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DEGRADATION PRODUCTS OF A PHENYLUREA HERBICIDE, DIURON: SYNTHESIS, ECOTOXICITY, AND BIOTRANSFORMATION

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Abstract—The degradation products of diuron (photoproducts and metabolites), already described in the literature, were synthesized in order to carry out further investigations. Their ecotoxicity was determined using the standardized Microtox® test, and most of the derivatives presented a nontarget toxicity higher than that of diuron. Therefore, the biotransformation of these compounds was tested with four fungal strains and a bacterial strain, which were known to be efficient for diuron transformation. With the exception of the 3,4-dichlorophenylurea, all the degradation products underwent other transformations with most of the strains tested, but no mineralization was observed. For many of them, the biodegradation compound for which the toxicity was important was 3,4-dichlorophenylurea. This study underlines the importance of knowing the nature of the degradation products, which has to be kept in mind while analyzing natural water samples or soil samples.

Keywords—Phenylurea herbicides Diuron Degradation intermediates Biotransformation Toxicity

INTRODUCTION

Since their discovery in the early 1950s, phenylurea derivatives have grown into one of the most prominent and diversified groups of pesticides. One of the most important such compounds, diuron, N-3,4-dichlorophenyl-N′,N′-dimethylurea, is principally employed for selective control of germinating grass and broad-leaved weeds in many crops (mainly cereals) but also is used for weed control on noncultivated areas (maintenance of roads, railways, parks, etc.).

This herbicide is applied on soil, where it shows great persistence, four to eight months, depending on humidity and soil type [1,2]. However, several studies have reported the presence of diuron in surface water [3,4]. In the face of growing concern over its potential to contaminate surface and ground water, a large amount of data has been collected on the direct and indirect effects of diuron on target and nontarget organisms (for a review, see [1]), and diuron is suspected of being genotoxic [5]. Most toxicity studies have focused on the herbicide [6–9], but the degradation products can also be involved in environmental pollution. It is thus essential to collect data on the fate of this herbicide after spreading on soils. In parallel to field studies carried out directly in contaminated areas, information at the laboratory scale is particularly interesting because it concentrates on the molecular aspect of degradation by characterization and quantification of intermediate products. Two major degradation paths have to be considered, those being phototransformation by sunlight and biodegradation by soil microorganisms.

Since its commercialization, numerous studies have investigated both of these aspects for diuron. The main identified degradation products are presented in Figure 1. The phototransformation has been reported by Jirkhovsky et al. [10], and several photoproducts have been identified after irradiation under different conditions. In aqueous solution, the main primary photoproducts (products 1 and 2) resulted from the substitution of the chlorine atom by a hydroxyl group. Irradiation after dispersion on solid support mainly led to the formation of the N-(3,4-dichlorophenyl)-N′,N′-formylmethylurea (product 3) and the demethylated products 4 and 5.

Other studies have also been carried out on the microbial degradation of diuron. Most of this work has consisted of monitoring the disappearance of diuron or finding microorganisms able to degrade it, with no identification of metabolites [11–15]. A few studies have described the nature of the metabolites; the main metabolites observed were the mono- and didemethylated derivatives (products 4 and 5) and 3,4-dichloroaniline (product 6) [16–19].

In previous work, we reported toxicity studies carried out on metabolites 4 and 5 showing that the degradation products present nontarget toxicity higher than that of the parent molecule [20].

Because the degradation compounds may reach surface or ground water, the aim of the present work was to study the compounds coming from the initial transformation of diuron. Different aspects were successively considered, including synthesis and determination of the toxicity (Microtox® test, Azur Environmental, Carlsbad, CA, USA) of all these derivatives, biotransformation assays on these compounds using five strains efficient for diuron degradation (four fungal strains and a bacterial strain), isolation and identification (nuclear magnetic resonance [NMR], mass spectrometry [MS]) of the new metabolites obtained, and comparison of metabolites toxicity with that of the parent molecules.

All the toxicity studies were performed with the Microtox test. This system, using the bioluminescent bacterium *Vibrio fischeri*, is a standardized test that is widely used. It is cost efficient, is not time consuming, and requires little substance for several replicate tests [21].

MATERIALS AND METHODS

Synthesis of metabolites and photoproducts

The synthesis of the dealkylated compounds 4 and 5 have been described elsewhere [20].

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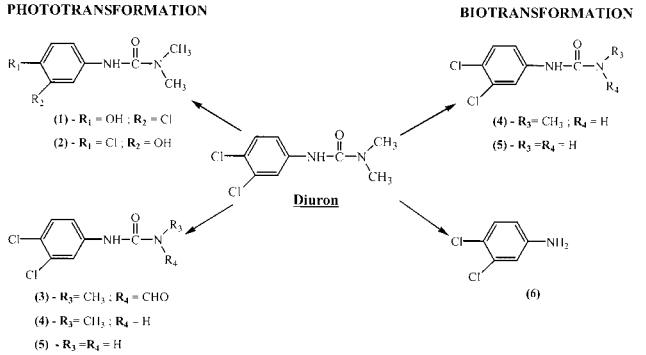

Fig. 1. General scheme of the photo- and biotransformation of diuron.

The herbicide diuron (purity, 98%) was obtained from Rhône-Poulenc (Lyon, France).

Chemicals. The 3,4-dichlorophenylisocyanate, methylamine, N-methylformamide, 2-methoxy-4-nitroaniline, *tert*-butylnitrite, dimethylcarbamylchloride, and 3,4-dichloroaniline were purchased from Aldrich (Saint-Quentin-Fallavier, France). The 2-chloro-4-nitrophenol was obtained from Lancaster (Bischheim, France).

Syntheses. For synthesis of N-(3,4-dichlorophenyl)-N′-formyl-*N′*-methylurea (product 3), an equimolar solution of 3,4-dichlorophenylisocyanate and N-methylformamide was prepared as a 30% (w/w) acetone solution. The mixture was stirred under reflux for 24 h. After filtration, the white solid obtained was purified on silica gel (eluent was ether:cyclohexane:ethanol [75:20:5]).

