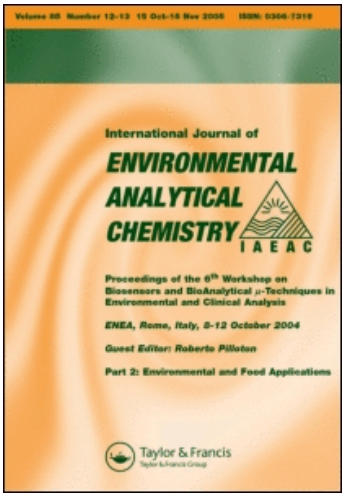

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METABOLISM OF ISOPROTURON IN SOILS ORIGINATING FROM DIFFERENT AGRICULTURAL MANAGEMENT SYSTEMS AND IN CULTURES OF ISOLATED SOIL BACTERIA

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To assess the biodegradation pathway of the phenylurea herbicide isoproturon [N-(4- isopropylphenyl)-N',N'-dimethylurea] in soils, acetone extracts of the herbicide and its metabolites in four soils originating from different agricultural management systems were analyzed by HPLC. Furthermore, the metabolite pattern in media of mixed and pure cultures of bacteria isolated from these soils was investigated after 14 days of incubation with isoproturon.

After 56 days of soil incubation with [ring-U-^{14}C-]-labelled isoproturon, polar metabolites represented up to 80% of the total integrated area. In addition to the main metabolite monodesmethyl-isoproturon, 2-OH-isoproturon, 2-OH-monodesmethyl-isoproturon, 2-OH-didesmethyl-isoproturon, and didesmethyl-isoproturon were also identified in soil extracts. In mixed bacterial cultures, 1–4.2% of the total integrated area corresponded to polar metabolites within 14 days, whereas in pure bacterial cultures only negligible amounts of degradation products were found.

Based on this extensive characterization of metabolites, a concise model for the pathway of isoproturon degradation in soils is suggested.

KEY WORDS: Biodegradation, metabolism, phenylurea herbicides, isoproturon, soil microorganisms, bacterial cultures

INTRODUCTION

The biodegradation process of xenobiotics can be elucidated using different approaches. Some studies describe the disappearance of the initially applied compound or its mineralization to CO_2, in general. Others investigate the pathway of pesticide decomposition in detail, including the identification of metabolites. However, since pesticide metabolites should be evaluated for their potential environmental hazard, only a knowledge of both variables, mineralization rates and detailed degradation pathways, will provide extensive information needed for an ecotoxicological risk assessment.

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Microbial degradation represents the most important process in soils for removing isoproturon [N-(4-isopropylphenyl)-N',N'-dimethylurea] (G, Figure 1), a phenylurea herbicide commonly used in cereals against annual grasses[1,2]. Moreover, the mineralization of isoproturon to CO_2 is generally correlated to the microbial biomass in arable soils[3]. The degradation pathway of isoproturon has been described to proceed via initial cometabolic steps followed by metabolic processes[4,5]. Based on TLC-analysis of soil extracts, Mudd *et al.*[6] identified monodesmethyl-isoproturon (F, Figure 1), didesmethyl-isoproturon (E, Figure 1), 2-OH-monodesmethyl-isoproturon (B, Figure 1), 2-OH-didesmethyl-isoproturon (A, Figure 1), isopropyl-aniline and 2-OH-isopropyl-aniline, and proposed two pathways of isoproturon degradation in soils: after a common

