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Biodegradation of the Phenylurea Herbicide Isoproturon and its Metabolites in Agricultural Soils

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Abstract

Degradation of the phenylurea herbicide isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea) and several phenylurea and aniline metabolites was studied in agricultural soils previously exposed to isoproturon. The potential for degradation of the demethylated metabolite 3-(4-isopropylphenyl)-1-methylurea in the soils was much higher compared to isoproturon. In the most active soil only 6% of added ^{14}C-labelled isoproturon was mineralised to $^{14}CO_2$ within 20 days while in the same period 45% of added ^{14}C-labelled 3-(4-isopropylphenyl)-1-methylurea was mineralized. This indicates that the initial *N*-demethylation may be a limiting step in the complete mineralization of isoproturon. Repeated addition of 3-(4-isopropylphenyl)-1-methylurea to the soil and further subculturing in mineral medium led to a highly enriched mixed bacterial culture with the ability to mineralize 3-(4-isopropylphenyl)-1-methylurea. The culture did not degrade either isoproturon or the didemethylated metabolite 3-(4-isopropylphenyl)-urea when provided as sole source of carbon and energy. The metabolite 4-isopropyl-aniline was also degraded and utilised for growth, thus indicating that 3-(4-isopropylphenyl)-1-methylurea is degraded by an initial cleavage of the methylurea-group followed by mineralization of the phenyl-moiety. Several attempts were made to isolate pure bacterial cultures degrading 3-(4-isopropylphenyl)-1-methylurea or 4-isopropyl-aniline, but they were not successful.

Abbreviations: IPU – isoproturon; MDIPU – 3-(4-isopropylphenyl)-1-methylurea; DDIPU – 3-(4-isopropylphenyl)-urea; 4IA – 4-isopropyl-aniline.

Introduction

Isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea) (IPU) is a phenylurea herbicide used for pre- and post-emergence control of annual grasses and broad-leaved weeds in spring and winter cereals. IPU is heavily used in the United Kingdom and in Western Europe and has been detected in ground and surface waters in concentrations above the limit level of 0.1 μg l^{-1} for pesticides set by the European Community (Nitchke & Schussler 1998; Spliid & Køppen 1998). In Germany, IPU has recently been detected in concentrations as high as 42 μg l^{-1} in effluents from rural wastewater treatment plants following biological treatment (Nitchke & Schussler 1998)

Most of the previously published studies on the biodegradation of IPU in soil do not focus on the fate of the degradation products. These metabolites may be more toxic than IPU itself (Mansour et al. 1999; Remde & Traunspurger 1994), and have been shown to be persistent and contribute to contamination of groundwater and surface waters (Johnson et al. 1998; Schuelein et al. 1996). Although photochemical and chemical processes may be involved in the degradation of IPU (Kulshrestha 1983; Kulshrestha & Mukerjee 1986; Mansour et al. 1999), biodegradation is reported to be the most significant mechanism for its dissipation from soil (Cox et al. 1996; Mudd et al. 1983). However the mineralization of the phenyl-moiety, defined as the complete degradation to CO_2, is slow and its

extent is often limited. Typically, in soil 5–25% of added ^{14}C-phenyl-labelled IPU is recovered as $^{14}CO_2$ within about 2–3 months at 20 °C (Kubiak et al. 1995; Larsen et al. 2000; Lehr et al. 1996; Pieuchot et al. 1996; Reuter et al. 1999) The present study compares the potential biodegradation of IPU and several of its known metabolites in soils from two agricultural fields previously treated with IPU. The purpose was to determine the limiting steps for complete mineralization of IPU.

Materials and methods

Soil samples

Soil from the plough layer (0–25 cm) was collected from an agricultural field near Græse, Denmark (designated soil G1) and from three different plots 1 meter apart within an agricultural field near Flakkebjerg (designated soil F1, F2 and F3), Denmark. The field near Græse had previously been treated with IPU for 9 consecutive years while the field near Flakkebjerg was only treated with IPU the year before sampling. Characteristics of the soils are presented in Table 1. The samples were stored at 5 °C until required.

Chemicals

IPU was obtained from Riedel-de Häen (Seelze, Germany). The metabolites 3-(4-isopropylphenyl)-1-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU) and 4-isopropyl-aniline (4IA) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All chemicals were of analytical grade purity. [^{14}C-U-phenyl]-labelled IPU (4.43 MBq mg^{-1}, 97% radiochemical purity) was obtained from Amersham Life Science (Buckinghamshire, United Kingdom). [^{14}C-U-phenyl]-labelled MDIPU (4.42 MBq mg^{-1}, >99% radiochemical purity) was purchased from the Institute of Isotopes (Budapest, Hungary). The chemical structures of IPU, MDIPU, DDIPU and 4IA are shown in Figure 1.