For product 3, the yield was 70%, rate of flow (Rf) was 0.49, melting point (mp) was 145 to 146°C: for ^{1}H NMR (dimethylsulfoxide [DMSO] d_6-δ), 3.45 ppm (s, 3H), 7.54 ppm (dd, 1H, J = 2.4 Hz and J = 8.8 Hz), 7.62 ppm (d, 1H, J = 8.8 Hz), 7.90 ppm (d, 1H, J = 2.4 Hz), 9.15 ppm (s, NH), 9.87 ppm (s, CHO); for ^{13}C NMR (DMSO d_6-δ), 27.2 ppm (CH_3), 120.4 ppm (C_6), 121.7 ppm (C_2), 125.3 ppm (C_4), 130.6 ppm (C_5), 130.9 ppm (C_3), 138.7 ppm (C_1), 153.1 ppm (CO), 162.8 ppm (CHO). Analysis was done for $C_9H_8Cl_2N_2O_2$ (C 43.75; H 3.26; N 11.33), and we found C 43.55, H 3.23, N 11.44.

Hydroxylated compounds 1 and 2 were synthesized by reaction of the appropriate chlorohydroxyaniline (obtained by catalytic hydrogenation of the nitro compound on PtO_2 in absolute ethanol) with an excess of dimethylcarbamylchloride (5 equivalents) under an argon atmosphere in anhydrous tetrahydrofurane. The mixture was stirred overnight at room temperature. The reaction mixture was then diluted with ether and washed several times with water. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was purified on silica gel using the mixture ether:cyclohexane:ethanol (75:20:5) as eluent.

N-(3-chloro-4-hydroxyphenyl)-*N′,N′*-dimethylurea (compound 1) was synthesized from 4-amino-2-chlorophenol obtained from the corresponding commercial nitroderivative.

The yield for compound 1 was 30%, Rf was 0.23, mp was 119 to 120°C: for ^{1}H NMR (CD_3OD-δ), 3.20 ppm (s, 6H), 7.01 ppm (d, 1H, J = 8.7 Hz), 7.27 ppm (dd, 1H, J = 2.5 Hz and 8.7 Hz), 7.54 ppm (d, 1H, J = 2.5 Hz); for ^{13}C NMR (CD_3OD-δ) 37.0 ppm (2 CH_3), 117.5 ppm (C_5), 121.4 ppm (C_3), 123.3 ppm (C_6), 125.0 ppm (C_2), 133.8 ppm (C_1), 150.7 ppm (C_4), 159.4 ppm (CO). Analysis was done for $C_9H_{11}ClN_2O_2$ (C 50.36, H 5.16, N 13.05, O 14.90), and we found C 50.20, H 5.28, N 13.04, O 14.74.

N-(4-chloro-3-hydroxyphenyl)-*N′,N′*-dimethylurea (compound 2) was obtained from 5-amino-2-chlorophenol, previously synthesized from 2-methoxy-4-nitroaniline.

To synthesize 2-chloro-5-nitroanisole, 4.8 g (36 mmol) of $CuCl_2$ and 5.45 ml (36 mmol) of *tert*-butylnitrite were mixed in 70 ml of acetonitrile. The resulting stirred mixture was cooled to 0°C in an ice bath. A solution of 2-methoxy-4-nitroaniline (5 g, 30 mmol) in 50 ml of acetonitrile was slowly added. After the complete addition of the amine, the reaction mixture was left at 0°C for 2 h and then allowed to reach room temperature. The reaction solution was then poured into 80 ml of 20% (v/v) aqueous HCl and extracted with ether. The organic layer was washed once with 20% (v/v) aqueous HCl and dried over $MgSO_4$. The ether phase was removed under reduced pressure and, after recrystallization in aqueous ethanol, 3.8 g of an orange solid was obtained.

The yield of 2-chloro-5-nitroanisole was 67%, mp was 80 to 82°C (mp value from the literature [24] was 82–83°C): for MS, 187 to 189 ($M^{+\cdot}$), 141 and 143 (M-NO_2), 126 and 129 (M-NO_2-CH_3), 111 and 113 (M+1-NO_2-OCH_3); for ^{1}H NMR ($CDCl_3$-δ), 4.00 ppm (s, 3H), 7.53 ppm (d, 1H, J = 8.5 Hz), 7.79 ppm (d, 1H, J = 2.5 Hz), 7.90 ppm (dd, 1H, J = 8.5 Hz and J = 2.5 Hz); for ^{13}C NMR ($CDCl_3$-δ), 56.7 ppm (CH_3), 106.8 ppm (C_6), 116.3 ppm (C_4), 130.0 ppm (C_2), 130.5 ppm (C_3), 147.4 ppm (C_5), 155.5 ppm (C_1).

To synthesize 2-chloro-5-nitrophenol, 5 g (27 mmol) of 2-chloro-5-nitroanisole was refluxed in 30 ml of aqueous HBr (48%, w/v) for 24 h. After cooling to room temperature, the reaction mixture was extracted with diethyl ether (total volume = 200 ml). The organic phase was then washed twice with 50 ml of distilled water and extracted three times with a Na_2CO_3 (10%, w/v) solution. The aqueous yellow phase was then acidified with concentrated HCl to pH 1 and extracted with Et_2O. The combined ether extracts were dried over $MgSO_4$ and the solvent was evaporated to dryness, with 2.6 g of yellow crystals being obtained.

The yield of 2-chloro-5-nitrophenol was 56%, mp was 110 to 112°C (mp value from the literature [24] was 118–119°C): for MS, 173 and 175 ($M^{+\cdot}$), 143 and 145 (M-NO), 127 and 129 (M-NO_2), 115 and 117 (M-NO-CO), and 46 (base, NO_2); for ^{1}H NMR ($CDCl_3$-δ), 6.00 ppm (s, OH), 7.51 ppm (d, 1H, J = 8.8 Hz), 7.79 ppm (dd, 1H, J = 8.8 Hz and J = 2.6 Hz), 7.90 ppm (d, 1H, J = 2.6Hz).

2-Chloro-5-aminophenol (compound 8) was obtained after catalytic hydrogenation (on PtO_2 in absolute ethanol) of the 2-chloro-5-nitrophenol. The yield was 90%, Rf was 0.20, mp was 158 to 160°C with the following results: for MS, 143 to 145 ($M^{+\cdot}$), 105, 80, 77, 50; for ^{1}H NMR (400.13 MHz, $CDCl_3$-δ), 3.70 ppm (s, NH_2), 5.45 ppm (s, OH), 6.22 ppm (dd, 1H, J = 2.5 Hz and J = 8.7 Hz), 6.36 ppm (d, 1H, J = 2.5 Hz), 7.06 ppm (d, 1H, J = 8.7 Hz).

