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## Enhanced isoproturon mineralisation in a clay silt loam agricultural soil

T. EL-SEBAI<sup>a\*</sup>, B. LAGACHERIE<sup>a</sup>, J.F. COOPER<sup>b</sup>, G. SOULAS<sup>a\*\*</sup>, F. MARTIN-LAURENT<sup>a\*\*\*</sup>

<sup>a</sup> UMR Microbiologie et Géochimie des Sols, INRA/Université de Bourgogne, 17 rue Sully, BP 86510, 21065 Dijon Cedex, France  
<sup>b</sup> LCBE, Centre de Phytopharmacie, Université de Perpignan, 58 avenue Paul, ALDUY, 66860 Perpignan Cedex, France

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**Abstract** – <sup>14</sup>C-ring-labelled isoproturon mineralisation was investigated in a French agricultural soil previously exposed to isoproturon. 50 different soil samples collected every 2 m along a transect of 100 m in length were treated one or two times with isoproturon under laboratory conditions and analysed by radiorespirometry. 94% of the soil samples showed a high ability to mineralise isoproturon with a relatively low variability in the cumulative percentage of mineralisation ranging from 30 to 51% of the initially added radioactivity for the samples treated once with the herbicide. About 45 to 67% of the initially added radioactivity was transformed into <sup>14</sup>CO<sub>2</sub> in soil samples treated twice with isoproturon. Fifty-one to 30% of the radioactive pesticide formed bound residues 120 days after the first isoproturon treatment. The radioactive compound incorporated in the microbial biomass ranged from 3 to 4% of the initially added radioactivity. The methanol-extractable radioactivity was negligible and in the majority of soil samples no metabolites could be detected by high performance liquid chromatography analyses. However, in one soil sample showing low ability to mineralise isoproturon, the mono-demethyl isoproturon derivative represented about 12% of the methanol-extractable fraction. After the second isoproturon treatment, the rate of isoproturon mineralisation was enhanced in most soil samples and the number of soil samples showing a low isoproturon mineralisation capacity decreased. A significant relationship (correlation coefficient, 0.89) between the logarithm of the mineralisation rate (ln k) and the soil pH was found, with a particularly strong positive effect on isoproturon mineralisation for pH > 6.5.

### isoproturon / biodegradation / soil microflora

## 1. INTRODUCTION

Herbicides belonging to the family of substituted urea are extensively used in agriculture, particularly in France, where the annual consumption reached 4665 tons in 1998 (Tixier et al., 2002). These substances enter the plant via the roots and inhibit the photosynthesis (Ducruet, 1991). They are mainly employed to selectively control weeds in cereal cultures. Phenylurea herbicides are transformed in soil by dealkylation, followed by cleavage of the phenylurea bridge yielding aniline derivatives (Sorensen et al., 2003). These metabolites can form bound residues (Azam et al., 1988) or be condensed into the corresponding azobenzenes (Pieuchot et al., 1996). Hydroxylation of alkyl side chains and mineralisation of the phenyl ring have also been observed (Mudd et al., 1983; Lehr et al., 1996; Perrin-Ganier et al., 1996). Photodecomposition leads to demethylation and ring hydroxylation (Faure and Boule, 1997; Jirkovsky et al., 1997). Although the transformation mechanisms are very similar for all phenylureas, their transformation rates are quite dif-

ferent, with DT<sub>50</sub> varying from 10 to 150 days (Kidd and James, 1991; Sorensen et al., 2003).

Isoproturon [3-(4 isopropylphenyl)-1,1-dimethylurea] is one of the phenylurea herbicides which is widely used for pre- and post-emergence control of annual grasses and broad-leaved weeds in spring and winter cereals (Fournier et al., 1975). Approximately 3300 tonnes were applied on 3 million hectares of agricultural land in the UK in 1997, making isoproturon the most widely used organic pesticide in this country. As a result of its intensive and repeated use as well as its properties (i.e. moderate persistence and relatively low adsorption), isoproturon is often detected in ground and surface water in Europe at levels exceeding the EU drinking water limit, fixed to 0.1 µg·L<sup>-1</sup> (Nitchke and Schussler, 1998; Spliid and Koppen, 1998; Stangroom et al., 1998). Ecotoxicological data suggest that isoproturon and some of its metabolites are harmful to aquatic invertebrates, freshwater algae and microbial activity (Mansour et al., 1999; Pérès et al., 1996; Remde and Traunspurger, 1994). Isoproturon and its metabolites such as azobenzene are

\* Present address: NRC, Pests and plant protection, El-Tahrair street, Dokki – Cairo 12622, Egypt.

