Fungal biodegradation of a phenylurea herbicide, diuron: structure and toxicity of metabolites

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Abstract: Microbial degradation, organic synthesis and ecotoxicology were used to investigate the fate of diuron after spreading on soils. Quantitative biodegradation assays were performed with fungal strains, showing that diuron was degraded but not entirely mineralized. The modifications observed consisted in demethylation of the terminal nitrogen atom. The identified metabolites were synthesized in sufficient amounts to confirm their structures and determine their non-target toxicity using four biotests. The two metabolites exhibited higher effects than parent diuron. This limited biodegradability and potential aquatic toxicity suggest that diuron is of higher environmental concern than previously recognized.

Keywords: diuron; biodegradation; metabolites synthesis; toxicity

1 INTRODUCTION

Among the herbicides used to prevent the growth of undesirable plants, phenylurea derivatives have been widely used since their discovery in 1950: the French consumption of substituted ureas was 4665 tons (active ingredient) in 1998. These substances enter the plant by the roots and are translocated to the leaves where they inhibit photosynthesis.[1] They are principally used for selective control of germinating grass and broad-leaved weeds in many crops (eg cereals). One of these derivatives, diuron (*N*-(3,4-dichlorophenyl)-*N*′,*N*′-dimethylurea; Fig 1,**1**), is also employed for total weed control on non-cultivated areas (maintenance of roads, railways, parks, etc).

This herbicide is applied to soil, where it tends to accumulate because of its low solubility in water (42 mg litre^{-1} at 25 °C). In the face of the growing concern over the potential of herbicides to contaminate surface and ground water, many data have been collected on the direct and indirect effects of diuron on target and non-target organisms,[2] and diuron is suspected to be genotoxic.[3] Accordingly, a French regulation published in the 'Journal Officiel' of 4 July 1997 limits its utilization on cultivated areas to 1.8 kg ha^{-1} year^{-1}. On non-cultivated areas, it is now forbidden to market and use specialities containing diuron alone, and the use of herbicides containing diuron associated with other xenobiotics is limited to 3 kg ha^{-1} year^{-1}.

Since its commercialization, numerous studies have investigated the microbial degradation of diuron. However, most of these works consisted of monitoring the disappearance of diuron or finding micro-organisms able to degrade it, with no identification of metabolites.[4–8] A few studies[9–12] describe the nature of the metabolites: the main observed were the mono- and didemethylated derivatives, and 3,4-dichloroaniline. To date, no kinetic or quantitative studies have been carried out.

Most non-target toxicity studies have focused on the herbicide, but the degradation products can also be involved in environmental pollution. It is, thus, of great importance to study the fate of diuron after spreading on soils and the toxicity of corresponding metabolites.

Microbial degradations were performed with several fungal species which have already been reported in phenylurea herbicides biodegradation studies.[12–14] The metabolites obtained were isolated and identified by [^{1}H] and [^{13}C]NMR and by mass spectrometry. Reference compounds were then synthesized in amounts sufficient to confirm their structure and prepare standard solutions for a quantitative study of the biodegradation. In order to compare the known

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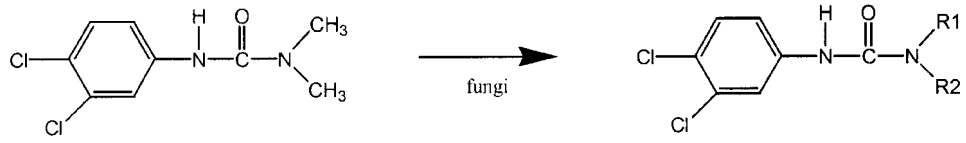

1 Diuron

2 $R_1 = CH_3$, $R_2 = H$

3 $R_1 = R_2 = H$

Figure 1. General scheme of the biodegradation of diuron.

toxicity of diuron with that of its degradation products, the toxicity of metabolites was evaluated *via* different microbiotests involving prokaryotic and eukaryotic cells: Microtox® test, *Tetrahymena pyriformis* Ehrenberg population test, *Tetrahymena pyriformis* Ehrenberg FDA method and *Spirostomum teres* Claparède and Lachmann population test.