For *N*-(4-chloro-3-hydroxyphenyl)-*N′,N′*-dimethylurea (compound 2), the yield was 28%, Rf was 0.25, mp was 220 to 221°C: for ^{1}H NMR(CD_3OD-δ), 3.20 ppm (s, 6H), 6.97 ppm (dd, 1H, J = 2.5 Hz and 8.8 Hz), 7.30 ppm (d, 1H, J = 2.5 Hz), 7.32 ppm (d, 1H, J = 8.8 Hz); for ^{13}C NMR (CD_3OD-δ), 37.0 ppm ($2CH_3$), 109.7 ppm (C_2), 114.4 ppm (C_6), 115.9 ppm (C_4), 130.6 ppm (C_5), 141.2 ppm (C_1), 154.4 ppm (C_3), 158.9 pp, (CO). Analysis was done for $C_9H_{11}ClN_2O_2$ (C 50.36, H 5.16, N 13.05, O 14.90), and we found C 50.57, H 5.23,N 13.13, O 15.11.

N-(4-chloro-3-methoxyphenyl)-*N′,N′*-dimethylurea (compound 7) was prepared according to the process already used for the hydroxylated compounds. The starting aniline was obtained from the 2-chloro-5-nitroanisole, the synthesis of which is described above.

The yield of compound 7 was 72%, Rf was 0.33, mp was 175 to 177°C: for MS, 228 and 232 ($M^{+\cdot}$), 183 and 185 (M-$NH(CH_3)_2$), 156 and 158 (M-1-$CON(CH_3)_2$); 72 ($CON(CH_3)_2$); for ^{1}H NMR (400.13 MHz, acetone d_6-δ), 3.00 ppm (s, 6H, $N(CH_3)_2$), 3.82 ppm (s, 3H, OCH_3), 7.08 ppm (dd, 1H, J = 2.3 Hz and J = 8.5 Hz), 7.17 ppm (d, 1H, J = 8.5 Hz), 7.51 ppm (d, 1H, J = 2.3 Hz), 7.84 ppm (s, NH); for ^{13}C NMR (100.61 MH_z, acetone d_6-δ), 35.6 ppm ($N(CH_3)_2$), 55.1 ppm (OCH_3), 103.9 ppm (C_2), 111.5 ppm (C_6), 114.1 ppm (C_4), 129.2 ppm (C_5), 141.1 ppm (C_1), 154.8 ppm (C_3), 155.4 ppm (CO).

Microbiology

Microbial strains. Fungal strains were commercially available. *Beauveria bassiana* American Type Culture Collection (ATCC) 7159, *Cunninghamella echinulata* var. *elegans* ATCC 9245, *Aspergillus niger* ATCC 9142, and *Mortierella isabellina* were obtained from Northern Regional Research Laboratory 1757 (Peoria, IL, USA). *Arthrobacter* sp. was provided by N. Truffaut (Université de Technologie de Compiègne, France) after isolation from a soil treated with diuron for many years.

Culture conditions. Microorganisms were all laboratory grown. Preculture and culture conditions for fungi have already been described elsewhere [22]. The bacterial strain *Arthrobacter* sp. N2 was grown for 24 h (preculture and culture) on Trypcase soya broth (bioMerieux-Marcy-l'Etoile, France). Cultures in 500-ml conical flasks containing 100 ml of medium were incubated in the dark at 27°C (or at 30°C for *Arthrobacter* sp.) on a shaking platform (200 rpm).

Biodegradation conditions. Cell cultures were harvested under sterile conditions by filtration on a sintered glass filter or by centrifugation (15 min, 8000 rpm). Cells were washed twice with Knapp buffer (1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 4 mg/L $FeCl_3.6H_2O$, 40 mg/L $MgSO_4.7H_2O$; pH 6.6) and suspended in this buffer at a final concentration of 50 g/L wet weight cells (or 25 g/L for *Arthrobacter* sp.). Stock solutions of diuron or degradation products in dimethylsulfoxide (40 mg/ml, or 30 mg/ml for product 3) were filter sterilized through a 0.2-μm pore size membrane. The final xenobiotic concentration was 40 mg/L (or 30 mg/L for product 3).

Incubation of cells under the same conditions in the presence of dimethylsulfoxide alone constituted a negative control. Solutions of the xenobiotic in Knapp buffer at a final concentration of 40 mg/L (or 30 mg/L for compound 3) were used for abiotic references.

Each biodegradation study included assays with diuron to check that the degradation conditions were comparable.

Analyses

Analytical method. The HPLC analyses were performed using a Waters 600E chromatograph fitted with a normal phase column (Nucleosil 3 μm, 150 × 4.6 mm; Interchim-Montluçon, France) at room temperature. Detection was performed with a Waters 486 ultraviolet detector set at 254 nm. The NMR spectra were performed on a Bruker AC 400 (^{1}H, 400 MHz, ^{13}C, 100 MHz). Mass spectra were recorded on a spectrometer Hewlett-Packard 5959B (Avondale, PA, USA) by electronic impact. Microanalyses (determination of the carbon, hydrogen, and oxygen content from a given weight of synthesized compound) were made at the Laboratoire Central de Microanalyse du CNRS in Vernaison (France).

Sample analyses. Samples (7 ml) were periodically removed aseptically and centrifuged (15 min, 4,000 rpm). Four milliliters of supernatant were extracted twice with 1 ml of ethyl acetate, and the combined crude extracts were then evaporated to dryness in a rotary evaporator under reduced pressure. The residues were then 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) at a flow rate of 0.5 ml/min. The recovery of the extraction method was around 90% for diuron and compounds 1, 2, and 8 and 95% for compounds 3 through 7.

Quantitative analyses. Standard solutions of diuron and compounds 1 to 8 were prepared at various concentrations in Knapp buffer. These solutions were then analyzed by the method described above to plot standard curves for the quantitative analysis of the samples.

Isolation of the metabolites

To isolate all the metabolites observed (compounds 4–8), quantitative biodegradation assays with a given strain were carried out on 10 conical flasks. After one incubation week, the supernatants of each assay were combined and continuously extracted for 48 h with ethyl acetate. After concentration of the organic layers, the residues were purified by column chromatography using silicagel (eluent, ethylacetate:cyclohexane [60:40], except for compounds 7 and 8, which used the eluent diethylether:cyclohexane:ethanol [75:20:5]).