\*\* Present address: UMR Oenologie-Ampéologie, Université Victor Segalen Bordeaux 2, 351 cours de la libération, 33405 Talence Cedex, France.

\*\*\* Corresponding author: fmartin@dijon.inra.fr

**Table I.** Soil physico-chemicals properties determined from 50 soil samples collected from the transect (\*: soil centrifuge moisture at 1000 g; \*\*: mMol.100<sup>-1</sup> g). †: granulometries properties determined from sub-samples made of 10 soil samples. EH for equivalent humidity, OM for organic matter content and EC for exchangeable cations.

	Clay %	Silts		Sand		EH	OM	N	C/N	pH	EC
		fine	coarse	fine	coarse	*	%	%	AU	AU	**
		%	%	%	%	%	%	%	AU	AU	AU
Higher value						21.6	29.90	2.1	9.45	7.8	29.90
Lower value						20.5	20.60	1.4	8.06	5.5	20.60
Mean value	17.1	29.8	44.4	8.8	0.5	21.3	26.8	1.78	8.75	7.1	26.8
Standard error	1.0	0.4	1.1	0.7	0.1	0.4	2.1	0.16	0.31	0.4	2.1

also suspected of being carcinogenic (Behera and Bhunya, 1990; Hoshiya et al., 1993; Brown, 1999).

Microbial degradation is considered to be the primary mechanism for isoproturon dissipation from soil (Fournier et al., 1975; Mudd et al., 1983; Gaillardon and Sabar, 1994; Cox et al., 1996). Recently, Turnbull et al. (2001) have isolated an *Arthrobacter* sp. able to degrade phenylurea (chlorotoluron, diuron, isoproturon, linuron, monolinuron and monuron) in their respective aniline derivatives by hydrolysis of the urea side chain in the carbonyl group. Another bacterial strain, *Sphingomonas* sp. (strain SRS2), has been isolated by Sorensen et al. (2001). It initiates isoproturon metabolism by two successive N-demethylations, followed by the cleavage of the urea side chain and finally, by the mineralisation of the phenyl structure.

Previous experiments have shown that accelerated degradation of isoproturon in soils can be induced by repeated application of the herbicide (Cox et al., 1996). Repeated application of some organic compounds such as diazinon, carbofuran, iprodione and vinclozolin, butylate, EPTC, alachlor, metolachlor, fenamiphos and ethoprophos has led to enhancing their breakdown, which can be so efficient that the pesticide may become ineffective (Sethunathan, 1971; Felsott et al., 1985; Walker and Brown, 1986; Dowler et al., 1987; Stirling et al., 1992; Karpouzias et al., 1999). However, bioavailability and physical parameters such as pH and soil type strongly influence the degradation rate of pesticides (Aislabie and Lloyd-Jones, 1995). A significant negative linear relationship ( $r^2$ , 0.746) between the DT50 values of isoproturon and the soil pH has previously been reported (Walker et al., 2001a, b).

Detailed examination of isoproturon persistence and movement in agricultural soil revealed that it is quite persistent in the environment, being degraded by up to 40% after 3 months (Nicholls et al., 1993; Harris et al., 1994). Examples of lack of efficacy have been reported in France (Yassir et al., 1999). The aim of the present study was to characterise accelerated biodegradation of isoproturon in soil samples collected from the field of Le Souich (France), yearly treated with this herbicide for the last 10 years and characterised by a significant weed infestation. Isoproturon mineralisation kinetics were determined from soil samples of Le Souich and modelled using the modified Gompertz model. The influence of soil physicochemical parameters such as pH on isoproturon mineralisation parameters resulting from the modelling of isoproturon mineralisation kinetics was estimated.