The Microtox® system using the bioluminescent bacterium *Vibrio fischeri* (Bejerinck) Lehmann and Neumann is a standardized test widely used: it is cost-efficient, not time-consuming and requires little substance for several replicate tests.[15] Significant inter-species relationships have been obtained between *V fischeri* bacteria test data and bioassay data for other organisms.[16]

The two protozoan ciliates, *T pyriformis* and *S teres*, have been acknowledged as efficient test organisms for the detection of pollutants in aquatic ecosystems.[17–19] *T pyriformis* is cultured in axenic conditions and its generation time is low (about 160 min at 28 °C). Therefore the effects of pollutants can be studied through several generations in a reasonable time (less than 24 h). Fluorescein diacetate (FDA) has previously been proposed for toxicity testing: this method estimates the activities of non-specific esterases of *T pyriformis* cells. It offers many advantages, including experimental simplicity, rapidity and high sensitivity. The ciliate *S teres* was selected for its ubiquitous distribution, large size for a unicellular species, moderate generation time (average 39 h) and high sensitivity to pure toxicants.

2 MATERIALS AND METHODS

2.1 Microbiology

2.1.1 Fungi

Strains were commercially available: *Beauveria bassiana* (Balsamo) Vuill. ATCC 7159, *Cunninghamella elegans* Lendner ATCC 9245, *Fusarium oxysporum* Schlecht, CBS 24861, *Geotrichum candidum* Link, CBS 23376 and *Mortierella isabellina* Oudem, NRRL 1757.

2.1.2 Culture conditions

Fungi were all laboratory-grown. Preculture and culture conditions have already been described elsewhere[20] except for *F oxysporum*. For this strain, preculture (48 h) and culture (48 h) were carried out in a medium containing glucose (20 g litre^{-1}), malt extract (10 g litre^{-1}; bioMerieux - Marcy-l'Etoile - France), yeast extract (5 g litre^{-1}; Difco) and tryptone (5 g litre^{-1}; Difco). Cultures, in 500-ml conical flasks containing 100 ml of medium, were incubated at 27 °C and 200 rev min^{-1} on a rotary shaker.

2.1.3 Biodegradation conditions

The herbicide diuron was obtained from Rhône-Poulenc (purity: 98%).

Growing cells. Stock solution of diuron in dimethylsulfoxide (40 mg ml^{-1}) was sterilized by filtration through a 0.2-μm membrane and added aseptically to the culture at the end of the exponential phase. The final diuron concentration was 40 mg litre^{-1}.

Resting cells. Cell cultures were harvested under sterile conditions by filtration on a sintered glass filter or by centrifugation (15 min, 8000 rev min^{-1}). Cells were washed twice with Knapp buffer (KH_2PO_4, 1; K_2HPO_4, 1; $FeCl_3.6H_2O$, 0.004; $MgSO_4.7H_2O$, 0.04 g litre^{-1}; pH 6.6) and suspended in this buffer at a final concentration of 50 g litre^{-1} wet weight cells. The herbicide was then added as described above.

Controls. In both cases, incubation of cells under the same conditions in the presence of dimethylsulfoxide alone constituted a negative control. Solutions of diuron in Knapp buffer or in complex medium at a final concentration of 40 mg litre^{-1} were used for abiotic references.

2.2 Chemicals

3,4-Dichlorophenylisocyanate and methylamine were purchased from Aldrich (Saint-Quentin-Fallavier – France).

*2.2.1 Synthesis of N-(3,4-dichlorophenyl)-N′-methylurea **2***

An excess (15 mmol) of methylamine was added dropwise, under argon atmosphere, to a 300 g kg^{-1} solution of 3,4-dichlorophenylisocyanate (10 mmol) in anhydrous THF. The solution was stirred at room temperature for 2 h and after evaporation of THF, the white solid obtained was purified on silica gel (eluent: ether + cyclohexane + ethanol (75 + 20 + 5 by volume)).