Toxicity tests (Microtox assays)

The toxicity of degradation products was determined with a Microtox test. This test consists of determining the concentration of a toxic compound that inhibits 50% of the natural bioluminescence (EC50) of the marine bacterium *V. fischeri.* The emission is measured after various exposure times (5, 15, and 30 min). A decrease of EC50 corresponds to an increase of toxicity. The tested compounds were dissolved in dimethylsulfoxide (maximal final concentration of DMSO was 6.4 mM). All the materials for analysis (test reagent, diluent, osmotic adjusting solution, reconstitution solution) were supplied by Azur Environmental. For each compound, assays were performed four times with a Microbics M 500 analyzer coupled to a PC using 500 DOS software for Microtox.

RESULTS

Syntheses of degradation products

In order to perform complementary studies on the degradation products, the first part of our work required the synthesis of all the degradation products not commercially available.

The syntheses of the dealkylated products 4 and 5 have already been described elsewhere [20]. The *N*-(3,4-dichlorophenyl)-*N′,N′*-formylmethylurea (product 3) was obtained from a method proposed by Crosby and Tang [23] after reaction of 3,4-dichlorophenylisocyanate with N-methylformamide.

Hydroxylated photoproducts 1 and 2 were synthesized from two chloronitrophenols, i.e., commercial 2-chloro-4-nitrophenol for product 1 and 2-chloro-5-nitrophenol for product 2. The latter was obtained from 2-methoxy-4-nitroaniline according to Figure 2.

We failed to obtain 2-chloro-5-nitrophenol from 2-amino-

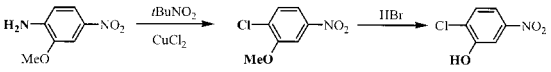

Fig. 2. Synthesis of 2-chloro-5-nitrophenol.

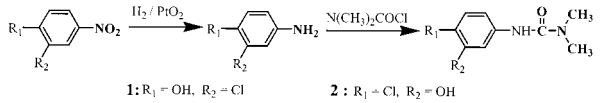

Fig. 3. Synthesis of photoproducts 1 and 2.

5-nitrophenol, so we chose to work with 2-methoxy-4-nitroaniline as described by Bonilha et al. [24]. They obtained 2-chloro-5-nitroanisole with a low yield due to the first step, which was the addition of a diazonium salt to the cuprous halide (Sandmeyer reaction: 10%). The use of *tert*-butylnitrite (as nitrosating agent) and anhydrous copper II halide, as described by Doyle et al. [25], gave a 67% yield for this compound.

Once the two chloronitrophenols had been obtained, photoproducts 1 and 2 were prepared as described Figure 3.

Microtox test

Diuron underwent both photo- and biodegradation, but the degradation was not complete. Because the derivatives obtained may be more toxic than parent molecule, the toxicity of each identified degradation product was evaluated using the standard Microtox test and compared with that of diuron. The values obtained are shown in Table 1; all compounds were tested in the same experimental conditions. The hydroxylated compounds 1 and 2 presented a nontarget toxicity similar to that of diuron, whereas all the other degradation products were more toxic than parent molecule. The more important biological effects on the bacteria were observed with the 3,4-dichloroaniline (compound 6) and the compound presenting a formamide function (compound 3) (EC50 = 3 and 30 μM, respectively). The mono- or didemethylated compounds 4 and 5 presented a three times higher toxicity than diuron. However, this biotest showed very small differences between EC50 values of these last two compounds.

Biodegradation studies

The Microtox test clearly showed that most of the degradation products of diuron were much more toxic than the parent compound. So, to assess the fate of these degradation products in the environment, the biodegradability of the derivatives, products 1 through 6, was tested with four fungal strains, including *A. niger, Beauveria bassiana, C. echinulata* var. *elegans*, and *Mortierella isabellina,* and a bacterial strain, *Arthrobacter* sp., isolated from soil treated with diuron for many years. The degradation studies were carried out using resting cells in buffer with a concentration of the compound tested of 40 mg/L (or 30 mg/L for product 3). Analyses of the abiotic references showed that the depletion observed for the different compounds was only due to biotransformation. The percentage of transformation was regularly estimated for the various degradation products by HPLC analysis; Table 2 shows the results obtained after six incubation days, the time period corresponding to a diuron depletion of more than 50% according to a previous study [20]. The same experimental conditions were used for all the compounds; however, the concentration in compound 3 was only 30 mg/L (as opposed to 40 mg/L for the others) due to the low water solubility of this product.

Among the strains tested, *M. isabellina* appeared to be the most efficient for the transformation of degradation products, each compound (except 5) being transformed at a rate higher than 50%, whereas this strain moderately transformed diuron. The *B. bassiana* and *C. elegans* strains were also efficient. On the contrary, the bacterial strain *Arthrobacter* sp. seemed to be very specific since this strain only transformed two compounds (2 and 3) out of the six degradation products tested to a great extent (80 and 90%, respectively).

The formylated product 3 was the most easily transformed; four strains were able to degrade more than 90% of this compound after six incubation days. On the contrary, the didemethylated compound 5 seems to be recalcitrant with all the microorganisms tested. The 3,4-dichloroaniline (product 6) was transformed by all the fungal strains, and the monodemethylated compound (product 4) was biotransformed by three of them. Furthermore, the strains did not present the same degradative behavior for both hydroxylated compounds; compound 2 was generally more easily transformed than its isomer, compound 1. The presence of a hydroxyl group in the *meta* position of the aromatic ring seemed to favor the transformation by the strains tested.

