the herbicide chlornitrofen in soil under flooded conditions. They found that the herbicides was reduced by Fe^{2+}, but they also demonstrated that the Fe^{2+} content in the soil depended on the microbial activity. In our experimental conditions 1.2 ppm of $FeSO_4 \cdot 7H_2O$ was present in the medium for microbial growth, but reduction of acifluorfen to aminoacifluorfen was observed only in the presence of microorganisms, indicating the importance of the microflora in this type of reaction.

The reduction of the nitro group of acifluorfen is not a major detoxification step in plants. Frear et al. (1983), studying the metabolism of acifluorfen in soybean, observed cleavage of the diphenyl ether bond and formation of conjugates with homoglutathione, cysteine, and glucose.

Further metabolism of aminoacifluorfen was observed with formation of two metabolites, namely 5-[[2-chloro-

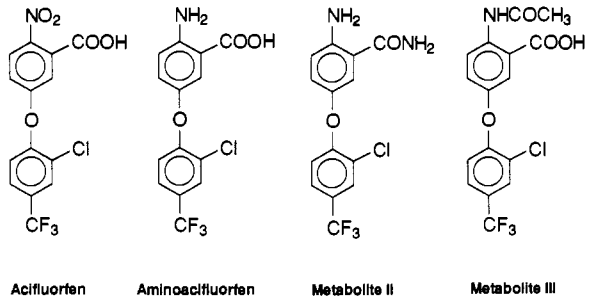

Figure 2. Structures of acifluorfen and its metabolites.

4-(trifluoromethyl)phenyl]oxy]-2-aminobenzamide (metabolite II) and 5-[[2-chloro-4-(trifluoromethyl)phenyl]-oxy]-2-(acetylamino)benzoic acid (metabolite III). The structures of acifluorfen and its metabolites are given in Figure 2.

Acylation of aromatic amines has frequently been observed, and it is regarded as a detoxification process used by soil micoorganisms, higher plants, and animals (Tweedy et al., 1970; Bollag et al., 1978; Parris, 1980). The acylation of chlomethoxynil in soil has been reported by Niki and Kuwatsuka (1976b). They also found trace amounts of 2,4-dichlorophenol and its dechlorinated derivative. In our study neither cleavage of the ether bond nor reductive dechlorination was observed. Incorporation of an amino group was observed in our study (metabolite II). Some microorganisms are known to add nitrate to aromatic compounds (Sylvestre et al., 1982), but no evidence has been found in the literature on biotransformations involving conversion of carboxylic to amidic groups.

From this study, it appears that reduction and conjugation of acifluorfen occur but these processes result in only a minor modification of the molecule and not its complete decomposition. This observation has significance as to the transport and fate of acifluorfen in soil, since the long-lived products of transformation might be adsorbed on the surface of colloidal soil particles or leached through the subsurface to groundwater. On the other hand, the amino derivative of acifluorfen was reported to be non-mutagenic against *Salmonella typhimurium* (strain TA 100) even after metabolic activation (+S9) (Draper and Casida, 1983b).

Further studies are in progress to assess the capability of mixed and pure cultures to degrade acifluorfen more extensively under different cultural conditions.

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