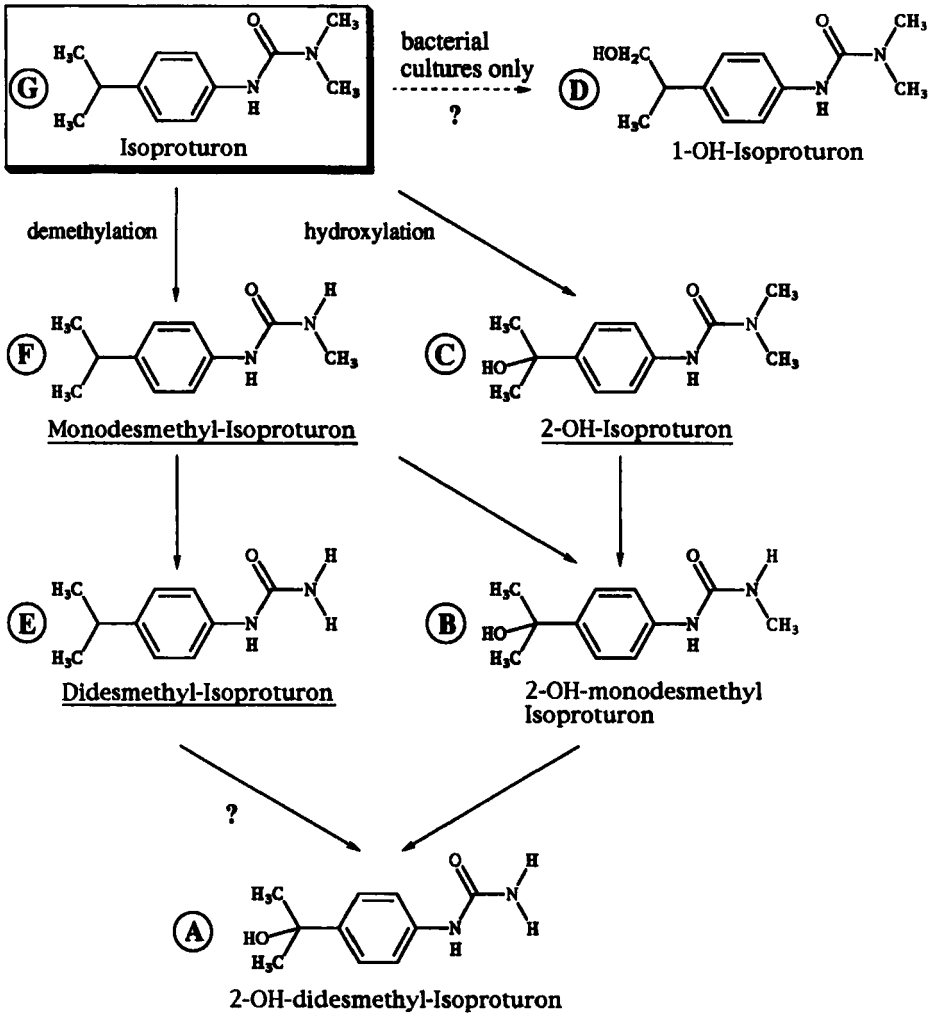

Figure 1 Proposed pathways of isoproturon biodegradation in soil or in media of bacterial cultures. Underlined compounds are present in both systems.

initial step of N-demethylation of isoproturon to monodesmethyl-isoproturon, the main pathway consisted of subsequent hydroxylation, demethylation and cleavage of side chains resulting in the metabolite 2-OH-isopropyl-aniline. An alternative pathway represented the demethylation of monodesmethyl-isoproturon to didesmethyl-isoproturon. However, the resolution in TLC-analysis was not high enough to characterize a considerable number of non-identified polar isoproturon metabolites in soil extracts, which indicate that the degradation pathway is even more complex than described above[7]. When soil extracts were analysed by HPLC using a gradient elution, some polar substances were clearly separated and identified as hydroxylated isoproturon metabolites[8].

In the present study, an improved gradient elution and the use of six reference substances enabled us to extensively characterize and identify isoproturon metabolites formed in soils, as well as to investigate the relative quantitative ratios between metabolites in soil extracts. To examine the influence of agricultural management systems and soil microbial properties on the isoproturon biodegradation pathway, the metabolite pattern was determined in acetone extracts of four soils originating from different farming systems. In former studies, the $^{14}CO_2$-production from [ring-U-^{14}C]-labelled isoproturon differed significantly between these four soils and correlated in three of them to the soil microbial biomass C_{mic}. Only the copper contaminated soil Hop, originating from a former hop yard, did not fit this correlation and showed a considerable mineralization capacity for isoproturon despite a very low microbial biomass[3]. Therefore, a futher objective of this study was to elucidate a possible coincidence of the different mineralization rates in the four soils with differences in the composition of metabolites in the soil extracts. Additionally, cultures of bacterial consortia and pure strains of bacteria isolated from the four soils were incubated with isoproturon. The metabolites of isoproturon were analyzed in the media of these cultures and compared with the metabolite pattern in the soil extracts.

MATERIALS AND METHODS

Chemicals

[Ring-U-^{14}C]-labelled isoproturon (specific activity 3.2 MBq mg^{-1}) for the degradation studies in soil was obtained from International Isotopes (Munich, Germany; radioactive purity: > 99.5%). For soil experiments, it was dissolved in acetone and mixed with the commercial formulation "Arelon" diluted in water as according to the manufacturer. Non-labelled isoproturon for the experiments with cultures of bacterial consortia and pure strains of bacteria was purchased from Promochem (Wesel, Germany; purity > 99.5%). Monodesmethyl-isoproturon [N-(4-isopropylphenyl)-N'-methylurea] and didesmethyl-isoproturon [N-(4-isopropylphenyl)urea] were gifts from Rhone-Poulenc (Lyon, France). Ciba Geigy AG (Basel, Switzerland) furnished the two demethylated substances as well as the three hydroxylated compounds 1-OH-isoproturon [N-(4-(1-hydroxyisopropyl)-phenyl)-N',N'-dimethylurea], 2-OH-monodesmethyl-isoproturon [N-(4-(2-hydroxyisopropyl)-phenyl)-N'-methylurea] and 2-OH-didesmethyl-isoproturon [N-(4-(2-hydroxyisopropyl)-phenyl)urea]. 2-OH-isoproturon [N-(4-(2-hydroxyisopropyl)-phenyl)-N',N'-dimethylurea] was isolated from media of wheat cell suspension cultures and identified by NMR and MS[9].