Identification and toxicity of new metabolites

Biotransformation of the hydroxylated photoproducts 1 and 2. Except with *M. isabellina,* the transformation percentage of product 1 by the fungal strains (Table 2) was always lower than that of the herbicide. Figure 4a presents degradation kinetics of product 1 with the four fungal strains. Although *C. elegans* was able to transform half of the added amount of product 1 in 1 d, the degradation quickly stopped and, after two incubation weeks, 55% of product 1 still remained intact. With the other three fungal strains, the degradation seemed to be gradual; product 1 was continuously transformed within two incubation weeks and no metabolite was observed with the analytical method used. *Arthrobacter* sp. was unable to

Table 1. Evaluation of the toxicity of diuron and its degradation products (1–6) with Microtox® test (exposure time 5 min, 4 assays for each compound)

		Degradation product					
EC50[a]	Diuron[b]	1 (OH *para*)	2 (OH *meta*)	3 (CHO)	4[b] ($NHCH_3$)	5[b] ($CONH_2$)	6 (aniline)
(mg/L ± SD)	68[c] ± 5	71 ± 3	72 ± 6	7.3 ± 0.9	18 ± 0.9	15 ± 1	0.48 ± 0.08
(μM ± SD)	290[c] ± 23	330 ± 16	330 ± 28	30 ± 4	84 ± 4	73 ± 6	2.98 ± 0.49

[a] EC50 = effective concentration 50% of the population; SD = standard deviation.
[b] Results obtained in the same experimental conditions during a previous study [20].
[c] Extrapolated values (higher than solubility).

Table 2. Extent of transformation (%) of diuron and degradation products (1–6) after six incubation days with the four fungal strains and *Arthrobacter* sp. Conditions of incubation were an initial concentration of 40 mg/L (or 30 mg/L for product 3) and wet weight of cells of 50 g/L

	Diuron[a]	Degradation product					
		1	2	3	4[a]	5[a]	6
Aspergillus niger	75	25	60	30	0	0	65
Beauveria bassiana	58	40	100	100	50	0	60
Cunninghamella elegans	100	45	38	100	80	0	75
Mortierella isabellina	58	63	55	88	83	0	91
Arthrobacter sp.	100	0	80	90	0	0	0

[a] Results obtained in the same experimental conditions during a previous study [20].

transform this photoproduct, which remained intact after two incubation weeks.

Except with *B. bassiana,* the transformation percentage of product 2 was lower than that of parent molecule (Table 2). *Beauveria bassiana* was the only strain able to transform the whole amount of product 2, and the transformation was very fast as no trace of product 2 was detected after two incubation days (Fig. 4b). No new metabolite was observed with *B. bassiana, C. elegans,* and *M. isabellina.*

However, for product 2, according to the HPLC analyses, new metabolites were detected with *A. niger* and *Arthrobacter* sp., eluted at 31 and 9.5 min, respectively. After isolation, the metabolite obtained with *A. niger* was identified by NMR and MS as the N-(4-chloro-3-methoxyphenyl)-N′,N′-dimethylurea (product 7) and with *Arthrobacter* sp. as the 2-chloro-5-aminophenol (product 8) (Fig. 5).

To confirm the structure of the two isolated metabolites and monitor their formation during a degradation study, the products 7 and 8, which are not commercially available, were synthesized. The compounds 7 and 8 were obtained from the 2-chloro-5-nitroanisole prepared during the synthesis of 2. Comparison of the spectral data (NMR, MS) showed that the synthesized compounds and the metabolites had identical structures.

Figure 6 presents the biotransformation kinetics of product 2 by *A. niger* (Fig. 6a) and *Arthrobacter* sp. (Fig. 6b). For both strains, the transformation was almost quantitative, and the metabolites accumulated in the medium. However, the transformation was slower with the fungal strain; within 1 d, only 10% of product 2 disappeared, while with *Arthrobacter* sp., the transformation rate was 50%.

According to the Microtox test, both compounds 7 and 8 present a toxicity higher than that of photoproduct 2 (EC50 = 330 μM), i.e., 140 and 149 μM, respectively.

Biotransformation of degradation product 3. The photoproduct 3 was the compound the most easily transformed by all the strains tested (Table 2). Figure 4c presents the time courses of transformation with the four fungal strains. This photoproduct quickly disappeared from the medium after incubation with *C. elegans* and *B. bassiana*; no trace of product 3 was detected after six incubation days. With *M. isabellina,* 80% of product 3 disappeared within 1 d, but the degradation rate abruptly decreased; after two incubation weeks, 7% of product 3 still remained intact. The biotransformation with *A. niger* was less efficient; after 12 incubation days, the depletion rate of product 3 only reached 19%.

The biotransformation of product 3 by the fungal strains led to the formation of two metabolites identified after isolation as the N-demethylated compounds (products 4 and 5) (Fig. 7).

Figure 8 presents the degradation kinetics of product 3 for the different fungal strains. With *A. niger* (Fig. 8a), the biotransformation consisted of quantitative formation of compound 4, but the transformation percentage was very low, around 30% after 12 incubation days. According to Figure 8b, *B. bassiana* was able to transform almost quantitatively the whole amount of product 3 within 6 d, and the formation of metabolite 4 corresponded to 83% of transformation. With *M. isabellina* (Fig. 8c), compound 4 was also the main metabolite observed, but its concentration only reached 35 μM, while only a small amount of product 3 was observed in the medium. During the biotransformation with *C. elegans* (Fig. 8d), the concentration of compound 5 was the highest attained, reaching more than 80 μM after 12 incubation days. In the first days, the formation of compound 5 seemed to correspond to the disappearance of compound 4, the concentration of compound 5 increasing concomitantly with the decrease in levels of compound 4. But after six incubation days, the concentration of compound 5 increased while concentration of compound 4 remained stable.

If we consider the toxicity data obtained for compounds 3, 4, and 5 (Table 1), the biotransformation of product 3 (Microtox, EC50 = 30 μM) into product 4 (Microtox, EC50 = 84 μM) or 5 (Microtox, EC50 = 73 μM) leads to a slight decrease in the biological effects on *V. fischeri.*

The biotransformation of product 3 by *Arthrobacter* sp. was accompanied by the formation of two metabolites identified as compounds 4 and 6. Both metabolites appeared simultaneously (Fig. 9); compound 6 seemed to come directly from cleavage of the amide bond of compound 3. The biotransformation of product 3 was very slow in comparison with that of diuron with this bacterial strain. No trace of diuron was detected after 10 h, whereas the whole amount of product 3 was transformed after two incubation weeks. As compound 6 presented high toxicity (Microtox: EC50 = 3 μM), the biotransformation of product 3 into compounds 4 and 6 was accompanied with an increase of the nontarget toxicity estimated with the Microtox test.

