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Abstract

Nutrient-agar plates containing isoxaben (500 mg l^{-1}) were used to isolate isoxaben-metabolizing bacteria from four European soils incubated with the herbicide under laboratory conditions. In flask experiments, inoculation of a basal salts medium containing nitrogen and [*phenyl*-UL-^{14}C]isoxaben with an isolate (B2b) resulted in a 33% recovery of the initial radioactivity as $^{14}CO_2$ after two weeks. A major metabolite identified by GC-MS and NMR analysis as 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole accumulated both in basal salts and nutrient broth media. 2,6-dimethoxybenzoic acid, a suspected metabolite of isoxaben, was not detected in either liquid media. However, the capability of the B2b isolate to use 2,6-dimethoxybenzoic acid as a source of carbon was demonstrated. Soil inoculation with the B2b strain isolate resulted in an increase in the recovery of $^{14}C0_2$ from both *phenyl*-UL and 5

isoxazolyl-^{14}C-isoxaben. The previous metabolite identified as 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole only accumulated if the soil was autoclaved before inoculation. This metabolite was rapidly mineralized by the microflora of a natural soil without history of isoxaben treatment. Homology patterns of sequenced 16S rDNA between isoxaben-transforming isolates and reference strains show that the four isolates identified belong to the genus *Microbacterium*.

Key words: actinomycetes; biodegradation; isoxaben; *Microbacterium*

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1 INTRODUCTION

Isoxaben (*N*-[3-(1-ethyl-1-methylpropyl)-5 isoxazolyl]-2,6-dimethoxybenzamide, compound 1, Figure 1), is a selective pre-emergence herbicide used to control annual broadleaf weeds in winter cereals, ornamentals, turfgrass, trees and vines.[1,2] Isoxaben has a very low water solubility (1-2 mg litre^{-1} at 25°C), is stable to hydrolysis at pH 5-9[3] and is susceptible to photodegradation in aquatic systems.[4] Primary breakdown in soil is achieved through microbial degradation. First report on soil degradation of isoxaben[5] refers to previous unpublished data from D.P. Rayney and L.K. Graper 1984-85 (Lilly Research Laboratories). These authors observed that *N*-[3-(1-hydroxy-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide, the major metabolite detected during their laboratory soil study, reached levels of approximately 20% of the applied isoxaben. In their study, Rouchaud and co-workers[6] identified demethoxy-isoxaben, 5-isoxazolone (3-[1-ethyl-1-

methylpropyl]isoxazolin-5-one) and 2,6-dimethoxybenzamide as the three main metabolites of isoxaben in the soil of winter wheat crops treated with the herbicide. Three minor compounds, 5-aminoisoxazole [5-amino-3-(1-ethyl-1-methylpropyl)isoxazole], 2-hydroxy-6-methoxybenzamide and 2,6-dimethoxybenzoic acid were also detected. A relatively low mineralization of ring-labeled ^{14}C-isoxaben in an acidic soil was also recently reported[7] but metabolites were not definitively identified.

Application timing affects the dissipation of isoxaben from field soils and the duration of weed control.[8-11] Herbicide persistence was less pronounced in wheat plots treated with organic fertilisers.[8] In addition, repeated isoxaben treatments enhanced soil pesticide biodegradation in several field experiments.[12]

The present work was carried out to further identify the key parameters governing isoxaben degradation in fields. This paper presents preliminary investigations on the isolation and characterization of isoxaben-transforming bacteria, as well as the identification of a major transformation product of the pesticide.

2 MATERIALS AND METHODS

2.1 Chemicals

Technical grade isoxaben, [*isoxazole*-5-^{14}C]isoxaben (specific activity 3.71 MBq mmol^{-1}) and [*phenyl*-UL-^{14}C]isoxaben (specific activity 17.80 MBq mmol^{-1}) were kindly provided by Dow AgroSciences Ltd (Norfolk, England). Radioactive and unlabelled isoxaben were purified by thin-layer chromatography (TLC) or crystallization by mixing a highly concentrated solution of isoxaben in methanol with water.