## 2. MATERIALS AND METHODS

### 2.1. Soil

The soil was collected in April 2002 from an agricultural field (0–10 cm layer) located in “le Souich” (50°13’21”N; 02°21’22”E). 50 separate soil samples were collected every 2 m along a transect 100 m in length. This field was continuously cropped with winter wheat and treated with 1.8 kg ha<sup>-1</sup> of isoproturon [3-(4-isopropylphenyl)-1,1 dimethyl urea] over the last decade. The soil physicochemical characteristics are shown in Table I. The moisture content of each soil sample and the water holding capacity of one average mixed sample were estimated before beginning the experiment. Soil samples were stored at 4 °C until used.

### 2.2. Isoproturon mineralisation

Isoproturon (analytical grade purity > 99%) was purchased from Riedel-de-Haen (Germany). <sup>14</sup>C-labelled isoproturon (specific activity 666 MBq/mmol; 99% radiochemical purity) was purchased from Amersham-Life Science (United Kingdom). Two series of 50 soil samples (40 g equivalent dry weight) were treated with 1.5 mg of isoproturon per kg of soil. <sup>14</sup>C-isoproturon (2kBq per sample) was added to the two series. Soil samples were moistened to 100% of water holding capacity and incubated at 20 ± 0.5 °C in the dark for 120 days in closed respirometer jars (Soulas, 1993). After 14 days of incubation, one of the two series of soil samples was treated again with <sup>14</sup>C-labelled isoproturon as previously described. <sup>14</sup>CO<sub>2</sub> resulting from mineralisation of <sup>14</sup>C-labelled isoproturon was trapped in 5 mL of 0.2 M NaOH solution placed in the respirometer. NaOH traps were changed after 1, 2, 3, 4, 6, 8, 10, 14, 17, 21, 24, 28, 31, 35, 42, 49 and 63 days of incubation. They were analysed for radioactivity content by liquid scintillation counting using ACSII (Amersham) scintillation fluid. The modified Gompertz growth model [ $y=(ae^{-k(t-t_i)} + ct)$ ] was fitted to the isoproturon mineralisation data using Sigma Plot 4.0. Four parameters were determined:  $a$ , the plateau or maximum percentage of mineralisation  $t_i$ , the abscissa of the inflexion point  $k$ , the mineralisation rate constant, and  $c$ , the rate of <sup>14</sup>C turnover due to late isoproturon mineralisation and/or microbial carbon turnover.

### 2.3. $^{14}\text{C}$ -isoproturon residues.

At the end of the incubation (120 days), all the samples of the first series were extracted as follows: aliquots (20 g dry soil equivalent) were extracted with 40 mL methanol under agitation (350 rpm, 16 h at 20 °C in the dark). Samples were centrifuged for 15 min at 6000 g. An aliquot (1 mL) of the supernatants was analysed for extracted radioactivity by liquid scintillation counting. The remaining supernatant was evaporated at 40 °C to dryness using a Sample Concentrator (Brinkman SC/48). The dried residues were dissolved in 2 mL of acetonitrile (Chromasolv for HPLC gradient grade) and analysed by reverse phase high performance liquid chromatography using a LC Star system (Varian) equipped with a Microsorb-MV C18 column (length 25 cm, internal diameter 4.6 mm, Varian) using an acetonitrile-water (75/25, V/V) solvent system delivered at a flow rate of 0.75 ml·min<sup>-1</sup>. Isoproturon was detected at 240 nm.

Quantitative determination of non-extractable radioactivity, mainly corresponding to the bound residues, was performed by combustion of 0.5 mg of dried soil samples extracted with methanol under O<sub>2</sub> flow at 900 °C for 4 min (Biological Oxidiser OX-500, EG&G Instruments, France). The  $^{14}\text{C}$ -CO<sub>2</sub> was trapped in 15 mL of mixture (Oxysolve C – 400 scintillate) and the radioactivity was determined by liquid scintillation counting.