Yield 72%; R_f 0.33; mp 165–166 °C [^{1}H]NMR (400.13 MHz) in hexadeuteroacetone (ref. CD_3-CO-

CHD_2 at 2.04 ppm): 8.23 (s)(NH); 7.91 (d)(1H); 7.36 (d)(1H); 7.30 (dd)(1H); 5.80 (s)(NH)); 2.70 (d)(3H). [^{13}C]NMR (100.61 MHz) in hexadeuteroacetone (ref. CD_3-CO-CD_3 at 206 ppm): 155.6 (CO); 140.9 (C_1); 131.6 (C_3); 130.2 (C_5); 123.3 (C_4); 119.3 (C_2); 117.8 (C_6); 26.0 (CH_3). Anal: Calculated for $C_8H_8Cl_2N_2O$ C 43.26, H 3.68, N 18.79; Found C 43.87, H 3.78, N 18.71.

2.2.2 Synthesis of N-3,4-dichlorophenylurea ***3***

As in section 2.2.1, except that ammonia bubbling was used instead of methylamine.

Yield 65%; R_f 0.26; mp 156–158 °C [^{1}H]NMR (400.13 MHz) in hexadeuteroacetone (ref. CD_3-CO-CHD_2 at 2.04 ppm): 8.48 (s)(NH). 7.94 (d)(1H); 7,37 (d)(1H); 7.32 (dd)(1H); 5.70 (s, NH_2). [^{13}C]NMR (100.61 MHz) in hexadeuteroacetone (ref. CD_3-CO-CD_3 at 206 ppm): 155.8 (CO); 140.8 (C_1); 131.6 (C_3); 130.3 (C_5); 123.6 (C_4); 119.6 (C_2); 117.0 (C_6). Anal: Calculated for $C_7H_6Cl_2N_2O$ C 41.00, H 2.95, N 13.66; Found C 40.98, H 3.01, N 13.50.

2.3 Analyses

2.3.1 Analytical methods

HPLC analyses were performed using a Waters 600E chromatograph fitted with either a reverse-phase column (LiChroCART C_{18} – Purospher – 250 × 4 mm – Merck) or a normal phase column (Nucleosil 3 µm – 150 × 4.6 mm – Interchrom) at room temperature. Detection was performed with a Waters 486 UV detector set at 254 nm.

2.3.2 Sample analyses

Samples (7 ml) were periodically removed aseptically and centrifuged (15 min, 4000 rev min^{-1}).

Cells were washed with Knapp buffer (2 × 7 ml) and, after centrifugation, the supernatants were combined and analysed by HPLC by the following two methods:

method A. Twenty microlitres of supernatant were injected on to a reverse-phase column. The mobile phase was acetonitrile + water (60 + 40 by volume) and the flow rate was 0.5 ml min^{-1}.

method B. Four millilitres of supernatant were extracted with ethyl acetate (2 × 1 ml) and the combined crude extracts were then evaporated to dryness in a rotary evaporator under reduced pressure. The residues were dissolved in ethyl acetate (2 ml) and 20 µl injected on a normal phase column, using a mobile phase of ethyl acetate + cyclohexane (60 + 40 by volume), at a flow rate of 0.5 ml min^{-1}.

2.3.3 Cell treatment

Cells were triturated in ethanol (50 ml) for 30 min. The ethanol phase was removed by filtration on a sintered glass filter and the cells were treated again in the same way. The combined ethanol extracts were evaporated to dryness and the residues resuspended in water (20 ml).

The water phase was then extracted with ethyl acetate (2 × 20 ml) After concentration, the residues were dissolved in ethyl acetate (2 ml) and analysed by normal phase HPLC.

2.3.4 Quantitative analyses

Standard solutions of compounds **1, 2** or **3** were prepared at various concentrations in Knapp buffer or complex medium. These solutions were then analysed by method A or B to plot standard curves for the quantitative analysis of the samples.