2.2 Enrichment, isolation and testing of isoxaben-degrading strains

The soils were obtained from 4 field sites in Europe with known histories of isoxaben treatments and from one site never previously treated with the herbicide (Table 1). Samples were collected to a depth of 15 cm, sieved below 4 mm and stored at 4°C for 3-6 weeks prior to use. For each soil, duplicates samples (50 g dry weight) were moistened to 90% of their moisture-holding capacities and treated successively with 250 µl of methanolic solutions containing isoxaben (0.5 and 5 mg per sample) at a one-month interval. The samples were incubated at 20°C in a 1-litre-hermetic glass jar. Thirty days after the last treatment, soil (10 g) was suspended in a sterile aqueous solution (100 ml) containing tetrasodium pyrophosphate ($Na_4P_2O_7$; 01 g) and shaken for 10 min. Serial ten-fold dilutions were prepared and a fraction of each dilution (0.1 ml µl) was spread onto nutrient-agar plates (Difco, Detroit, USA) supplemented with isoxaben (500 mg $litre^{-1}$) in methanol (5 ml $litre^{-1}$). The agar plates were cloudy as a result of particulate isoxaben in the medium, and visual inspection of the clearing zone around isoxaben-degrading colonies was possible after a one week incubation period at 28°C. Isolates were maintained on slope tubes of nutrient agar supplemented with isoxaben (250 mg $litre^{-1}$).

Primary testing of isoxaben-degradation by the selected isolates was carried out in 50 ml Erlenmayer flasks with nutrient broth (Difco) medium (10 ml) complemented with [*isoxazole*-5-^{14}C]isoxaben (250 mg and 50 kBq $litre^{-1}$). The pesticide was added as filter-sterilised solutions in methanol (0.1 ml per sample). The medium was inoculated with approximately 10^8 bacteria (0.1 ml of each isolate suspension) and incubated on a rotary shaker (150 rpm) in the dark at 28°C. Separate experiments with nutrient broth medium complemented with 250 mg $litre^{-1}$ of unlabelled isoxaben were also carried out in such a way to produce metabolites useful for the identification tests.

Isoxaben-mineralization testing by the B2b isolate were carried out in 50 ml Erlenmeyer flasks (four replicates) containing basal salts (BS) medium[13] with NO_3NH_4 (0.15

g.litre^{-1}) or nutrient broth medium (10 ml). Both media exhibited a pH of 6.9. The [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben (50 mg and 50 kBq litre^{-1}) was added as filter-sterilised solutions in methanol (0.1 ml per sample) in an empty-sterilised flask, plugged with cotton whole, and the solvent was air-evaporated at 30°C in the dark for 24 hours before adding sterile medium and inoculate to the different flasks as previously described. The flasks were enclosed in 1-litre glass jars and incubated on a rotary shaker (150 rpm) in the dark at 28°C for two weeks. NaOH (5 ml of 1 M solution in vials) was used to trap the $^{14}CO_2$ released in the incubation jars and the radioactivity was measured by liquid scintillation counting (Tricarb TR 1900, Packard) after mixing with a scintillation cocktail (ACSII, Amersham Pharmacia Biotech, Orsay, France).

The capability of B2b isolate to use 2,6-dimethoxybenzoïc acid as a source of carbon was determined using the BS-nitrogen medium. Diluted sodium hydroxide was used to make the acidic compound (2500 mg litre^{-1}) soluble and to maintain neutral pH of the medium.

2.3 Soil incubation studies

Soil incubation experiments were carried out using 25 g samples of natural or autoclaved (1 h, 120°C) soil from Dijon. Four of the eight replicates of each series were inoculated with a suspension of 10^9 isoxaben-degrading bacteria in sterile water (isolate B2b). Control samples received sterile water only. All soil samples were treated with a methanolic solution of [*isoxazole*-5-^{14}C] or [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben (25 µl per sample) to fortify the samples with the active ingredient (0.025 mg) and radioactive material (5 kBq). Similar experiments were carried out with a chromatographically-purified radioactive metabolite of isoxaben. In both cases, the soil moisture was adjusted to 90% of its water-holding capacity. Sample-containing bottles were put into 1-litre glass jars and incubated in

the dark at 20°C for 61 days. Vials with NaOH (0.2 M; 5 ml) were used to trap the $^{14}CO_2$ released in the incubation jars and the radioactivity was measured as previously described.