Mineral medium

The mineral salt (MS) medium used was modified from the HCMM2 medium described by Ridgway et al. (1990). Each litre contained 1.36 g KH_2PO_4, 1.78 g $Na_2HPO_4{\cdot}2H_2O$, 0.5 g KNO_3, 2.38 g $(NH_4)SO_4$, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2$, 2.86 mg H_3BO_4, 1.54 mg $MnSO_4{\cdot}H_2O$, 0.04 mg $CuSO_4{\cdot}5H_2O$, 0.021 mg $ZnCl_2$, 0.041 mg $CoCl_2{\cdot}6H_2O$, 0.025 mg $Na_2MoO_4{\cdot}2H_2O$. pH was adjusted to 7.2 with NaOH (1.0 M). The medium was autoclaved at 121 °C for 20 min. After cooling, 1.0 ml filter-sterilised $FeCl_3{\cdot}6H_2O$ solution (5.14 mg l^{-1}) was added. The effect of nitrogen availability on the biodegradation of IPU was studied by omitting KNO_3 and $(NH_4)SO_4$ from the MS medium. Nitrogen availability was studied due to the potential use of IPU as a nitrogen source by the soil microorganisms. The enrichment cultures were grown in a MS medium containing 10% of the normal amount of KNO_3 and $(NH_4)SO_4$ (0.05 g l^{-1} and 0.005 g l^{-1}, respectively).

Analysis for IPU and metabolites

The concentration of IPU, MDIPU, DDIPU and 4IA was analysed using a Hewlett Packard Series 1050 HPLC system equipped with a Hypersil C18 5μ ODS 3 column (250 × 2 mm). The analyses were conducted at 45 °C using a water:acetonitril (60:40 vol/vol) eluent at a flow rate of 0.7 ml min^{-1}. The compounds were detected by UV absorbance at 245 nm. Further details on the method are given in Juhler et al. (2001). Prior to analysis, 750 μl sub-samples were filtered using a 0.45 μm pore Titan syringe filter (Scientific Resources, Eatontown, USA).

Soil degradation studies

The IPU and MDIPU mineralization potential was determined by adding 1.25 mg or 0.025 mg of unlabelled compound and approx. 25 000 DPM ^{14}C-labelled compound in methanol stock solutions to sterile 100 ml flasks. The solvent was evaporated with a stream of filter-sterilised air and 50.0 ml MS medium then added. Dissolution of the compounds was aided by sonicating the flasks for 5 min in a Branson 2210 sonicator followed by 24 hours incubation at 20 °C. The flasks were then inoculated with 1.0 g (20 mg l^{-1}) or 10.0 g (200 mg l^{-1}) of wet soil. A small test tube containing 2 ml 0.5 M NaOH was placed in each flask and mineralization was measured as $^{14}CO_2$ trapped in the alkaline solution. At regular intervals the alkaline solution was replaced in a laminar flow bench with the flasks being left open for 5 minutes to replenish oxygen. At the end of the experiment 10 ml Wallac OptiPhase HiSafe 3 scintillation fluid (Turku, Finland) was added to each sample and the samples for counted 10 minutes using a Wallac 1409 liquid scintillation counter. Cumulative $^{14}CO_2$ production was corrected for quenching and background radioactivity. We have

Table 1. Characteristics of the agricultural soils from Græse (soil G1) and Flakkebjerg (soils F1, F2 and F3). Values of culturable heterotrophic bacteria (CFU) are mean and standard deviation of triplicates

Soil	pH[1]	Total organic carbon (wt. %)	Silt and clay (wt. %)	Sand, fine (wt. %)	Sand, medium (wt. %)	Sand, coarse (wt. %)	Gravel (wt. %)	CFU (R2A[2]) (g^{-1} wet soil)	CFU (water agar[3]) (g^{-1} wet soil)
G1	6.8	1.22	32.95	35.24	21.04	5.38	5.39	$1.6 \pm 0.4 \times 10^7$	$6.8 \pm 0.1 \times 10^7$
F1	5.6	1.12	46.19	28.11	17.24	4.75	3.70	$1.5 \pm 0.4 \times 10^7$	$2.3 \pm 0.5 \times 10^6$
F2	6.1	1.09	41.55	26.27	16.48	5.01	10.69	$2.2 \pm 0.2 \times 10^7$	$2.0 \pm 0.4 \times 10^6$
F3	6.0	1.13	38.90	23.56	15.39	4.31	17.82	$2.2 \pm 0.5 \times 10^7$	$2.8 \pm 0.5 \times 10^6$

[1] pH of a soil and 0.01 M $CaCl_2$ (1:2.5 vol/vol) suspension.
[2] R2A agar medium (Remel, Lenexa, KS 66215, USA).
[3] Water agar (Difco Bitek agar 15 g l^{-1}, Difco laboratories, Detroit, USA).