Soil samples, incubation and soil extraction

The biodegradation of isoproturon was investigated in four soils from different agricultural management systems. Soil Bio (15 y) originated from a long term biological management system without any pesticide treatment for 15 years, soil Bio (2 y) and soil Hop, a soil from a former hop plantation, had been under a biological cropping system for 2 years. Soil Conv was collected from a conventional cropping system with regular pesticide treatments. The physical and chemical properties of the four soils are listed in Table 1. Soil samples were collected from two different sites in Southern Germany from the upper horizon (0–10 cm), sieved (2 mm) and stored at –20°C. One week before application of the herbicide, the soil samples were preincubated at 22°C and at 50% of their maximum moisture holding capacity. At the start of the incubation with isoproturon, four 60 g samples of each soil were filled in 100 ml beakers. Water diluted commercial formulation "Arelon" (Arelon/H_20, 1:160 v/v) was mixed with [ring-U-^{14}C]-labelled isoproturon dissolved in acetone, and the mixture was applied dropwise on the soil surface using a Hamilton syringe. Each soil sample was treated with a radioactivity of 55 kBq and the recommended field application rate of 1250 g isoproturon ha^{-1}. Immediately after herbicide application, the soils were thoroughly mixed, placed in a desiccator and incubated at 25°C ± 2°C in the dark. To maintain soil humidity, the bottom of the desiccator was filled with distilled water and aerated continuously with air. After 32 days of incubation, one of the four replicates of each soil was analysed, whereas the remaining three were analysed after 56 days.

The soil samples were extracted for 6 h with 250 ml acetone in a soxhlet apparatus, then soil residues were dried at 100°C and ground in a mortar. The non-extractable radioactivity was determined by combustion of five aliquots of extracted soil in a Packard Oxidizer 306, with trapping of $^{14}CO_2$ in Carbo-Sorb (Packard) and counting in a liquid scintillation counter. The radioactivity in the soil extracts was determined by liquid scintillation counting (Packard Tri-Carb 1900). For chromatographic

Table 1 Physical, biological and chemical properties of the four soils used in the study.

Soil	*Bio (15 y)*	*Conv*	*Bio (2 y)*	*Hop*
Cropping system	*Biological*[a] *for 15 years*	*Conventional*[b]	*Biological*[a] *for 2 years*	*Former hop plant*[c] *biological for 2 years*
Clay (%)	17	16	18	13
Silt (%)	44	34	38	36
Sand (%)	39	50	44	51
pH ($CaCl_2$)	6.7	5.6	6.0	6.1
C_{org} (% dry matter)	1.40	1.17	1.73	1.71
N_{tot} (% dry matter)	0.15	0.12	0.17	0.18
C/N	9.33	9.75	10.18	9.50
Cu (mg kg^{-1} soil)	10	14	18	200
C_{mic} (mg kg^{-1} soil)[d]	529	316	438	230
$^{14}CO_2$ (%)[e]	23 ± 1.9	14 ± 1.9	19 ± 0.6	18 ± 2.0

[a] Biological cropping without chemical plant protection and only manuring.
[b] Conventional cropping with regular chemical plant protection and mineral fertilizer application.
[c] Regular treatment with $CuSO_4$.
[d] Soil microbial biomass C_{mic} determined by microbial heat production[13].
[e] $^{14}CO_2$ production from [ring-U-^{14}C]-labelled isoproturon [% of the initially applied radioactivity] during 67 days of incubation in a closed laboratory system[3] (n = 3, ± SEM).

determination of isoproturon and its metabolites, the extracts were concentrated on a rotary evaporator to 2 ml and then purified by application on a silica gel cartridge (LiChrolut SI60, Merck) conditioned with 9 ml acetone, followed by elution with 15 ml hexane:chloroform:acetone:ethanol (8:8:4:1 v/v/v/v) and finally with 2 ml acetone. The total recovery of radioactivity in the extracts after this purification step was > 90%. The purified soil extracts were evaporated to dryness on a rotary evaporator, redissolved in 500 µl methanol (100%) and centrifuged (2 min, 16000 rpm). The supernatants were removed and concentrated under a continuous nitrogen flow. The residues were redissolved in 100 µl methanol, centrifuged again and 50 µl of the supernatant were chromatographed by HPLC. To rule out a specific loss of degradation products during purification, aliquots of purified and non-purified soil acetone extracts were compared using HPLC. The chromatograms showed no differences between purified and non-purified samples.

Since sterilization may change the physico-chemical properties of soils[10,11], and soil sterility is difficult to maintain over 56 days, samples of sterile quartz sand were used as controls in soil degradation experiments. For this, 60 g quartz sand were sterilized at 140°C for 40 minutes at three subsequent days, moistened with 10 ml of sterile distilled H_2O, treated with [ring-U-^{14}C]-labelled isoproturon in the same way as the soil samples and then incubated for 56 days. Sterility was verified at the end of the experiment by incubation of a sand suspension on R_2A agar plates[12] at 30°C for one week and by microcalorimetry according to Sparling[13].