2.4 Extraction and analysis of pesticide residues from culture and soil experiments

Axenic cultures in liquid or agar media were extracted twice by stirring with an equivalent volume of dichloromethane for 60 min. The organic solution was dried on $MgSO_4$, filtered, and concentrated to dryness under a N_2 stream. Soil samples were extracted under the same conditions with methanol (200 ml per sample). For complementary analysis on the presence of 2,6-dimethoxybenzoic acid in liquid media, culture samples (10 ml) were introduced on 12 ml SPE tubes containing reversed phase packing LC 18 (Supelco, Bellefonte, PA USA). After elution with methanol (10 ml), the recovered eluate was concentrated to dryness as previously described.

The different extracts were analyzed by TLC using silicagel 60F254 (20 x 20 cm, 0.25 mm thickness) plates with a concentration zone (Merck, Nogent-sur-Marne, France). The following solvent systems were used: I, toluene:acetone (85+15 by volume; II, toluene:dioxan:acetic acid (135+20+1 by volume). Radioactive spots were visualised with Hyperfilm-max (Amersham, France).

Gas Chromatographic–Mass Spectrometric (GC-MS) analyses were carried out with a 5973 Mass Selective Detector coupled to a 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) fitted with a split-splitless injector and a DB™FFAP fused silica capillary column (30 m x 0.32 mm ID, 0.25 µm film thickness, J&W Scientific, Folsom, CA). The column was held at 40°C for 2 min and then the oven temperature was programmed to increase to 240°C at 10°C min^{-1}. The column was directly connected to the ion source of the spectrometer through a heated transfer line maintained at 250°C.

Electron Impact (EI) mass spectra were obtained at 70 eV with the instrument scanning from 29 to 350 amu and the source maintained at 230°C. Chemical Ionisation (CI) mass spectra were generated at 63 eV using methane as a reactant gas with the instrument scanning from 70 to 350 amu and the source temperature set at 250°C.

Fourier Transform Infrared (FTIR) data were collected with a Bio-Rad Digilab FTS-60A spectrometer (Cambridge, MA). It was connected by a Digilab Tracer® direct deposition interface to a Hewlett-Packard HP 5890 series II gas chromatograph (Palo Alto, CA). The GC instrument was equipped with the column already described for GC-MS analysis and operated in similar conditions. GC eluates were deposited onto a ZnSe window cooled with liquid nitrogen. The spectral resolution was 8 cm^{-1}, and real-time spectra were obtained by co-addition of four scans. A narrow-band (4000-700 cm^{-1}) mercury-cadmium-telluride (MCT) detector was used.

NMR data (^{1}H, 300 MHz; ^{13}C, 75 MHz) were recorded on a Gemini 300 instrument (Varian, Les Ulis, France). All NMR spectra were obtained in deuteriochloroform ($CDCl_3$). Chemical shifts are reported in δ (parts per millions) relative to $CHCL_3$ ($CDCl_3$) as internal reference: 7.27 ppm for ^{1}H (77.14 ppm for ^{13}C). Multiplicities are recorded as s (singlet), t (triplet), m (multiplet) and br (broad).

2.5 Molecular characterisation of isolates

Bacteria for molecular analysis were grown for 2-3 days on isoxaben-agar slopes. The cells were suspended in sterilised ultrapure water (Millipore-Q reagent) to obtain the optical density of 1 U at 620 nm. The cells were then lysed by thermal shock (5 s in liquid nitrogen and 30 s in boiling water, six times) and immediately used or stored at -20°C until PCR analysis.