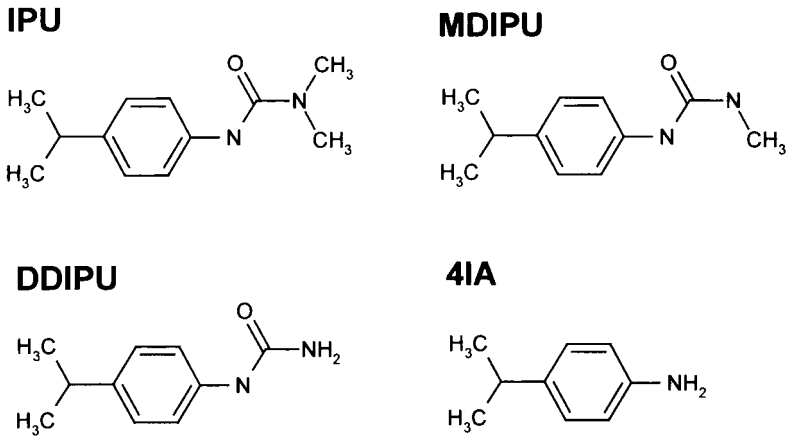

Figure 1. The chemical structures of isoproturon (IPU), 3-(4-isopropylphenyl)-1-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU) and 4-isopropyl-aniline (4IA).

shown that the recovery of added ^{14}C from this set-up range from 87–106% in mineralization studies of different ^{14}C-labeled herbicides following 258 days of incubation (Kristensen et al. accepted). Flasks containing sterile soil that had been autoclaved three times for 20 min at 121 °C at 24-hour intervals served as abiotic controls. Sterility was evaluated by plating on Nutrient agar (Difco Laboratories, Detroit, MI) and incubating at 20 °C for one week. All experiments were performed in the dark at 20 °C on an orbital shaker (IKA labortechnik KS 250 Basic, Staufen, Germany) at 100 rpm. Residual concentration of IPU and its metabolites was measured by HPLC at the end of the experiments.

Enrichment cultures

To establish an IPU- or MDIPU-mineralizing enrichment culture, soil slurries from Græse (soil G1) able to mineralize MDIPU or IPU were transferred to fresh MS medium containing MDIPU (25 mg l^{-1}) or IPU (25 mg l^{-1}) as the sole source of carbon and energy. Following several such transfers of 0.1 ml culture to 4.9 ml fresh medium in 20 ml screw-cap glass flasks a stable MDIPU-mineralizing enrichment culture was established. The enrichment culture was amended with glycerol (15% vol/vol) and frozen at −80 °C. Before use, the thawed culture was centrifuged at 7545*g* for 10 minutes and resuspended in fresh MS medium.

The involvement of fungi in the mineralization of MDIPU was examined by adding cycloheximide (200 mg l^{-1}) or chloramphenical (200 mg l^{-1}) to inhibit fungal and bacterial growth respectively. The antibiotics were added from ethanol solutions, with the solvents being evaporated before addition of the MDIPU. The enrichment culture was also tested for its ability to degrade IPU, DDIPU and 4IA provided as the sole source of carbon and energy. IPU, MDIPU

or DDIPU were added to sterilised flasks from stock solutions in methanol as described for the soil degradation studies. 4IA was added directly to the MS medium before autoclaving.

Growth was estimated by measuring optical density (OD_{600nm}) in a spectrophotometer (Perkin Elmer, Lambda 12) and correlating this to cell number determined by acridine orange direct counting (AODC) (Hobbie et al. 1977). The enrichments were performed in the dark at 20 °C on an orbital shaker, as previously described.

Isolation and screening of pure bacterial cultures

Attempts to isolate pure bacterial cultures were made by plating aliquots (0.1 ml) on a 4IA-containing agar (4IA-agar), an MDIPU-containing agar (MDIPU-agar) or R2A agar (Remel, Lenexa, KS, USA). 4IA-agar and MDIPU-agar were made with 15 g l^{-1} Noble Agar (Difco, Detroit, MI) and MS medium provided MDIPU or 4IA (25 mg l^{-1}) as sole source of carbon and energy. Several colonies were transferred to fresh MDIPU- or 4IA-containing liquid media and their degradation activities tested.