2.4 Toxicity tests

For the four bioassays, the tested compound was added in dimethylsulfoxide solution.

2.4.1 Microtox® assays[15]

The inhibition (EC_{50}) of bioluminescence of *V fischeri* by the chemicals tested was measured after various exposure times (5, 15 and 30 min). All the materials for analysis (test reagent, diluent, osmotic adjusting solution, reconstitution solution) were supplied by Azur Environmental (Carlsbad CA, USA). Assays were performed with a Microbics M 500 analyser coupled to a PC computer using 500 DOS software for Microtox®.

2.4.2 Test methods for Tetrahymena pyriformis

2.4.2.1 Determination of IC_{50} by microplate method. This technique, adapted from the method developed by Sauvant *et al*,[21] was based on the determination of the median inhibitory concentration IC_{50}, which is the concentration of toxicants required to induce a 50% increase in the Relative Doubling Time (RDT).

A 15-h culture of *T pyriformis* strain was first prepared in PPYS medium.[22] This exponential phase culture (10^4 cells ml^{-1}) was dispensed in a 96-well microplate (200 µl per well). After 10 h, the tested substances were added (T_0), eight different concentrations being tested. The microplates were incubated at 28 °C in the dark for 14 h; the absorbance of each well was then measured spectrophotometrically against PPYS as a blank with a microplate reader (Biorad 500) fitted with a 540 nm test wavelength filter. The growth of *T pyriformis* populations in the microplate for each tested concentration of xenobiotic was evaluated by plotting the OD_{14}/OD_0 ratio, where OD_{14} and OD_0 are the optical densities at T_{14} and T_0.

T pyriformis populations were characterized by their Doubling Time (DT). In the presence of toxic substances, a decrease in cellular proliferation was noted and induced an increase in DT. For each tested concentration of substance, the DT was determined and expressed relative to the DT of a concurrently performed control culture (arbitrarily set at 100%), and named Relative Doubling Time (RDT, expressed as a percentage). Finally, the 50% inhibitory concentration (IC_{50}) was calculated by regression analysis

Table 1. Screening of strains for diuron degradation

Fungus	*Depletion (%)*
Cunninghamella elegans	100
Mortierella isabellina	58
Beauveria bassiana	58
Fusarium oxysporum	4
Geotrichum candidum	0

Conditions of incubation: Initial concentration 40 mg litre^{-1}; wet weight cells: 50 g litre^{-1}. Analysis by method A (Section 2.3.2).

(y = RDT(%) and x = concentration (mg litre^{-1})). Experiments were repeated four times for each substance.

2.4.2.2 FDA method. This biotest is based on *in vivo* non-specific esterase activities. The non-polar fluorescein diacetate (FDA) enters the cell where it is hydrolysed by esterases to yield fluorescein, which is retained in the cytoplasm since it is a polar compound. The degree of fluorescence depends on the physical and metabolic state of the cell and has been proven as a reliable indicator of the inhibitory effects of pollutants.[23,24] This FDA method has been used as biomarker in different organisms (ie. microalgae,[25] rotifers,[26] activated sludges[27]). Its application to *T pyriformis* has been described elsewhere.[17] In the FDA method, the median effective concentration (EC_{50}) is the concentration of xenobiotic required to induce a 50% decrease in the fluorescence compared with untreated cells. The EC_{50} values were quantified by regression analysis computed with StatView SE 1.03 according to this model:

$$\text{Decrease in fluorescence } (\%) = a + b\ (\log(\text{concentration}))$$

2.4.3 Spirostomum teres bioassay

This test, developed by Twagilimana *et al*,[19] was based on the determination of the 24-h LC_{50} (which is the concentration of the substance required to induce a 50% mortality compared with controls) after probit analysis according to Finney.[28]

3 RESULTS

3.1 Screening

A first series of tests was carried out with several fungal strains to choose the most efficient for diuron degradation. Commercially available micro-organisms were used: *Beauveria bassiana, Cunninghamella elegans, Fusarium oxysporum, Geotrichum candidum* and *Mortierella isabellina*.