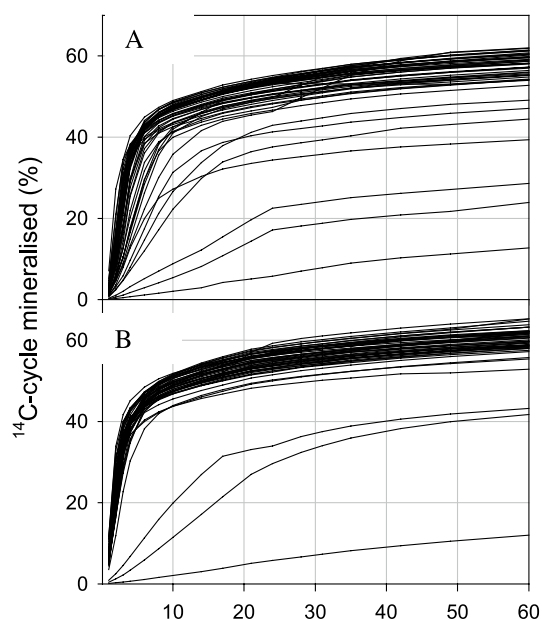
### 2.4. Incorporation of radioactivity in the soil microbial biomass

The  $^{14}\text{C}$  incorporated in the microbial biomass was determined using the fumigation-extraction method (Vance et al., 1987). For each soil sample, one aliquot (10 g dry soil equivalent) was fumigated overnight with ethanol-free chloroform vapours in a vacuum incubator. After fumigation, chloroform vapours were eliminated by 3 successive evacuations. A second aliquot of soil was left unfumigated. Organic carbon was extracted from both fumigated and unfumigated soil samples with potassium sulphate (0.025 M; 50 mL) by agitation on a rotary shaker for 45 min. Extracts were filtered on Whatman GF/C paper and 3 to 4 drops of phosphoric acid were added. The radioactivity of the extracts was measured by liquid scintillation counting. The  $^{14}\text{C}$  incorporated in the microbial biomass was determined using the formula: biomass  $^{14}\text{C}$  = (dpm fumigated extract – dpm unfumigated extract)/Kc. The Kc factor (0.37) was used to convert extractable carbon into biomass-carbon.

## 3. RESULTS AND DISCUSSION

### 3.1. Isoproturon mineralisation kinetics

The mineralisation kinetics of isoproturon were determined by quantifying  $^{14}\text{CO}_2$  trapped in NaOH after 1, 2, 3, 4, 6, 8, 10, 14, 17, 21, 24, 28, 31, 35, 42, 49 and 63 days of incubation. The majority of soil samples treated once with isoproturon showed a high ability to mineralise isoproturon. These samples showed a relatively low variability in the cumulative percentage of mineralisation, which ranged from 15 to 60% of the initially added radioactivity after 14 days of incubation (Fig. 1,