Results

Soil degradation studies

IPU degradation and the production of MDIPU, DDIPU and 4IA in the soil slurries from Græse (soil G1) is summarised in Table 2. The extent of mineralization was greatest in the *N*-deficient medium under which conditions no MDIPU, DDIPU or 4IA where detected. In contrast, abiotic transformation of IPU to MDIPU, DDIPU, and 4IA was observed in sterilised soil and uninoculated controls. Small amounts of MDIPU and DDIPU were also measured in soil added to the *N*-containing MS medium. In view of our finding of abiotic degradation of IPU and metabolite accumulation in abiotic controls, we also studied the potential for mineralization of the demethylated metabolite MDIPU. This proved to be very rapid in the soil G1 (Figure 2a). After an 8-day lag phase during which mineralization was very slow, the rate of $^{14}CO_2$ formation increased substantially. In contrast, IPU was mineralised at a constant rate during the 42 days experimental period. A similar degradation pattern for MDIPU was observed in the Flakkebjerg soils (Figure 2b). However, the mineralization was slower and the production of $^{14}CO_2$ was lower than observed in the Græse soil. Significant differences in the mineralization of MDIPU between the soils from Flakkebjerg was seen (Figure 2b). No mineralization of IPU was observed in either of the soils from Flakkebjerg (Figure 2b). Upon repeated addition of MDIPU to soil G1, mineralization was immediate and enhanced (Figure 3). HPLC analysis carried out before each re-addition of MDIPU revealed no MDIPU, DDIPU, 4IA or other unidentified peaks.

Enrichment of a mixed bacterial culture

Soil G1 showing rapid and enhanced mineralization of MDIPU were used in attempts to establish enrichment cultures degrading this metabolite. It was not possible to reestablish the mineralization activity following transfer of IPU-mineralizing soil to a fresh IPU-containing medium (data not shown). In contrast, several transfers of aliquots of MDIPU-mineralizing culture to fresh MDIPU-containing MS medium resulted in a stable MDIPU-mineralizing mixed culture. Provided MDIPU was the sole carbon source, this culture degraded 25 mg l^{-1} MDIPU within 7 days (Figure 4). 4IA accumulated transiently and growth was observed to an OD_{600nm} of 0.03, corresponding to 1.6×10^7 cells ml^{-1} as determined from the correlation between OD_{600nm} and cell number counted by AODC. A similar degradation pattern was seen with 4IA, when provided as the sole carbon source, but the culture did not degrade either IPU or DDIPU (Figure 4).

Selective inhibition of bacterial and fungal growth in the MDIPU-degrading enrichment culture revealed that only bacteria played a role in the degradation process (Table 3). Plating of the mixed cultures on R2A, MDIPU- and 4IA-agar resulted in several colony morphotypes, however transfer of colonies to fresh MDIPU or 4IA containing liquid MS medium did not result in any cultures degrading either of the compounds. Attempts to re-establish degradation activity by mixing together the isolated bacterial cultures also failed.

Discussion

The first step in the biodegradation of IPU in soil reportedly involves *N*-demethylation to MDIPU or hydroxylation to 3-(4-(2-hydroxyisopropyl)-phenyl)-1,1-dimethylurea (Juhler et al. 2001; Lehr et al. 1996; Mudd et al. 1983). These metabolites may