These degradation studies were carried out using resting cells in Knapp buffer with a diuron concentration of 40 mg litre^{-1}. The disappearance of diuron was evaluated by reverse phase HPLC (method A) after seven days incubation. Analyses of the abiotic references showed that the depletion observed for diuron was due only to biotransformation.

Among the fungal strains tested, only three were able to transform diuron to any great extent (up to 50%) after seven days incubation: *B bassiana, C elegans*, and *M isabellina* (Table 1). With *F oxysporum* and *G candidum*, no degradation occurred. *C elegans* was the most efficient, no diuron remaining after seven days' incubation.

Accordingly, a kinetic and quantitative study on the biodegradation of diuron by *B bassiana, C elegans* and *M isabellina* was performed.

3.2 Metabolite identification

Diuron disappearance was monitored by normal phase HPLC (method B).

Diuron degradation by the three fungal strains led to the formation of two metabolites obtained in different ratios according to the micro-organism. Figure 2 shows an example of degradation: it presents the normal phase HPLC chromatogram (eluent: ethyl acetate + cyclohexane (60 + 40 by volume); flow rate: 0.5 ml min^{-1}) of the ethyl acetate extracts from the culture of *B bassiana* after three days' incubation with diuron. Diuron eluted at a retention time of 14.6 min and two other products were observed at retention times of 17.7 and 33.7 min.

To isolate these metabolites, a quantitative assay was carried out on ten conical flasks. After one week's incubation, the supernatants were combined and continuously extracted for 48 h with ethyl acetate. After concentration of the organic layer, the residue was purified by column chromatography using silica gel (eluent: ethyl acetate + cyclohexane (60 + 40 by volume)).

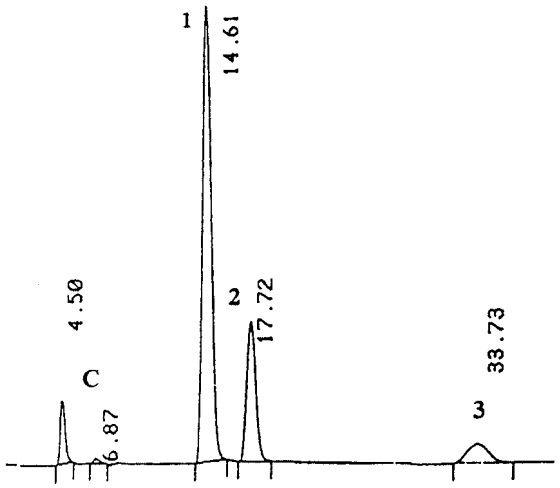

Figure 2. Biodegradation of diuron by *Beauveria bassiana*: HPLC analysis, after three days' incubation, of supernatant by method B. (Section 2.3.2). Conditions of incubation: initial diuron concentration: 40 mg litre^{-1}; wet weight cells: 50 g litre^{-1}. **C**: negative control; **1**- diuron; **2**-*N*-(3,4-dichlorophenyl)-*N'*-methylurea; **3**-N-3,4-dichlorophenylurea.

The unknown compounds were identified by NMR and mass spectrometry as the demethylation products **2** and **3** (Fig 1). After injection of these two isolated products on a normal phase column, *N*-3,4-dichlorophenyl-*N*′-methylurea **2** and 3,4-dichlorophenylurea **3** eluted at retention times of 17.7 min and 33.7 min respectively.

3.3 Syntheses of the metabolites

To confirm the structures of the different metabolites isolated, and monitor their formation during a degradation study, the demethylation products **2** and **3**, which are not commercially available, were synthesized according to a method adapted from the work of Crosby and Tang.[29]

Comparison of the spectral data (NMR, MS) showed that the synthesized compounds and the metabolites presented the same structures.