Isolation and identification of isoproturon degrading soil microorganisms

Two different enrichment cultures were used to isolate mixed bacterial populations of isoproturon degraders: one containing isoproturon as the only carbon source in order to investigate the metabolic degradation of the herbicide, and another with glucose as an additional carbon source to examine cometabolic degradation. The nutrient solution contained 20 ml of a vitamine stock solution[14] and 20 ml of a stock salt solution[15] per liter distilled water. The cometabolic cultures contained an additional 0.2% glucose. The isoproturon stock solution was prepared with ethanol (0.234 mg isoproturon/10 ml ethanol). Before isoproturon was added to the nutrient media, the ethanol from the stock solution was evaporated under continuous helium flow. The residual isoproturon was redissolved in nutrient solution, sonicated, vigorously shaken for 10 h in the dark and filter sterilized. Samples of each soil (5 g fresh weight) were inoculated in 50 ml nutrient medium. The enrichment cultures and two non-inoculated controls were incubated with shaking at 25°C in the dark. After 14 days, 10 ml of each culture were transferred to 40 ml fresh medium with isoproturon. At the end of the third cultivation period, the cultures were harvested by centrifugation (10 min, 5000 rpm), then isoproturon and its metabolites were determined in the supernatants of each culture using HPLC with UV-detection.

In order to identify microorganisms which degrade isoproturon, pure strains of soil bacteria were isolated from metabolic cultures of soil Bio (15 y) and soil Conv. Therefore, 100 µl aliquots of the bacterial cultures were transferred on R_2A agar plates[12]and incubated for 7 days at 25°C. Morphologically uniform bacterial colonies were picked, transferred to 20 ml isoproturon medium and incubated at 25°C for 14 days. After centrifugation, isoproturon and its metabolites were determined in the pure culture media using HPLC. The isolated bacterial strains were physiologically characterized with the Api-identification system (BioMerieux SA, Lyon, France).

High-Performance Liquid Chromatography (HLPC)

Isoproturon and its metabolites in soil acetone extracts and in bacterial culture media were separated on a LiChrospher 100 RP-18 column (250 × 4 mm, 5 μm, Merck, Darmstadt, Germany) connected to a model L-6200 Intelligent Pump (Merck Hitachi, Darmstadt, Germany). Elution was performed with a two-step linear gradient of bidistilled water (solvent A) and acetonitrile (solvent B) at a flow-rate of 1 ml min^{-1}: the percentage of solvent B in A+B increasing from 5% to 35% in 15 min and to 60% after 30 min followed by rinsing with 100% solvent B. The UV-detector (model 759A Absorbance Detector, Applied Biosystems, Weiterstadt, Germany) was operated at 240 nm to monitor isoproturon and its metabolites in bacterial culture media, whereas [ring-U-^{14}C]-labelled isoproturon and its radiolabelled degradation products in soil acetone extracts were detected using a Radioactivity Monitoring Analyser (RAMONA, Raytest, Münster, Germany).

Quantitative HPLC results are presented as percentage of peak areas relative to the total integrated area. As the initially applied radioactivity consisted of > 99.5% isoproturon, the conversion of the herbicide to polar metabolites was expressed as the sum of all metabolites [portion of peak areas of all polar metabolites (A–F) relative to the total integrated area].

RESULTS

Extractability of the radioactivity in soils

The radioactivity in soil acetone extracts and in the non-extractable fraction was determined after 32 and 56 days of incubation with [ring-U-^{14}C]-labelled isoproturon. At the end of the incubation period the major fraction of the ^{14}C remaining in soils could not be extracted with acetone. In three of the four soils the portion of acetone-extractable radiolabelled compounds decreased from 39.4–43.2% of the total radioactivity in the soil after 32 days to 9.9–21.7% after 56 days. Only in soil Hop, no decrease of extractable radioactivity could be determined during the last 24 days of incubation. Soil Bio (15 y) showed the most important decline of extractable radioactivity from 39.4% after 32 days to 9.9% after 56 days (Table 2). The fraction of non-extractable herbicide residues represented 56.4–60.6% of the radioactivity in each soil after 32 days with a further increase up to 90%, indicating a shift from the soluble to the non-extractable fraction during incubation. Only in soil Hop, the fraction of non-extractable herbicide residues remained nearly stable during the last 24 days (Table 2). Among the four soils tested, the

Table 2 Percent distribution of radioactivity in soil acetone extracts and in non-extractable residues (NER) in four soils after 32 days and 56 days of incubation with [ring-U-^{14}C]-labelled isoproturon. Values described as % of total radioactivity present in each soil sample.

Soil	*Incubation period (days)*	*Non-extractable residues*	*Acetone extractable residues*
Bio (15 y)	32 (n = 1)	60.6	39.4
	56 (n = 3)	90.1 ± 2.3	9.9 ± 2.9
Conv	32 (n = 1)	56.8	43.2
	56 (n = 3)	78.4 ± 1.8	21.7 ± 1.9
Bio (2 y)	32 (n = 1)	59.3	40.7
	56 (n = 3)	73.6 ± 9.9	26.4 ± 9.9
Hop	32 (n = 1)	56.4	43.6
	56 (n = 3)	54.0 ± 0.5	45.9 ± 0.5

highest amount of non-extractable residues (90% of the total radioactivity in soil) corresponding to the smallest extractable fraction (10% of the activity present in soil) was detected in soil Bio (15 y) after 56 days.