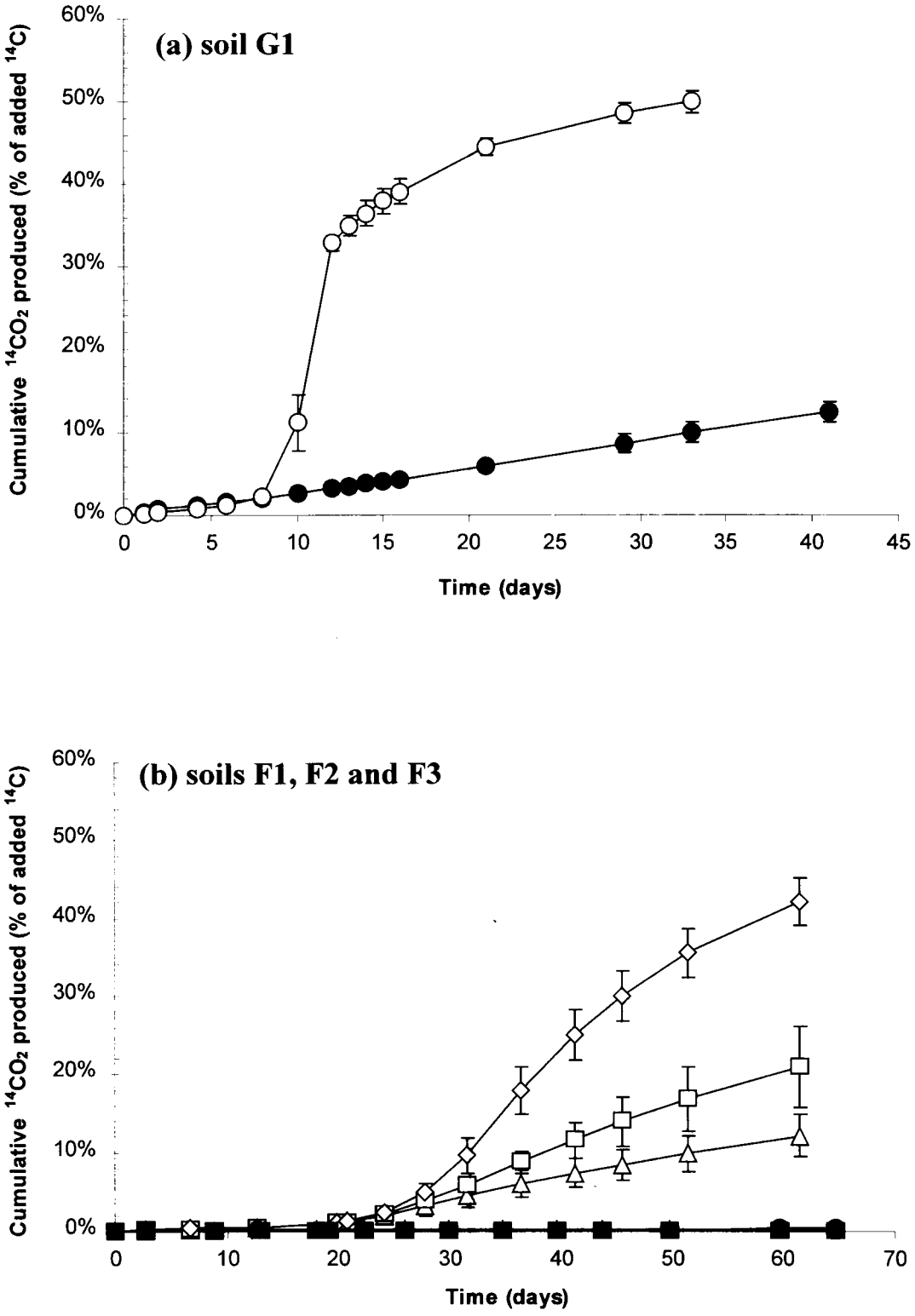

Figure 2. Mineralization of [^{14}C-U-phenyl]-isoproturon (IPU) (closed symbols) and [^{14}C-U-phenyl]-3-(4-isopropylphenyl)-1-methylurea (MDIPU) (open symbols) in concentrations of 0.5 mg l^{-1} in mineral medium inoculated with agricultural soil (200 mg l^{-1}) from (a) Græse (soil G1) or (b) Flakkebjerg (soil F1, F2 and F3). Mean values are shown with the standard deviation of triplicates as indicated by vertical bars. Symbols: soil G1 (●, ○), F1 (▲, △), F2 (■, □) and F3 (♦, ◊).

Table 2. Degradation of isoproturon (IPU) and accumulation of 3-(4-isopropylphenyl)-1-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU) and 4-isopropyl-aniline (4IA) in Græse soil (soil G1) after 135 days. Mean and standard deviation of triplicates expressed as percent of applied IPU (25 mg l^{-1})

Sample	IPU	MDIPU	DDIPU	4IA	Mineralized	Sum
N-containing medium						
Soil (20 mg l^{-1})	90.1 ± 5.1	0.3 ± 0.1	0.1 ± 0.1	nd[1]	3.7 ± 1.3	94.1 ± 5.2
Sterilized soil[2]	97.6 ± 0.4	0.2 ± 0.2	1.7 ± 0.1	3.6 ± 0.1	0.5 ± 0.1	103.6 ± 0.5
Uninoculated[2]	99.6 ± 6.0	nd	1.0 ± 0.1	4.8 ± 0.5	0.4 ± 0.1	105.8 ± 6.0
N-deficient medium						
Soil (20 mg l^{-1})	94.1 ± 2.5	nd	nd	nd	8.9 ± 0.8	103.0 ± 2.6
Soil (200 mg l^{-1})[3]	89.3 ± 1.7	nd	nd	nd	12.3 ± 0.2	101.6 ± 1.7

[1] Not detected.
[2] Two replicates only.
[3] Incubated for 45 days only.