Biotransformation of the N*-demethylated compound (compound 4).* Among the strains tested, three fungal strains were able to transform product 4, i.e., the strains *B. bassiana, C. elegans,* and *M. isabellina* (Table 2). After one incubation week with *A. niger* or *Arthrobacter* sp., compound 4 was recovered intact. Figure 4d presents the kinetics of transformation by the fungal strains. The *M. isabellina* and *C. elegans* were the most efficient strains tested since only around 20% of compound 4 remained intact after one incubation week. The transformation by *M. isabellina* was the fastest at the beginning, but the rate quickly slowed down. With *B. bassiana,* around 50% of compound 4 disappeared within 1 d, but the transformation rate stopped and 50% of compound 4 remained intact after five incubation days.

Only one metabolite was observed and, after isolation, it was identified as the didemethylated compound 5 (Fig. 10). Except with *C. elegans,* the concentration of compound 5 was very low and did not correspond quantitatively to the transformation of compound 4 (data not shown); other metabolites could not have been detected with the analytical method used.

According to the Microtox test, compound 5 presents a toxicity slightly higher than compound 4; therefore, the bio-

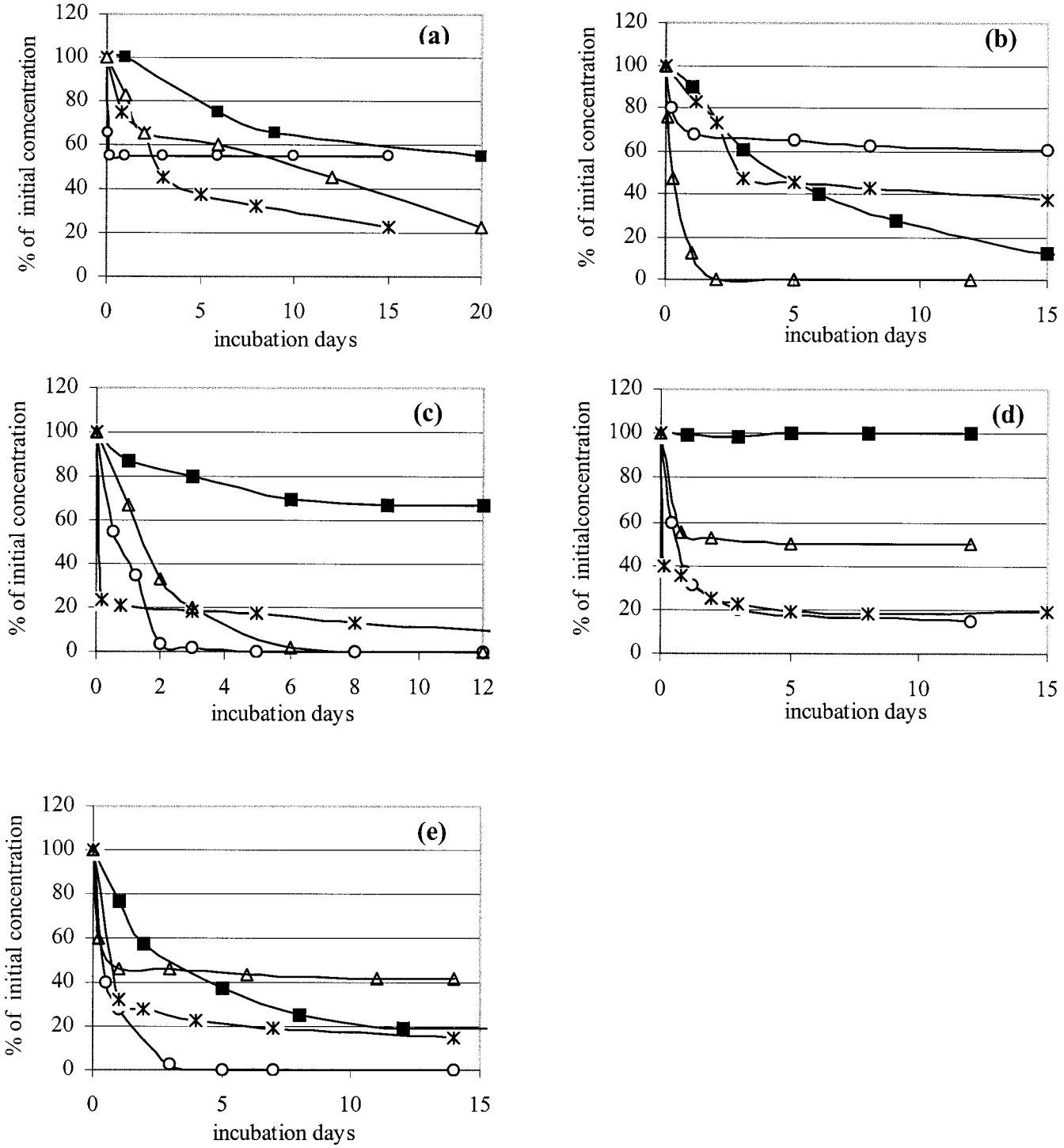

Fig. 4. Kinetics of degradation by the four fungal strains of compounds 1 (**a**), 2 (**b**), 3 (**c**), 4 (**d**), and diuron (**e**). ■, *Aspergillus niger*; ○, *Cunninghamella echinulata* var. *elegans*; △, *Beauveria bassiana*; *, *Mortierella isabellina*. Conditions of incubation were an initial concentration of 40 mg/L (or 30 mg/L for compound 3) and wet weight cells of 50 g/L.

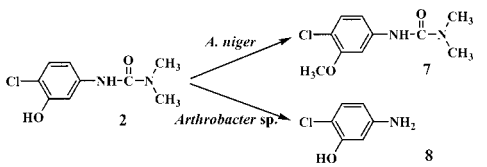

Fig. 5. Structure of the metabolites coming from the biotransformation of compound 2 by *Aspergillus niger* and *Arthrobacter* sp.

transformation of compound 4 was accompanied by a very slight increase in the potential ecotoxicity.