Extracting soil with methanol instead of acetone, Kubiak *et al.*[5] recovered more than 90% of the initial radioactivity from [ring-U-^{14}C]-labelled isoproturon in soil extracts after 100 days. Preliminary experiments using soil samples spiked with [ring-U-^{14}C]-labelled isoproturon showed that, after soxhlet extraction with acetone, no derivatives were formed, whereas the soxhlet extraction with methanol resulted in several derivatives of the herbicide (data not shown). This result confirms former studies which reported that reactions between methanol and phenylurea pesticides may occur during the extraction process[16,17]. For that reason, exclusively acetone was used for soil extraction in the present study.

After 56 days, 23–49% of the initially applied radioactivity were not recovered and are likely to represent the percentage of volatile degradation products as well as $^{14}CO_2$ from the herbicide. These degradation products of isoproturon were not monitored during soil incubation.

Identification of isoproturon metabolites in soil acetone extracts

HPLC analysis of soil acetone extracts revealed isoproturon and seven polar metabolites. Five of these were identified by comparison of their retention times with reference compounds as monodesmethyl-isoproturon (F), didesmethyl-isoproturon (E), 2-OH-isoproturon (C), 2-OH-monodesmethyl-isoproturon (B) and 2-OH-didesmethy-isoproturon (A) (systematic names see chemicals), whereas two minor metabolites remained unknown (M1 Rt = 17.3 min, M2 Rt = 22.7 min). The metabolite 1-OH-isoproturon (D) was not detected in soil extracts (Table 3, Figure 2b).

Table 3 [ring-U-^{14}C]-labelled isoproturon and its metabolites in acetone extracts from four different soils after 32 and 56 days of incubation.

Peak	*A*	*B*	*C*	*M1*	*M2*	*E*	*F*	*G*	*Sum of Metabolites*
Rt (min)	12.7	14.1	15.7	17.3	22.7	23.7	25.5	27.4	
Soil									
Bio (15 y)									
32 d	nd	10.3	10.1	2.6	nd	3.5	44.7	28.9	71.1
56 d	3.00	12.9	9.8	nd	nd	4.8	49.6	19.9	80.1
Conv									
32 d	nd	6.3	13.4	2.4	nd	2.8	32.8	42.3	57.7
56 d	nd	8.3	12.8	1.9	4.2	3.0	38.6	31.2	68.8
Bio (2 y)									
32 d	nd	5.9	12.9	2.8	nd	3.4	36.6	38.4	61.6
56 d	1.8	9.2	17.1	2.4	3.8	2.2	31.9	31.6	68.4
Hop									
32 d	nd	3.7	8.7	2.9	nd	2.8	32.1	49.8	50.2
56 d	nd	4.8	11.6	2.3	nd	2.6	33.4	45.3	54.7
Sterile Quartz Sand									
56 d	nd	nd	nd	nd	3.9	nd	nd	96.1	3.9

A = 2-OH-didesmethyl-isoproturon, *B* = 2-OH-monodesmethyl-isoproturon, *C* = 2-OH-isoproturon, *E* = didesmethyl-isoproturon, *F* = monodesmethyl-isoproturon, *G* = isoproturon. Values given as percentage of total integrated area of HPLC analysis with radiodetection. *Sum of metabolites* = portion of peak areas of all polar metabolites (*A*—*F*) relative to the total integrated area. Rt = Retention time (min), nd = not detected.