3.4 Kinetic and quantitative studies

The standard curves for **1, 2** and **3** enabled a quantitative study of diuron depletion and the formation of its metabolites to be carried out. Results were reproducible and the confidence limits were about 1 mg litre^{-1}.

For the three fungal strains, diuron degradation led to the formation of the demethylated products, but the rate of diuron disappearance and metabolite formation depended on the micro-organism used.

Table 2 presents the time course of the concentration of diuron and its metabolites during biodegradation assays with resting cells (initial diuron concentration 40 mg litre^{-1}).

Not all the micro-organisms tested had the same degradative behaviour. *C echinulata* var *elegans* was the most efficient, diuron being totally degraded within seven days; after one day's incubation, only 11 mg litre^{-1} of diuron was left. Although *B bassiana* and *M isabellina* degraded half of the diuron added in one day, the degradation slowed down, and, after two weeks' incubation, 17 mg litre^{-1} of diuron still remained.

In all cases, the major metabolite was the monodemethylated product **2**. The didemethylated product **3** was observed after a lag of two to seven days (according to the micro-organism tested) and its concentration remained very low (less than 2 mg litre^{-1} with *M isabellina*). The formation of **3** was accompanied by a significant disappearance of **2** in the case of *C elegans*. On the contrary, no relationship between **2** and **3** was observed with *M isabellina* and *B bassiana*.

Table 2. Quantitative study of diuron degradation

		1	**2**	**3**
Strain	*Incubation (days)*	(*mg litre*$^{-1}$)		
C elegans	1	11	15	0
	7	0	9	8
	14	0	8	10
M isabellina	1	24	5	0
	7	17	8	1
	14	17	10	2
B. bassiana	1	19	4	0
	7	17	7	3
	14	17	9	4

Conditions are in Table 1; analysis by method B.

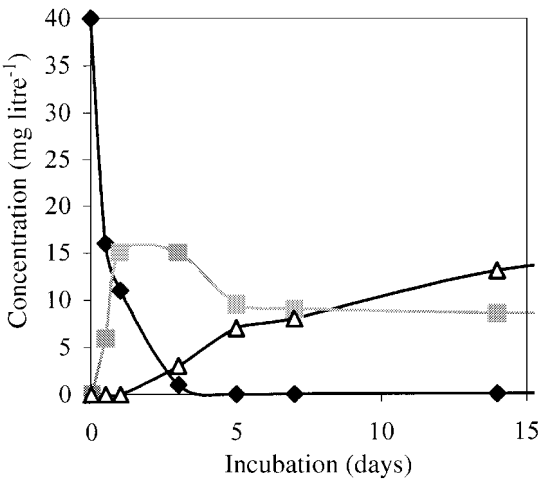

Figure 3. Kinetics of diuron degradation by *Cunninghamella elegans*. (◆) Diuron, (▤) **2;** (△) **3**. Conditions of incubation: initial diuron concentration: 40 mg litre^{-1}; wet weight cells: 50 g litre^{-1}.

Figure 3 shows the kinetics for the most efficient strain, *C elegans*. After four incubation days, no diuron was left and the two demethylated products **2** and **3** were detected. The concentration of **2** reached a maximum of 15 mg litre^{-1} after two days and then decreased slowly to 6 mg litre^{-1}. The depletion in **2** was accompanied with an increase in the concentration of metabolite **3**, which reached 10 mg litre^{-1} after two weeks' incubation.

3.5 Toxicity tests

Diuron was transformed by fungi, but the degradation was not complete in all cases. As the derivatives obtained may have been more toxic than diuron itself, four biotests were used to determine the toxicities of the identified degradation products and these were compared with that of diuron.

The toxicity data for diuron and its biodegradation products are presented in Table 3.

According to Microtox® test, the metabolites **2** and **3** presented a three times higher toxicity than that of diuron. Inhibition of bioluminescence of *V fischeri* by these compounds was not gradual and peaked after 5 min exposure. This biotest showed very small differences between EC_{50} values of monodemethylated product **2** and didemethylated one **3**.