DISCUSSION

Most of the diuron degradation products presented a non-target toxicity higher than that of the parent molecule. These results corroborate previous ones obtained with only a few degradation products [20]. Among all the products tested, the substituted anilines and the formylated compounds had the

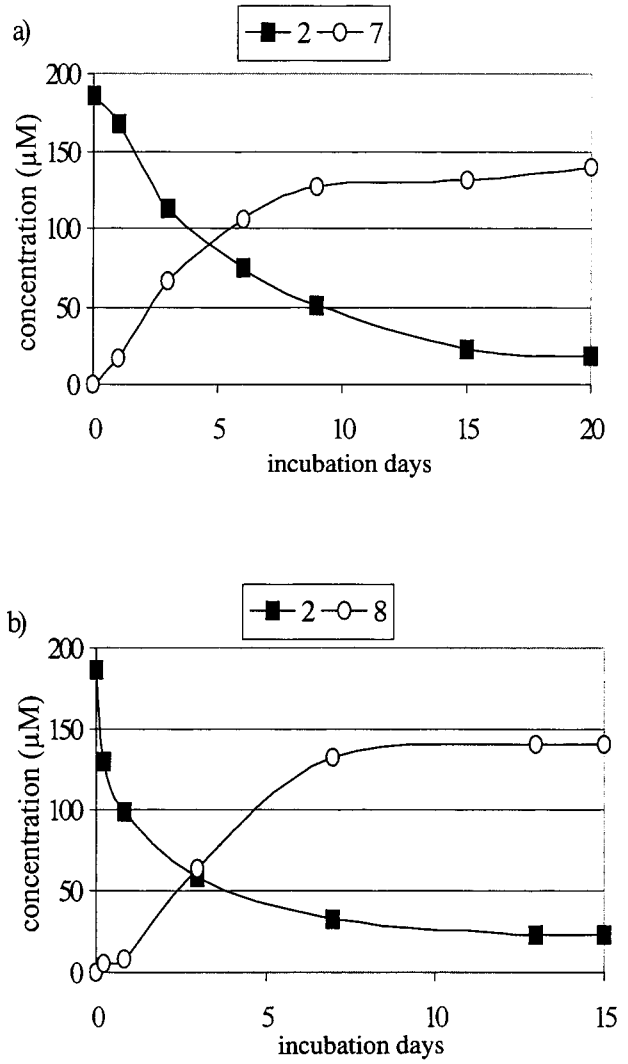

Fig. 6. Kinetics of degradation of compound 2 by (**a**) *Aspergillus niger* and (**b**) *Arthrobacter* sp. N2.

most toxic effects on the bacterium *V. fischeri.* These significant results show the importance of knowing the fate of these degradation products.

A previous study on the biotransformation of 3,4-dichloroaniline by various strains showed that this compound can be transformed into less toxic ones [26]. According to this present work, all the degradation products may be transformed by five strains, all of which were able to degrade diuron efficiently, including four fungal strains (*B. bassiana*, *C. echinulata* var. *elegans*, *A. niger*, and *M. isabellina*) and a bacterial strain (*Arthrobacter* sp).

The fungal strains appeared as the less specific ones; except for *A. niger*, they were able to transform six out of seven products tested. On the contrary, a small change in substrate

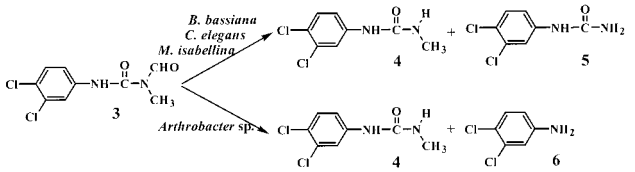

Fig. 7. Structure of the metabolites coming from the biotransformation of compound 3.

structure led to a great difference in the degradative behavior of *Arthrobacter* sp. Although this bacterial strain was able to transform diuron easily, only two degradation products were biotransformed with this strain, the N-formylated compound (product 3) and the chlorophenol (product 2). The substitution of one chlorine atom by a hydroxyl group slowed down or even stopped the degradation when the substitution was at the *meta* or *para* position, respectively.

Except for the N-didemethylated compound 5, all the degradation products underwent other transformations with most of the strains tested. It is also of great importance to identify these breakdown compounds in order to evaluate the residual toxicity. The biotransformation of compound 1 did not allow the identification of metabolites; within 1 d, up to 45% of the product disappeared with *C. elegans* but no degradation product was observed with the analytical method used. Compound 2 was also transformed with all the strains tested. The biotransformation of compound 2 by *A. niger* consisted almost quantitatively of the *O*-methylation of the phenol function. *O*-methylation is a significant transformation reaction, often observed with chlorophenols, both with fungal [27–29] and bacterial [30,31] strains. The *A. niger* seemed to be able to *O*-methylate only compound 2, which presents a hydroxyl group in the *meta* position. All the studies cited above also stress the role played by the substitution of the chlorophenol considered. Indeed, with several *Rhodoccocus* strains, the *O*-methylation reaction was favored when the hydroxyl group was flanked by two chlorine substituents; the methylation was slowed down when no halogen was present [31]. According to Häggblom et al. [31,32], *O*-methylation is an environmentally important transformation reaction because it increases the lipophilicity of the compound and thus the potential for bioaccumulation. This change in lipophilicity may explain the increase in the biological effects observed on *V. fischeri* (Microtox test) with the *O*-methylated compound (EC50 = 140 μM) in comparison with the parent compound (EC50 = 330 μM).

Arthrobacter sp. was also able to transform compound 2, presenting a hydroxyl group at the *meta* position. The metabolite obtained in stoichiometric quantities corresponded to the 5-amino-2-chlorophenol. The position of the hydroxyl group seemed to have great importance for the interactions with the enzymatic site since this strain cannot degrade compound 1.

Among all the products tested, compound 3 appeared to be the most easily transformed. This result is very interesting as the photoproduct 3 presents high toxicity according to the Microtox test. Moreover, other studies on the indirect phototransformation of diuron also reported the formation of this compound [33]. So, although photoproduct 3 may come from different degradative pathways of diuron, this compounds does not seem to be relevant as a pollutant agent because it appears to be transformed easily by microbial strains. Four out of five strains were able to degrade it up to 80% after six incubation days. The metabolites obtained with the fungal strains corresponded to the N-demethylated compounds 4 and 5. With *Arthrobacter* sp., another metabolite was identified as 3,4-dichloroaniline (compound 6). In the same way, the N-monomethylated compound 4 was biotransformed into the phenylurea compound 5 by three fungal strains. But compound 5 was further transformed by none of the strains tested. Thus, in the test conditions, the phenylurea compound 5 and 3,4-dichloroaniline compound 6 constitute the final metabolites of several products issued from the degradation of diuron. More-