Table 3. Effects of antibiotics on the mineralization of 3-(4-isopropylphenyl)-1-methylurea (MDIPU) (25 mg l^{-1}) in a mixed culture. Mean and standard deviation of triplicates expressed as percent of applied ^{14}C-MDIPU after 15 days of incubation

Treatment	% $^{14}CO_2$ produced
Mixed culture	55.7 ± 3.7
Mixed culture with cycloheximide	53.8 ± 0.3
Mixed culture with chloramphenical	1.1 ± 0.3

subsequently be degraded to several other metabolites, including DDIPU and 4IA. Although the hydroxylated metabolites previously described during degradation of IPU in soil (Lehr et al. 1996; Mudd et al. 1983; Schuelein et al. 1996) are not detectable using our HPLC method, the mass balance calculated on the basis of the measured amounts of IPU, MDIPU, DDIPU, 4IA and $^{14}CO_2$ indicates that neither hydroxylated nor other metabolites were produced in large amounts. It has previously been shown that MDIPU is the main metabolite periodically accumulating during the degradation of IPU in various soils (Cox et al. 1996; Gaillardon & Sabar 1994; Juhler et al. 2001; Lehr et al. 1996; Mudd et al. 1983; Pieuchot et al. 1996), as well as in pure cultures of IPU-degrading soil bacteria and fungi (Berger 1998; Roberts et al. 1998).

Our initial soil degradation study indicated that given the right conditions, indigenous microorganisms might degrade the phenylurea and aniline metabolites produced during degradation of IPU. Excluding KNO_3 and $(NH_4)SO_4$ from the MS medium enhanced the mineralization of IPU compared to that in the *N*-containing MS medium. Previous studies with enrichment of phenylurea-degrading soil bacteria have also successfully provided the phenylurea compounds as the sole nitrogen source (Cullington & Walker 1999; Roberts et al. 1998; Roberts et al. 1993). To avoid *N*-limitation during the enrichment procedures, however, we chose to include 10% of the normal *N* level in the MS medium used in our enrichments.

Comparison of $^{14}CO_2$ production from the mineralization of IPU with that of MDIPU revealed a substantial difference in the biodegradability of these two compounds. Since both compounds were labelled in the phenyl-moiety, this finding indicate that initial *N*-demethylation may be rate limiting for the complete mineralization of IPU to CO_2 in agricultural soils. The constant rate of IPU mineralization in the soil from Græse (soil G1) combined with the lack of activity on sub-culturing, indicate that the degraders involved do not grow on IPU. The nature of the initial degradation steps remains unknown. Our controls show that IPU is transformed abiotically to MDIPU, DDIPU and 4IA. Biotic processes in soil may also be involved, however. In contrast to our study, Roberts et al. (1998) enriched and isolated several soil bacteria capable of degrading IPU by stepwise demethylation to MDIPU and DDIPU, but mineralization was not demonstrated. Recently, Berger (1998) observed that several soil fungi can potentially transform IPU to MDIPU. This suggests the possibility of consortial mineralization of IPU involving an initial attack on the dimethylurea-group carried out by fungi or non-growing soil bacteria, followed by mineralization of the demethylated metabolite.