In the mean time, determination of acute toxicity on ciliated protozoan populations (*T. pyriformis* and *S teres*) revealed a higher biological effect of compound **2** *versus* compound **3**. Although diuron was less active than its metabolites on *S. teres* populations, a non-negligible value for its IC_{50} was observed on *T pyriformis* populations.

Microbiotest	1	2	3
	mg litre^{-1} ±SD (μM ±SD)		
Microtox®			
EC_{50} 5 min	68.16±5.44 (290±23)	18.50±0.86 (85±4)	15.02±1.16 (73±6)
EC_{50} 10 min	66.87±7.47 (280±32)	17.96±1.46 (83±7)	17.29±1.60 (85±8)
EC_{50} 30 min	58.07±6.71 (250±29)	16.52±1.66 (76±8)	17.24±1.45 (85±7)
FDA			
IC_{50} 1 h	>100 (>430)	>100 (>460)	18.70±3.32 (91±16)
Tetrahymena pyriformis			
IC_{50} 14 h	6.33±1.11 (27±5)	2.28±0.24 (10±1)	10.23±1.83 (48±9)
Spirostomum teres			
LC_{50} 24 h	40.20 (170)	6.17 (28)	10.18 (50)

Table 3. Comparison of toxicity values obtained for diuron (**1**) and its degradation products (**2, 3**) in four biotests

The FDA test appeared to be insufficiently sensitive for two of the tested substances (**1** and **2**), but this bioassay revealed a marked diminution of the degree of fluorescence in the presence of compound **3**. This indicated a great difference in the *in vivo* inhibition of esterases activities by the two demethylated metabolites.

4 DISCUSSION

The biodegradation of diuron by the three fungal strains tested produces the mono and didemethylation of the urea function (**2, 3**). These metabolites were first reported in 1966 by Dalton *et al*,[30] who analysed samples of soils treated annually with diuron and isolated the intermediate breakdown products. However, the identification of the degradation products was confirmed only by infrared absorption. Since then, other published degradation studies obtaining these metabolites have been reported,[8–11] but no quantitative studies are available.

Among the strains tested, *C elegans* was the most efficient at degrading diuron, no herbicide remaining after five days incubation. Tillmanns *et al.*[12] and Vroumsia *et al*[6] have already noted the high efficiency of this strain for phenylurea herbicide degradation.

The monodemethylated product **2** is the major metabolite obtained with resting cells of the three fungal strains especially with *M isabellina*. Assays were carried out with growing cells in complex medium to assess the influence of another energy source. Figures 4A and 4B show the degradation by *M isabellina* with resting cells and growing cells, respectively. Although the degradation is favoured by co-metabolism conditions, the proportion of the didemethylated compound remains very low.

During the degradation by the fungal strains, no balance between loss of diuron and appearance of