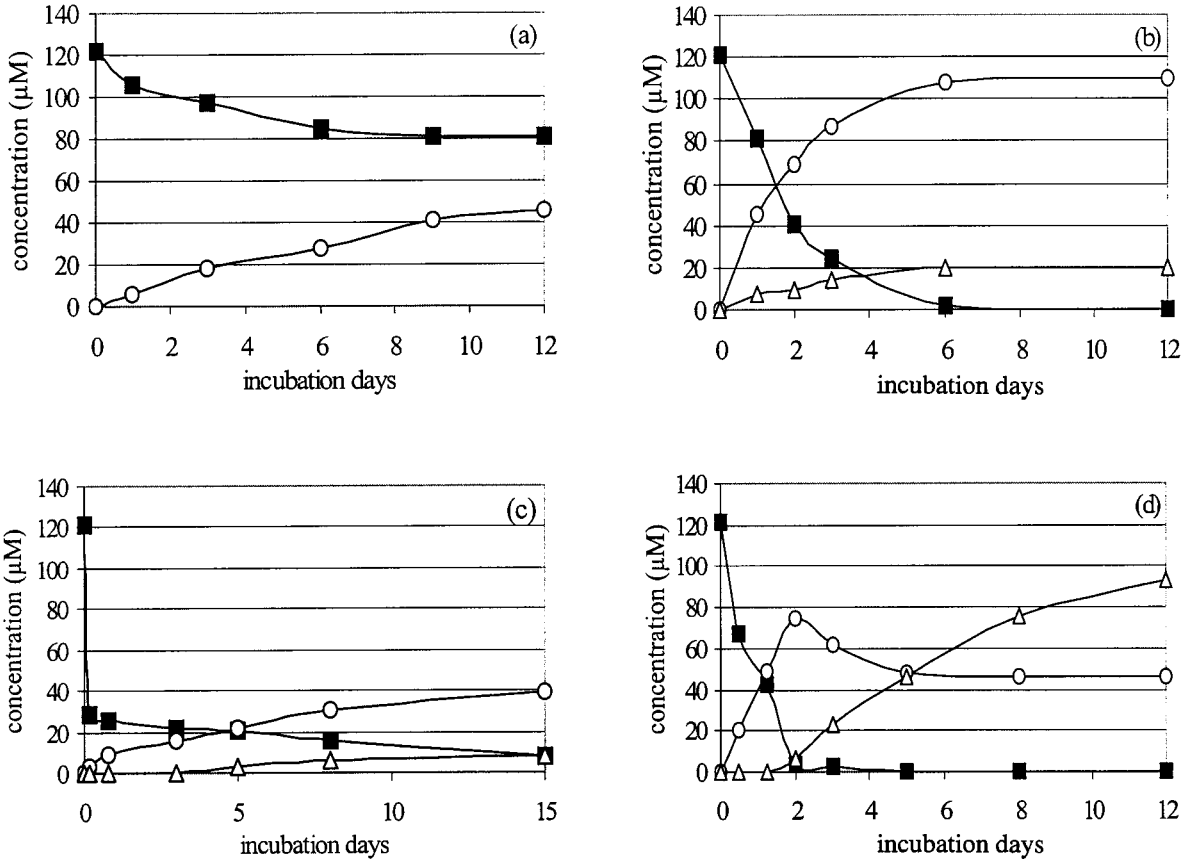

Fig. 8. Kinetics of degradation of compound 3 by (**a**) *Aspergillus niger*, (**b**) *Beauveria bassiana*, (**c**) *Mortierella isabellina*, (**d**) *Cunninghamella echinulata* var. *elegans*. ■, compound 3; ○, compound 4; △, compound 5.

over, according to the Microtox test, both these compounds present a toxicity higher than that of the original herbicide diuron.

CONCLUSION

These results show the importance of knowing the nature of the breakdown products to assess the impact of a polluting agent. Indeed, the pollutant may undergo reactions yielding compounds that present toxicity higher than that of the parent substance. Biodegradation studies therefore must not be limited to the disappearance of the pollutant if it is not mineralized; it is essential to identify the intermediate products in order to carry out further tests on them, such as toxicity evaluation or biodegradation studies.

According to this study, the herbicide diuron may undergo transformation leading to compounds having a higher toxicity than the diuron, according to the Microtox test. With the five strains tested, the products coming from the initial transformation of diuron were also transformed. Nevertheless, no mineralization was observed. In soils, a wide range of microorganisms is present, and we can expect that the microflora will succeed in mineralizing these compounds. Indeed, studies done on another type of herbicide, atrazine, have shown that mineralization of this compound may be achieved not only by pure atrazine-catabolizing cultures [34–37] but also by bacterial consortium [38–40], each member of the consortium dealing with one or several degradation steps [41]. However, the nature of the degradation products should be kept in mind while analyzing natural water samples or soil samples.

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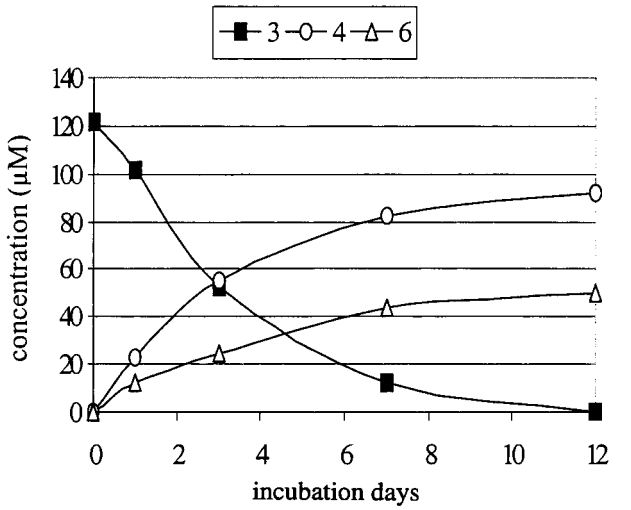

Fig. 9. Kinetics of degradation of compound 3 by *Arthrobacter* sp. N2.

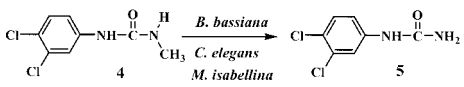

Fig. 10. Structure of the metabolite coming from the biotransformation of compound 4.

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