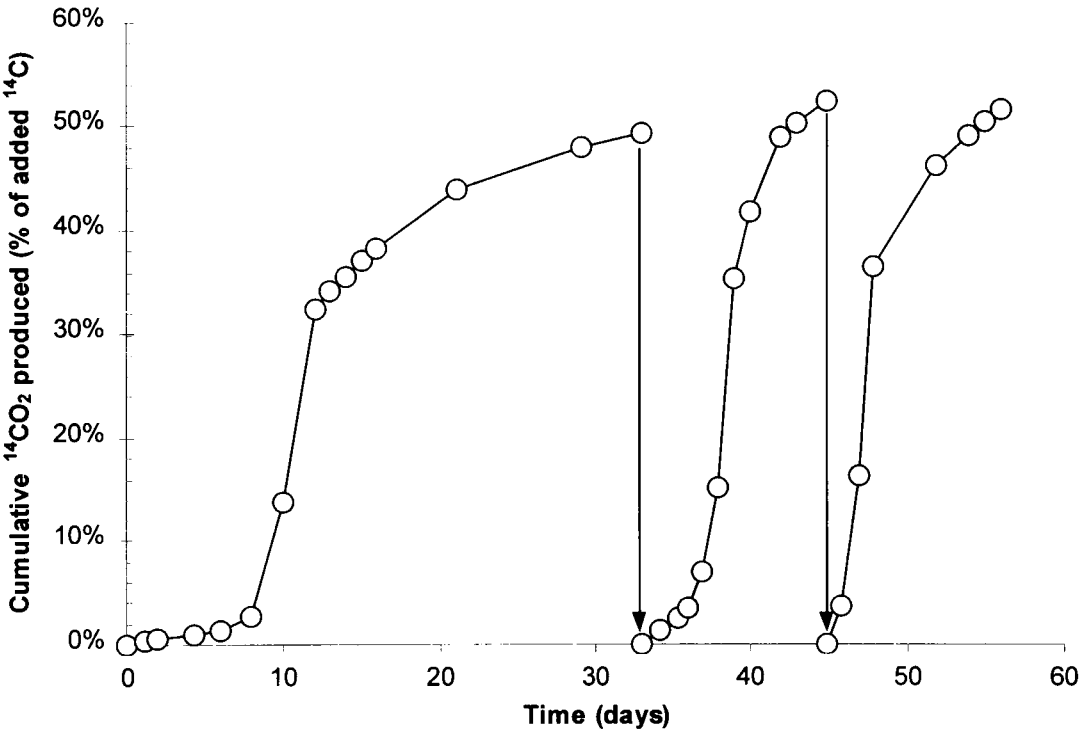

Figure 3. Enhanced mineralization of 3-(4-isopropylphenyl)-1-methylurea (MDIPU) in Græse soil (soil G1). Arrows indicate repeat addition of MDIPU to a final concentration of 0.5 mg l^{-1}. The $^{14}CO_2$ accumulated were calculated as percentage of MDIPU added each time following the first, second and third treatment only.

The mineralization pattern of MDIPU appears to be sigmoidal in the soils G1 and F3, thus suggesting that a growth or adaptation period occurs prior to any significant mineralization. Both the lag phase and subsequent rapid mineralization were virtually the same among the replicates. This suggests that the process does not involve random events such as mutation or gene transfer, but is more likely to be the result of selection for bacteria capable of growing on MDIPU as the carbon and energy source. The maximum recovery of ^{14}C as $^{14}CO_2$ was approximately 50% in the soils G1 and F3. Remaining ^{14}C may at least partly have been incorporated in the microbial biomass as was shown with our enrichment culture. Residues in the form of hydroxylated metabolites, or other intermediate metabolites not detectable with our HPLC method (Juhler et al. 2001) may, however, also be involved. Cox et al (1996) have previously shown that the potential for biodegradation of IPU in different soils was unrelated to pre-exposure in the field. It remains to be elucidated whether the potential for growth-linked mineralization of MDIPU observed in the present study involves ubiquitous pathways and hence is widespread, or whether it is related to the pre-exposure of the soils to IPU. Since the agricultural field in Flakkebjerg had received a uniform application of IPU, the differences observed in the mineralization of MDIPU between the three soils indicate that other factors besides pre-exposure are involved. Beck et al. (1996) found a considerable spatial heterogeneity in the dissipation of IPU within an agricultural field, with times for 50% reduction in concentration of IPU ranging from 31 to 483 days in 25 different plots. Walker et al. (2001) has also observed heterogeneity in the degradation of IPU within a previously treated agricultural field. They measured a considerable difference in the kinetics and rate of degradation among different plots and generally found a higher pH and number of culturable bacteria in the plots with fast degradation compared to those showing slower degradation. The soils we investigated all have approximately the same number of culturable bacteria and pH, which indicates that additional factors govern the observed differences in degradation behaviour.

It is well documented that a minor difference in chemical structure such as the presence of a methyl-