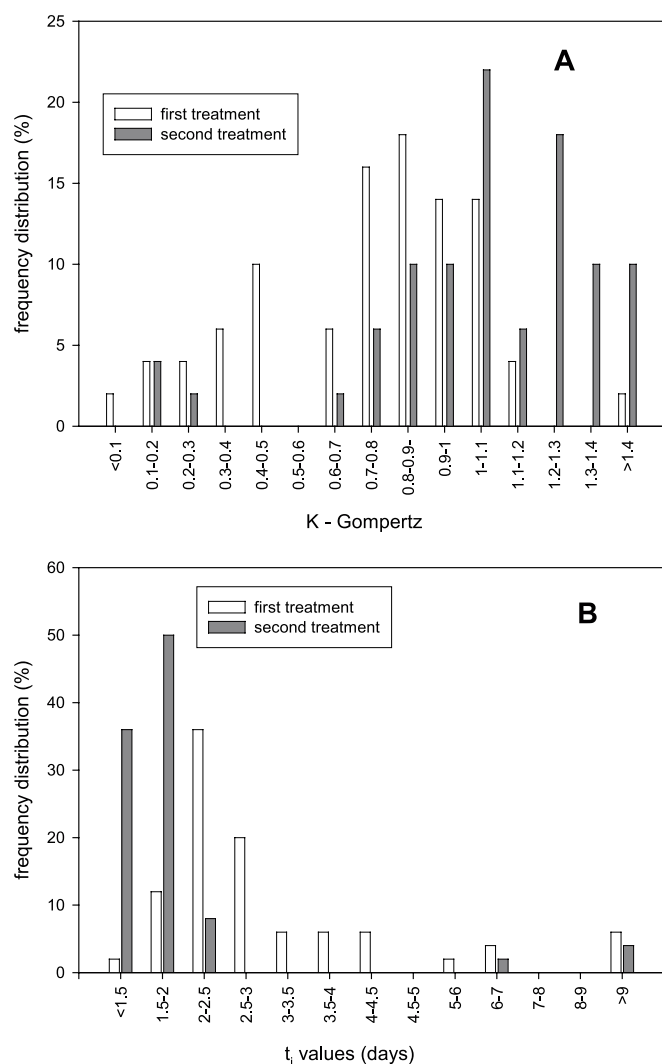


**Figure 1.** Kinetics of mineralisation of isoproturon determined for 50 soil samples collected from the field of Le Souich along a transect of 100 m in length, either treated once or twice with isoproturon under laboratory conditions.

panel A). Only three soil samples mineralised less than 20% of the initially added isoproturon after 14 days. Differences observed in isoproturon mineralisation may reflect the size of the isoproturon-degrading microbial communities initially present in the soil. According to the rate of mineralisation, soil samples could be separated into four categories for which the maximum rate of mineralisation was observed after (i) 2, (ii) 2.5, (iii) 4 and (iv) 5 days. After 120 days of incubation most of the soil samples exhibited cumulative percentages of mineralisation ranging from 50% to 70% of the initially added radioactivity. Only three samples mineralised less than 45% of the initially added isoproturon. The relatively low variability of isoproturon biodegradation may reflect a relatively low spatial heterogeneity of isoproturon-degrading microbes.

Most of the soil samples treated twice with isoproturon showed a maximum rate of mineralisation after 1.5 days of incubation while samples treated once exhibited maximal k values after only 2.5 days (Fig. 1, panel B). It suggests that the repeated application of isoproturon treatment to the soil enhanced its mineralisation rate. These results are in agreement with previous work reporting the isolation and the characterisation of *Sphingomonas* sp (SRS2), a bacterial strain able to mineralise isoproturon from an adapted experimental plot repeatedly treated with isoproturon (Sorensen et al., 2001, 2002).

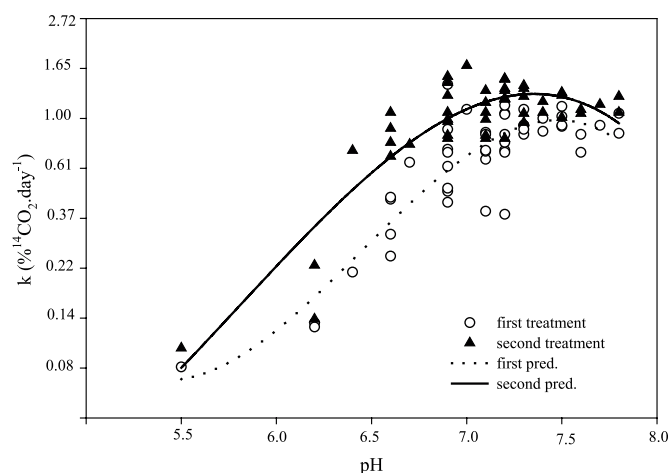
Isoproturon mineralisation kinetics was fitted using the modified Gompertz model. The distribution within classes of the mineralisation rate constant (Fig. 2, panel A) and of the abscissa of the inflexion point (Fig. 2, panel B) revealed that under laboratory conditions two successive isoproturon treatments applied to the soil significantly modified its mineralisation. This observation may explain the decreased efficacy of



**Figure 2.** Frequency distributions of the isoproturon mineralisation rate constant ( $k$ ) determined from the modelling of isoproturon mineralisation kinetics of soil samples either treated once or twice with isoproturon under laboratory conditions.

isoproturon and the presence of weeds, such as foxtail (*Alopecurus myosuroides* Huds), reported by the farmer on this field plot. The modification of the frequency distribution of the isoproturon mineralisation kinetics parameters in response to repeated herbicide treatment revealed differences in isoproturon-degrading activity between samples, which may result from the properties of the isoproturon-degrading microbial communities.