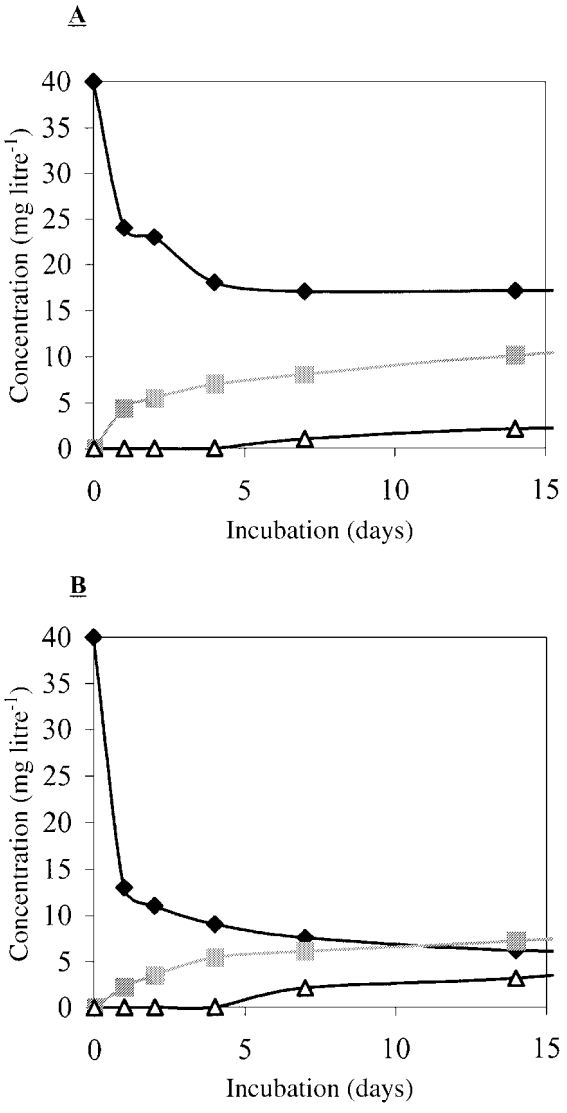

Figure 4. Kinetics of diuron degradation by *Mortierella isabellina*. (◆) Diuron; (▤) **2;** (△) **3**. A - with resting cells in mineral medium. B - with growing cells in complex medium. Initial diuron concentration: 40 mg litre^{-1}.

metabolites was observed, and analyses were therefore carried out on the whole content of a conical flask at each time of the kinetic study, instead of taking samples; however the same results were obtained. Moreover, cell extraction with ethanol showed that no retention in cells occurred. The mass balance might be explained by the formation of metabolites not detected at 254 nm or by the mineralization of part of the herbicide.

Although diuron is degraded by both sunlight and micro-organisms, the degradation is incomplete. As the degradation products may be more toxic than diuron itself, we determined their toxicity with four biotests: Microtox® test, *T pyriformis* population test, *T pyriformis* FDA method and *S teres* test.

These four tests thus focused on two different criteria: for the Microtox® test and the FDA method, the toxicity was determined by the evaluation of the direct inhibition of enzymatic activities. For the two other biotests, we assessed a global effect on a series of processes involved in cellular division. The degradation products showed toxicities different from that of diuron.

With the Microtox® test, diuron led to a lower inhibition of *Vibrio fischeri* bioluminescence than its metabolites. These results are in agreement with determination of antimicrobial activities against Gram-positive and Gram-negative bacteria by the conventional agar diffusion method (data not shown): the demethylated compounds exhibited the greatest bacterial growth inhibition. Although *T pyriformis* seemed to be very sensitive to diuron, determination of acute toxicity on eukaryotic ciliate protozoan populations also showed a higher toxicity of the metabolites compared to the parent herbicide. However, with these tests, we noted a marked effect of the monodemethylated compound **2**. Microbiotests using ciliated protozoan populations seemed to be particularly well suited to non-target toxicity studies of phenylurea herbicides in aquatic ecosystems.

Bioassays based on *in vivo* enzymatic inhibition are well known for their higher sensitivity than acute toxicity assays, but the *T pyriformis* FDA method (a sub-lethal biotest) seemed to be not sensitive enough for diuron and metabolite **2**. These compounds did not affect *in vivo* non-specific esterase activities. Differences in sensitivity of the two biotests on *T pyriformis* may be partially explained by the fact that the IC_{50} against population was determined after 14 h of exposure, whereas the FDA IC_{50} values were estimated only up to 1 h exposure. During this period, the tested substances might be partially bioconverted into more toxic compounds.

All these results show that the evaluation of the toxicity depends on the type of biotest, so it is of great importance to carry out various bioassays to estimate hazards due to xenobiotics.

The mono- and didemethylated metabolites are more toxic than diuron. These results stress the importance of knowing the nature of the breakdown products to assess the impact of a polluting agent. The pollutant may undergo reactions yielding compounds that present a non-target toxicity higher than that of the parent substance. Biodegradation studies must not therefore be limited to the disappearance of the pollutant if it is not mineralized: it is essential to identify the intermediate products and compare their toxicities with that of the pollutant.

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