ARDRA (Amplified Ribosomal DNA Restriction Analysis) of 16S rDNA and PCR-RFLP (Restriction Fragment Length Polymorphism) analysis of the 16S-23S rDNA spacer

region (IGS) have previously been described in detail.[14] Only 4 restriction endonucleases were used here: *Alu* I, *Dde* I, *Hae* III, and *Hinf* I.

Sequencing of 16S rDNA (1384 bp) was performed by Genome Express (Grenoble, France). Sequences were compared by using the Infobiogen server (Villejuif, France) with Fasta version 3.2t05 [15] and Align version 2.0u programs.[16] Partial 16S rDNA sequence of isoxaben-degrading isolates from this study were deposited in Genbank under the following accession number AY040877.

2.6 Phenotypic testing

Phenotypic tests were carried out with soil isolates and six reference strains of *Microbacterium*: *M. dextranolyticum* DSM 8607, *M. flavescens* DSM 20643, *M. ketoreductum* DSM 12510, *M.* sp. DSM 20028, *M. testaceum* DSM 20166 and *M. trichotecenolyticum* DSM 8608. Nutrient agar plates or slope tubes supplemented with isoxaben were inoculated with the test bacteria and incubated for one day at 28°C. The cells, harvested with sterile water and suspended to an optical density of 1 U at 620 nm, were then used as inoculum for the different tests. Biolog GP and GN (Biolog, Inc) microplates,[17,18] were inoculated with cell suspension (0.1 ml per well) and incubated at 28°C for 3 days. Colour development was automatically determined by means of a microplate reader (Thermomax, Molecular Devices Corp., MenloPark, CA). Api Coryne microplates (Api System S.A., La Balme les Grottes, France) were used according to the manufacturer's recommendations. Gram reactions were recorded on 2- to 3-day-old colonies using a fluorogenic substrate.[19]

3 RESULTS AND DISCUSSION

3.1 Isolation of isoxaben-transforming strains

All attempts to isolate isoxaben-degrading strains after soil enrichment and inoculation of the liquid and agar-BS media failed, probably because the low solubility of isoxaben in the medium. In contrast, on most of Nutrient-agar isoxaben plates, a few regular, brilliant, yellow, colonies of bacteria (10^6 CFU g^{-1} of soil, 1-6% of total counts), grew in clearing zones. The total colony count in soils I-IV incubated with isoxaben was approximately) Four to five colonies forming clearing zones from each soil source were isolated to further characterise the micro-organisms. No colony was observed in the soil from Dijon, suggesting that previous field and/or laboratory treatments with isoxaben were necessary for bacterial proliferation.

3.2 Isoxaben degradation in axenic cultures

Four of the nineteen randomly selected isolates were used in this study: F2b from the Langon French vineyard, A1j from the Serignac French orchard soil, Asp2 from the Bristol English soil and B2b from the St Amand Belgian soil. Isoxaben degradation was never observed when nitrogen was lacking in culture media suggesting that the pesticide cannot be used as sole source of nitrogen by the isolates. Kinetics of mineralization of [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben (50 mg litre^{-1}) inoculated by the B2b isolate are shown Figure 2. After two weeks, 33%f the labelled isoxaben were recovered as $^{14}CO_2$. A $^{14}CO_2$ release was also observed from flasks of nutrient broth medium with 250 mg litre^{-1} of pesticide. Under such conditions, 36% of the initial radioactivity was trapped as $^{14}CO_2$ after two weeks (data not shown). Less than 0.1% of the labelled isoxaben were recovered after two weeks both from BS-nitrogen and nutrient broth uninoculated media.

TLC analysis of culture extracts (NB medium with 50 or 250 mg isoxaben litre^{-1}) showed a decrease in the residual ^{14}C-isoxaben and the accumulation of a slightly yellow, non-radioactive spot with a 0.61 and 0.45 *rf* in solvent systems I and II, respectively (metabolite I). In the same analysis control isoxaben spots presented a 0.71 and 0.63 *rf* in

solvent systems I and II, respectively. Only low traces of other labelled metabolites were generally observed.