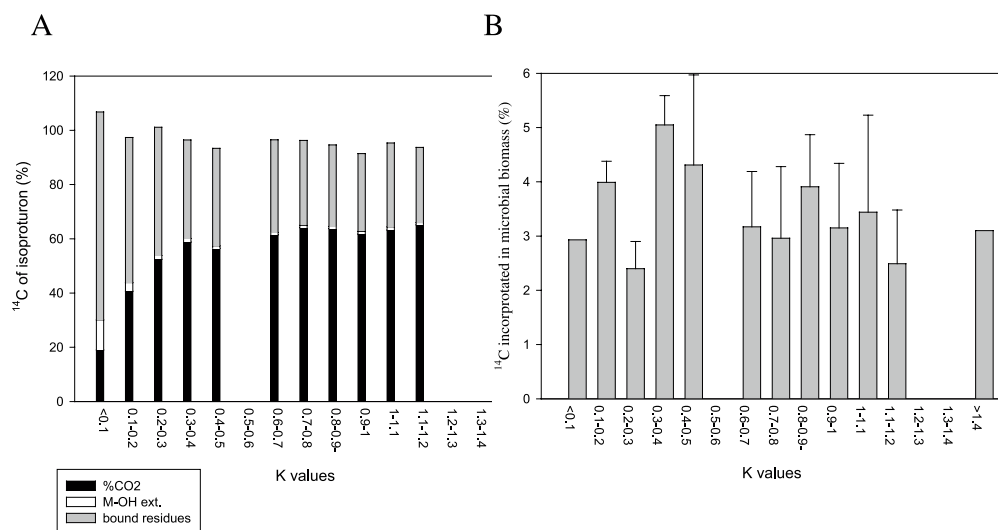
It has previously been reported that the soil microflora of British and Danish agricultural soils adapted to isoproturon degradation in response to repeated application of isoproturon (Cox et al., 1996; Soulas et al., 1993). Our results suggest that repeated application of isoproturon on the field of Le Souich, continuously cropped with winter wheat over the last decade, contributed to the adaptation of soil microflora which became able to rapidly biodegrade this herbicide. In fact, the median



**Figure 3.** Evidence for the existence of a significant correlation between the soil pH and the logarithm of the mineralisation rate constant ( $k$ ) determined from the modelling of isoproturon mineralisation kinetics. First pred. for prediction obtained for soil samples treated once with isoproturon and second pred. for prediction obtained for soil samples treated twice with isoproturon.

frequency of the abscissa of the inflexion point observed in soil samples treated twice with isoproturon is almost 2 times lower than those treated only once. In addition, it is noteworthy that the median frequency of the mineralisation rate ( $k$ ) observed in soil samples treated twice with isoproturon is lower than those treated once. As it has been previously suggested, biodegradation is one of the major processes contributing to pesticides' dissipation of their phytotoxicity from soil (Cox et al., 1996; Fournier et al., 1975; Gaillardon and Sabar, 1994; Mudd et al., 1983). In previous studies, it has been shown that accelerated biodegradation of isoproturon often takes place in soil repeatedly treated with this herbicide (Cox et al., 1996; Soulas et al., 1993). Since the pioneering work of Audus (1949) reporting on 2,4-D and other phenoxyacanoates, accelerated breakdown and an accompanying reduction in herbicide efficacy have been shown. As an example, decreased persistence of MCPA [(4-chloro-*o*-tolyl) oxy] acetic acid following two applications of MCPA has been described (Fryer and Kirkland, 1970). More recently, other chemicals such as carbofuran (Charnay and Fournier, 1994) and atrazine (Barriuso and Houot, 1996; Piutti et al., 2002) have been found to behave similarly. Another study revealed that soil microflora is also able to adapt to isoproturon biodegradation in response to prolonged periods of herbicide application.