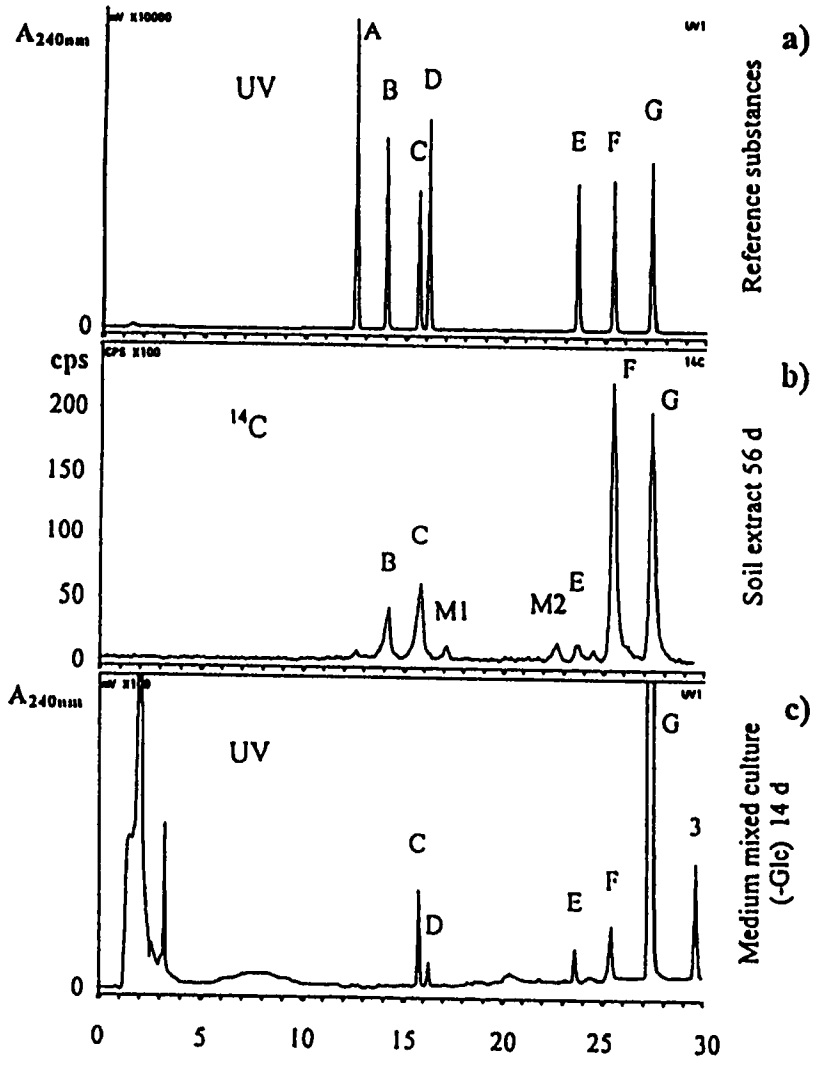

Figure 2 HPLC elution profiles of isoproturon and its metabolites with UV-detection at 240 nm (2a, 2c) or with ^{14}C-radiodetection (2b). a) mixture of reference compounds with A = 2-OH-didesmethyl-isoproturon, B = 2-OH-monodesmethyl-isoproturon, C = 2-OH-isoproturon, D = 1-OH-isoproturon, E = didesmethyl-isoproturon, F = monodesmethyl-isoproturon, G = isoproturon; b) acetone extract from soil Conv; c) culture medium of a mixed bacterial culture isolated from soil Conv without glucose amendment.

In all four soils, the percentage of isoproturon in acetone extracts decreased during the last 24 days of incubation in favour of increasing portions of polar metabolites. After 56 days, the sum of polar metabolites in soil extracts represented up to 80% of the total integrated area compared to 3.9% in extracts of controls with sterile quartz sand. Among soils, the lowest transformation rate after 56 days was found in soil Hop (54.7% of the

total integrated area as polar metabolites, Table 3). Monodesmethyl-isoproturon (F) was identified as the major degradation product in all soil extracts, representing 32–50% of the total integrated area, followed by 2-OH-isoproturon (C) with 9.8–17%. Only in soil Bio (15 y) the relative peak area of 2-OH-monodesmethyl-isoproturon (B) exceeded the relative peak area of 2-OH-isoproturon (C) at the end of the incubation (Table 3). Didesmethyl-isoproturon (E) was detected in all soil extracts in small amounts up to 4.8% of the total integrated area. In acetone extracts from soil Bio (15 y) and Bio (2 y), 2-OH-didesmethyl-isoproturon (A) represented after 56 days between 3 and 1.8% of the total integrated area, respectively. The non-identified compound M1 was found in all four soils (1.9%–2.9% of the total integrated area). An additional non-identified substance M2 was present with a relative peak area of 4.2% in soil Conv and of 3.8% in soil Bio (2 y), whereas the compound was not revealed in extracts from soil Bio (15 y) and soil Hop (Table 3). This unknown compound was also the only conversion product of isoproturon detected in controls with sterile quartz sand. Since the samples of soil or quartz sand were incubated in the dark to avoid possible photodecomposition of the herbicide[18], all metabolites identified in extracts of non-sterile soil samples (M1, A–F) were derived from biodegradation.

Metabolism of isoproturon in mixed and pure cultures of soil bacteria

At the end of the third cultivation period of the enrichment cultures, isoproturon and four of its metabolites were detected in culture media of bacterial consortia isolated from soils. In these media of mixed bacterial cultures, 2-OH-isoproturon (C), monodesmethyl-isoproturon (F), didesmethyl-isoproturon (E) and 1-OH-isoproturon (D) were identified by comparison of their retention times with reference compounds and by co-injection with the corresponding reference substances. The relative peak area of isoproturon in media of mixed bacterial cultures represented 95.8–99% of the total integrated area compared to > 99.4% in culture media of sterile controls. The highest transformation rate within 14 days was found in the cometabolic culture (with glucose amendment) of soil Hop and in the metabolic culture (without glucose amendment) of soil Conv with 4.2% and 3.5% relative peak area of all polar metabolites, respectively. Both cultures of soil Bio (15 y) as well as the cometabolic cultures of soil Bio (2 y) and soil Conv showed a sum of polar metabolites of 2–3% of the total integrated area. In metabolic cultures of soil Hop and soil Bio (2 y), nearly no isoproturon was degraded. Glucose amendment increased the transformation rate in these two cultures, but decreased the percentage of metabolites in enrichment cultures of soil Conv. Moreover, the presence of glucose as an additional carbon source had no positive effect on the transformation rate in bacterial cultures isolated from soil Bio (15 y) (Table 4).