In contrast to the previous results, release of $^{14}CO_2$ did not exceed 0.2% in two weeks when inoculated flasks of BS-nitrogen media were treated with [*isoxazole*-5-^{14}C]isoxaben (Figure 2) but as previously a major-radioactive metabolite with a TLC behaviour similar to metabolite I accumulated in the cultures. This metabolite was also present in the clearing zones extracts obtained from the culture of efficient isolates on the nutrient-agar plates with isoxaben. Similar results were observed in separate experiments with A1j, Asp2 and F2b isolates.

Labelled or unlabelled 2,6-dimethoxybenzoic acid (compound 2, Figure 1, 0.18 *rf* in solvent system II) neither accumulated in the different culture extracts although B2b rapidly grew on the BS-nitrogen medium supplemented with this compound as the sole source of carbon. This rapid growth could explain why this compound did not accumulate in the medium. However, as previously reported,[6] the presence of demethoxyisoxaben and of 2-hydroxy-6-methoxybenzamide in soil treated with isoxaben suggests a modification of the phenyl ring before isoxaben hydrolysis of the amide bond. This hypothesis is supported by the study of a chloridazon-degrading bacteria transforming metamitron to 2,-dihydroxy-metamitron which was then cleaved by *meta*-fission.[20] while a *Rhodococcus* sp. isolated from treated soil partly mineralized the phenyl ring of metamitron.[21]

3.3 Soil studies

Both [*isoxazole*-5-^{14}C] and [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben were poorly mineralized in natural and autoclaved samples of the soil from Dijon (Figures 3 and 4). Inoculation of natural samples with the suspension of the B2b isolate from soil IV greatly increased the mineralization rates of ^{14}C-isoxaben whatever the site of labelling.

Accumulation of the metabolite I was never observed in natural (non-autoclaved) soil. In contrast, the radioactive metabolite I was present in methanol extracts from autoclaved soil treated with [isoxazole-5-^{14}C]isoxaben] and inoculated with B2b. These results indicate that, as in liquid system, the B2b isolate is capable of mineralizing the phenyl ring of isoxaben but is unable to mineralize the isoxazole part of the pesticide. In separate experiments, addition of the radioactive metabolite I in the non-inoculated natural soil resulted in a rapid mineralization of the compound (Figure 5) while the compound was not mineralized in the autoclaved soil. These results suggest a high degrading ability of the indigenous microflora for metabolite I even if the soil has no previous history of isoxaben use. They also explain the similarity of $^{14}CO_2$ kinetics observed with *phenyl* and *isoxazole*-^{14}C-isoxaben in the non-autoclaved soil inoculated with B2b.

3.4 Identification of the main metabolite formed from isoxaben

The mass spectrum of metabolite I showed a base peak at m/z 139. The highest peak observed, although with a very low abundance of ion, was at m/z 168. This ion could be attributed to the molecular ion according to the results of chemical ionisation with methane as reagent gas. In that case, the spectrum exhibited a base peak at m/z 169, and other peaks at m/z 197 and m/z 209, due to the $[MH]^+$, $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ ions, respectively. The isotopic contribution associated with these pseudomolecular ions suggested the formula of $C_9H_{16}N_2O$ for metabolite I. The molecular weight of 168, even, was consistent with the presence of two nitrogen atoms.

The presence of an amine group in the infrared spectrum of metabolite I was confirmed by the two broad bands at 3327 and 3187 cm^{-1} (ν_{N-H} stretch). This low frequency (approximately 100 cm^{-1}) was attributed to the solid phase of the compound deposited on the zinc selenide window held at 77 K in the GC-FTIR interface.[22] The intense absorption band at

1665 cm^{-1} was attributed to the C-N stretching absorption of the imine function from the isoxazole cycle.