Only very few soil samples did not respond to repeated application of isoproturon. In order to determine why they behaved differently, soil physicochemical characteristics (pH, organic matter, N, C/N, equivalent humidity and exchangeable cations) were related to mineralisation potential estimated from the  $k$  value (i.e. mineralisation rate constant) using a polynomial regression procedure. Only one significant relationship between the mineralisation rate constant ( $k$ ) and the soil pH was observed with a relatively good correlation coefficient ( $r^2$ ) of 0.89 in soil samples treated with isoproturon under laboratory conditions (Fig. 3). These results therefore indicate that soil pH



**Figure 4.** Distribution of  $^{14}\text{C}$  in  $^{14}\text{CO}_2$ , microbial biomass, methanol extract and bound residues fractions in function of the mineralisation rate constant (k) determined from the modelling of isoproturon mineralisation kinetics determined from soil samples treated once with this herbicide. M-OH ext for methanol extract.

is a key physicochemical factor influencing the biodegradation rate of isoproturon. Studies of the fate of isoproturon in agricultural fields and contrasting soil types have revealed considerable spatial variability in degradation rates (Beck et al., 1996; Walker et al., 2001a, b). At two different sites in the United Kingdom, isoproturon half-life in soil was found to vary between 6 and 30 days, with the degradation rate varying according to the soil pH. Recent studies demonstrated that the soil pH affected the ease of induction of growth-linked metabolism, with low degradation rates at low soil pH which is apparently linked to co-metabolic degradation of the compound (Bending et al., 2001). In more recent studies estimation of the size of isoproturon microbial communities based on Most Probable Number (MPN) counting showed that rapid biodegradation of isoproturon was associated with the proliferation of isoproturon-degrading micro-organisms (Bending et al., 2003). On the contrary, low biodegradation of isoproturon was either linked to a delay in its proliferation or to apparent co-metabolic degradation. In addition, an isoproturon-degrading isolate (*Sphingomonas* sp.) has been shown to have a narrow pH optimum (7 to 7.5) for optimal metabolism of isoproturon. Variation in the size of isoproturon-degrading microbial communities as well as soil pH could largely account for spatial variation of isoproturon degradation rates.

### 3.2. Distribution of $^{14}\text{C}$ in different soil compartments

The distribution of the  $^{14}\text{C}$  in the different soil compartments was determined after 120 days of the incubation period (Fig. 4).  $^{14}\text{C}$  reported as a function of the value of the isoproturon mineralisation (k) was distributed as follows: (i) 45 to 67% in  $^{14}\text{CO}_2$ , (ii) 0.5 to 10% in methanol extract, (iii) 30 to 51% in bound residues and (iv) 3 to 4% in microbial biomass. When a high amount of isoproturon was mineralised, only low amounts of radioactivity remained in the soil methanol extract and in the

soil non-extractable fraction. The methanol-extractable radioactivity was very low in most soil samples analysed. Almost no metabolites could be detected by high performance liquid chromatography analyses. Only one soil sample showed a low ability to mineralise isoproturon. For this sample, the monodemethyl isoproturon derivative, the isoproturon and 4-isopropylaniline represented 12%, 5%, and 4% of the methanol-extractable radioactivity. In addition, three unknown isoproturon metabolites eluted after 3.9, 7.0 and 8.3 min represented 52, 18 and 7% of the methanol-extractable radioactivity, respectively. This set of data also indicated that the incorporation of  $^{14}\text{C}$  in the microbial biomass did not vary significantly among the different soil samples (Fig. 4, panel B). These results suggest that the catabolic pathway specific for telluric bacterial communities adapted to isoproturon biodegradation is probably very similar in the different soil samples collected from the field of Le Souich. It suggests either the existence of a dominant degrading bacterial species or the existence of a catabolic pathway spread over the adapted isoproturon-degrading microflora.

## 4. CONCLUSION

The present study reports for the first time isoproturon biodegradation in a French clay silty loam agricultural soil treated with this herbicide for at least ten years, as it has previously been shown for a British agricultural soil (Cox et al., 1996). It confirms that repeated application of isoproturon induced the establishment of isoproturon-degrading microbial communities which are able to rapidly biodegrade this herbicide in soil samples in laboratory assays. The cumulative percentage of mineralisation reached up to 60% over a 40-day incubation period. Isoproturon metabolites were non-detectable in most of the soil samples analysed, which all showed a high ability in isoproturon degradation. Soil pH was found to be positively



correlated with the rate of isoproturon mineralisation. These observations are in agreement with recent studies suggesting that enhanced biodegradation of isoproturon in agricultural soils resulted from successive herbicide applications. This work also underlined the positive correlation between the mineralisation rate and soil pH.

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