Similar to soil acetone extracts, monodesmethyl-isoproturon (F) and 2-OH-isoproturon (C) represented the main metabolites in culture media of all bacterial consortia, with 2-OH-isoproturon (C) prevailing in cultures of soil Bio (15 y). Didesmethyl-isoproturon (E) was present in all culture media, but hardly exceeded the values of the sterile controls. In some of the mixed cultures, also 1-OH-isoproturon (D) was detected, which was not detected in soil acetone extracts. A further non-identified substance detected in the culture media (peak 3, Figure 2c) was also determined in samples of culture media without any pesticide application. Therefore, it may represent a compound originating from the nutrient solution and not a conversion product from isoproturon. Glucose amendment to the mixed bacterial cultures had no clear effect on the composition of isoproturon metabolites in the culture media.

Table 4 Isoproturon and its metabolites detected in culture media of bacterial consortia from four different soils after 14 days of incubation with and without glucose amendment using UV-detection at 240 nm.

Peak	*C*	*D*	*E*	*F*	*G*	*Sum of Metabolites*
Rt (min)	15.7	16.3	23.7	25.5	27.4	
Mixed cultures from soil						
Bio (15 y)						
–glucose	1.2	0.7	0.2	0.7	97.2	2.8
+glucose	0.8	0.3	0.4	0.7	97.8	2.2
Conv						
–glucose	1.1	0.2	0.8	1.4	96.5	3.5
+glucose	0.9	nd	0.4	1.0	97.7	2.3
Bio (2 y)						
–glucose	0.4	nd	0.3	0.3	99.0	1.0
+glucose	1.1	nd	0.3	1.0	97.6	2.4
Hop						
–glucose	0.4	nd	0.3	0.6	98.7	1.3
+glucose	1.7	0.9	0.4	1.2	95.8	4.2
Sterile Control						
–glucose	nd	nd	0.5	0.3	99.2	0.8
+glucose	nd	nd	0.4	0.2	99.4	0.6

C = 2-OH-isoproturon, *D* = 1-OH-isoproturon, *E* = didesmethyl-isoproturon, *F* = monodesmethyl-isoproturon, *G* = isoproturon. Values given as percentage of total integrated area (n = 2). *Sum of metabolites* = portion of peak areas of all polar metabolites (*C–F*) relative to the total integrated area. Rt = Retention time (min), nd = not detected.

A total of six pure strains were isolated from mixed bacterial cultures of soil Bio (15 y) and soil Conv. These strains were identified using the Api-identification system as *Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas aureofaciens, Rhodococcus equi, Corynebacterium aquaticum and Oerskovia spp.* In the media of all isolates, isoproturon and its metabolites monodesmethyl-isoproturon (F) and didesmethyl-isoproturon (E) were detected after 14 days, but the sum of all polar metabolites represented less than 1.1% of the total integrated area and hardly differed from the sterile control (data not shown).

DISCUSSION

To date, the metabolite 2-OH-isoproturon (C, Figure 1) has not been available as a reference compound. Therefore, this substance has been recently isolated from media of wheat cell suspension cultures and identified by MS and NMR[9]. In the present study, 2-OH-isoproturon was identified in all soil metabolites as well as in culture media of isolated soil bacteria and is reported as an isoproturon metabolite in soils for the first time (Figure 2). Based on the determination of 2-OH-isoproturon as one of the major metabolites in soil extracts, we suggest that the decomposition of isoproturon in soils

includes a third important pathway in addition to the two degradation pathways described by Mudd *et al.*[6]: the hydroxylation of the initial compound to 2-OH-isoproturon (Table 3, Figure 1). Furthermore, the former model for the pathway of isoproturon degradation[6] was confirmed by the detection of considerable amounts of monodesmethyl-isoproturon and 2-OH-monodesmethyl-isoproturon as well as minor amounts of didesmethyl-isoproturon in the acetone extracts of the four soils (Table 3). During the process of isoproturon degradation, 2-OH-didesmethyl-isoproturon is likely to be the last metabolite formed, since this compound was only detected within the last 24 days of incubation in two of the soils (Table 3, Figure 1). Although 2-OH-isopropyl-aniline and isopropyl-aniline were reported as degradation products of isoproturon in soil[6,19], these compounds were not detected in the acetone extracts (Table 2). With regard to their high affinity to soil humic substances[20,21], we suggest that the two isoproturon metabolites are immobilized in the fraction of the non-extractable herbicide residues in the four soils.