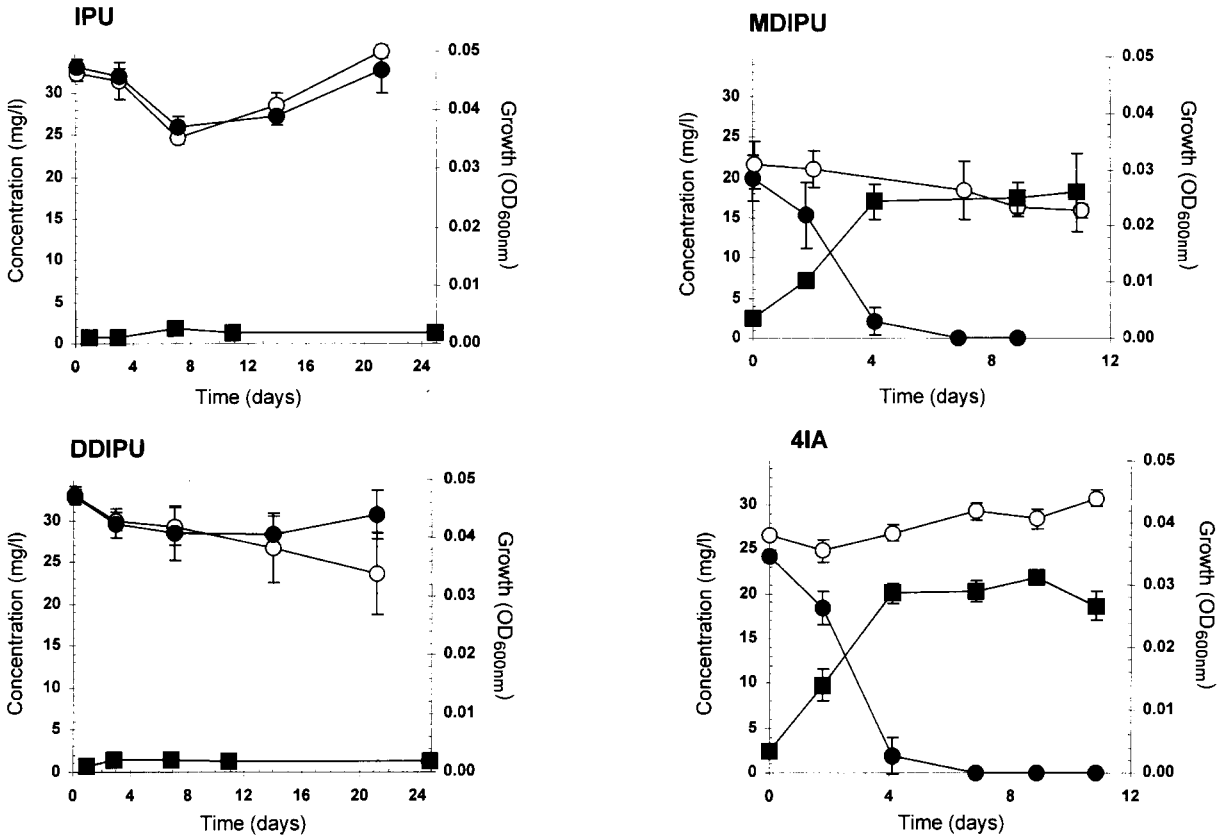

Figure 4. Degradation of isoproturon (IPU), 3-(4-isopropylphenyl)-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU) and 4-isopropyl-aniline (4IA) by the mixed bacterial culture (closed circles) and in uninoculated controls (open circles). Growth of the bacterial culture is shown during degradation (closed squares). Mean values of triplicates are shown with the standard deviation indicated by vertical bars.

group may render a compound more recalcitrant towards biodegradation (Alexander 1994). The lack of a DDIPU-degrading capacity and the transient accumulation of 4IA during degradation of MDIPU suggest that the MDIPU-degrading pathway is initiated by cleavage of the methylurea-group rather than by demethylation to DDIPU. Such deamination-decarboxylation has previously been observed for two linuron-degrading soil bacteria yielding the metabolite 3,4-dichloroaniline (Berger 1998; Engelhardt et al. 1973), as well as by an IPU-degrading soil bacterium resulting in the accumulation of 4IA (Cullington & Walker 1999). None of these bacteria were capable of performing any further degradation of these aniline metabolites, however. No intermediates were observed when our mixed bacterial culture was grown on 4IA indicating complete mineralization to CO_2 and biomass.

We failed to isolate pure cultures capable of degrading either MDIPU or 4IA. Mixing of colonies also failed to result in defined mixed cultures degradation these compounds. Roberts et al. (1993), who studied a mixed bacterial culture capable of mineralizing linuron, suggested that failures in both isolating pure cultures and reestablishment of the degradation activity when mixing colonies might be explained by synergistic interactions among the involved bacteria in the mixed culture. However, the lack of isolation of pure cultures with any activity towards MDIPU may also suggest, that one or more unculturable bacteria are involved in the mineralization. Recently, Forlani et al. (1999) found that unculturable bacteria was involved in the degradation of the herbicide glyphosate in soil and it is generally accepted that the majority of bacteria in the soil environment is unculturable by commonly used methods (Torvik et al. 1990).

Conclusions

The present study shows that the initial *N*-demethylation in the degradation of IPU in agricultural soils might be critical, giving rise to MDIPU that apparently is more readily mineralised by soil bacteria than IPU itself. This is the first report on mixed bacteria cultures capable of performing growth-linked mineralization of MDIPU and 4IA. Isolation and screening of several bacteria strains from the mixed cultures failed to result in any pure cultures capable of degrading MDIPU or 4IA, and the interactions among the involved bacteria still remains to be elucidated.

Acknowledgements

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