The metabolite pattern in acetone extracts showed only little differences among soils. Even the copper contaminated soil Hop (Table 1), with the lowest microbial biomass and the highest mineralization capacity for isoproturon per unit biomass[3], showed the same metabolite pattern as the other three soils. By contrast, the conversion rates of isoproturon to polar metabolites differed considerably among soils (Table 3). Soil Bio (15 y) showed the highest portion of polar metabolites in the extractable fraction after 56 days and thus corresponded with the highest $^{14}CO_2$-production (Table 1). In the other three soils no relationship was obvious between their mineralization capacity and the conversion of isoproturon to polar metabolites in the extractable fraction.

Beyond that, a considerable portion of isoproturon and its metabolites formed in soils could not be extracted with acetone (Table 2). In general, these non-extractable herbicide residues in soils result in part from irreversible adsorption of the compound or its metabolites to soil particles or from covalent binding to soil humic substances. Soil microorganisms also contribute to the formation of non-extractable pesticide residues by immobilization in soil organic matter or by direct incorporation in the microbial biomass[7,22]. Former studies revealed a decrease of acetone-extractable residues from ^{14}C-urea-labelled isoproturon in soils decreasing from 35–57% after 20 days to 2–27% after 132 days[2]. At the same time, 8–31% higher portions of non-extractable residues from ^{14}C-labelled isoproturon were detected in non-sterile soil than in autoclaved soil[2]. This agrees with the decrease of the extractable radioactivity during incubation with a concomitant increase of the non-extractable fraction in three of the four soils used in the present study. Moreover, the soil with the highest microbial biomass, soil Bio (15 y) (Table 1), showed at the end of the incubation the highest portion of non-extractable isoproturon residues (Table 2) suggesting that the biological processes described above play a dominant role during the formation of non-extractable isoproturon residues in arable soils.

To characterize microorganisms which are able to degrade isoproturon, soil bacteria were isolated by enrichment cultures from the four soils used in the present study. In media of mixed bacterial cultures, polar metabolites represented 1–4.2% of the total integrated area within 14 days, whereas nearly no conversion was observed in culture media of six isolated pure bacterial strains (relative peak area of all polar metabolites $< 1.1\%$). These results confirm former studies showing that linuron, another phenylurea herbicide, was degraded in mixed bacterial cultures, but not in cultures of pure bacterial strains[23]. Fournier and Catroux[24] reported a modest degradation rate of isoproturon in cometabolic cultures of *Pseudomonas putida* in contrast to a considerable conversion of the herbicide in cultures of pure fungal strains. This indicates that isoproturon

degradation in soils requires the presence of a microbial community which consists of different groups of microorganisms such as soil bacteria, fungi or algae. Furthermore, the number of isolated soil microoganisms is restricted by external conditions during the isolation process e.g. the composition of the culture media, and represents only a small part of the total soil microflora. For this reason it is also possible that single strains of microorganisms which are able to degrade isoproturon were not isolated by the present enrichment cultures. As isoproturon degradation is supposed to include initial cometabolic processes followed by metabolic transformation, the conversion of the herbicide in bacterial cultures is probably also influenced by a substrate amendment to the culture media[4,5]. In the present study, isoproturon was converted at low rates in mixed bacterial cultures without glucose addition (Table 4), indicating that some of the isolated strains were able to use at least side chains of the compound to a low extent as carbon source. The glucose amendment enhanced the isoproturon degradation only in mixed bacterial cultures of soil Bio (2 y) and soil Hop, but not in bacterial cultures of soil Bio (15 y) and Conv. These results support the hypothesis that isoproturon degrading microorganisms generally depend on substrates as additional energy source which are more complex than glucose[24]. Hence, the lack of a suitable substrate in the present bacterial cultures may also be a reason for the low conversion rate of isoproturon.

The metabolite pattern in the present culture media revealed a similar pathway of isoproturon degradation in soil and liquid cultures of isolated microorganisms. The metabolic pathway in soil and bacterial cultures, based on the results of this study, is shown in Figure 1. 1-OH-isoproturon was the only isoproturon metabolite detected in media of mixed bacterial cultures but not in soil extracts. This suggests that the metabolite is either derived from an additional metabolic pathway in bacterial cultures (Figure 1) or is strongly absorbed to the soil matrix and, therefore, cannot be extracted with acetone.

We conclude that in soils from various agricultural management systems, different transformation rates of isoproturon to polar metabolites are not accompanied by an alteration of the qualitative metabolite pattern. In comparison to the degradation of isoproturon in soils, the degradation in cultures of isolated soil bacteria was considerably reduced, but the metabolite pattern was similar in both systems. Since pure strains of soil bacteria were not able to degrade the initially applied pesticide, different strains of bacteria and further groups of microorganisms are likely to be involved in the biodegradation process of isoproturon in soils. Therefore, further investigations will focus on the contribution of bacteria and fungi to isoproturon degradation in soils. The extensive characterization of isoproturon metabolites in soil extracts and culture media by HPLC demonstrated that the pathway of isoproturon degradation described until now[6] has to include the formation of 2-OH-isoproturon and 1-OH-isoproturon.

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