Taken together, these results, based on mass spectrometry, on calculation of isotopic contribution, and on IR analysis, suggested that the major metabolite of isoxaben observed in liquid or agar cultures and in inoculated soil was 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole (compound 3, Figure 1). A definitive proof was provided by NMR analysis. Chemical shifts δ measured by ^{1}H NMR were: 4.97 (s, 1 H, H-4), 4.36 (br s, 2 H, NH_2), 1.68-1.46 (m, 4 H, 2 CH_2), 1.19 (s, 3 H, CH_3), 0.80 (t, J = 7.4 Hz, 6 H, 2 CH_3), whereas these for ^{13}C NMR were: 171.8(C), 168.0 (C), 78.9 (CH), 39.4 (C), 33.3 (2 CH_2), 21.7 (CH_3), 8.7 (2 CH_3). They were consistent with the structure proposed for metabolite I.

In their previous study, Rouchaud and co-workers[6] also reported the presence of 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole in soil extract but the compound did not accumulate and they therefore considered the opportunity of an unusual mechanism for amide hydrolysis with 5-isoxazolone accumulation. Our results suggest that 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole could be an important intermediary product of the microbial degradation of isoxaben in soil. Alternative metabolic pathways correlated with specific microflora could therefore be proposed. Comparison with Camper's results[7] is more difficult because authors used a very acidic soil for their experiments. This could explain the relatively slow mineralization of labelled isoxaben. In contrast our results rather suggest the capability of certain microbial species to rapidly transform isoxaben and use part of the pesticide molecule as a source of carbon.

3.5 Characterization and identification of isolates

All the 19 isolates exhibited analogous RFLP profiles, whilst only one isolate from the French orchard soil (A1j) exhibited a slightly different 16S-23S profile with a single restriction endonuclease: *Dde I.* Only F2b, A1j, Asp2 and B2b isolates were retained for further characterization: The 16S rDNA sequences analysed from the 4 selected isolates were identical and consistent with the *Microbacterium* genus (98.3% identity with *Microbacterium trichotecenolyticum* DSM 8608, 98.1% identity with *M. flavescens* DSM 20643, 97.6% with *M. testaceum* DSM 20166, 97.6% with *M.* sp. DSM 20028, 97.3% with *M. dextranolyticum* DSM 8607 and 97.4% with *M. ketoreductum* DSM 12510). All isolates and reference strains exhibited a Gram positive reaction, as well as similar characteristics with Biolog and Api Coryne tests

The *Microbacterium* genus belongs to the *Microbacteriaceae* family which is reported to include *Microbacterium*, *Agrococcus*, *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curtobacterium, Rathayibacter, Leucobacter* and *Cryobacterium* genera.[23] The union of the *Microbacterium* and *Aureobacterium* genera[24] and a reclassification of *Brevibacterium oxydans* as *Microbacterium oxydans* have recently been proposed.[25]

Actinomycetes are phylogenetically and taxonomically related Gram positive bacteria with a high G+C content in their DNA.[26] The degradation of pesticides by actinomycetes has recently been reviewed.[27] Many pesticides, including phenoxyacetates, organochlorines, N-methylcarbamates, *N*-phenylcarbamates, organophosphates or *s*-triazines can be degraded by actinomycete species notably in the *Arthrobacter*, *Brevibacterium* and *Clavibacter* genera which belong to the same suborder (*Micrococcineae*) as *Microbacterium.*

It can be surprising that all isolates from very different origins turn out to have the same degradation intermediate and turn out to belong to the same genus and probably species. Such presence of pesticide degrading strains with similar ribotypes in very different soils was already observed with carbofuran[14]. However the high degree of genetic diversity within this

population possibly explained fortuitous similarities. Preliminary experiments did not suggest such high diversity within isoxaben degrading bacteria and did not allow locating isoxaben-catabolic genes in the *Microbacterium* sp. genome. Therefore, there is a need for further research on the genetic and enzymatic mechanisms involved in isoxaben transformation. Studies of actual involvement of microbial populations such as *Microbacterium* sp. strains on the field behaviour of isoxaben are in progress. Effects of repeated isoxaben treatments are considered at the present time by analogy with the microbial degradation of different amide herbicides such as napropamide, carbetamide or propyzamide.[28-30] In other respects there is an increasing environmental interest for natural strains such as *Microbacterium* sp isolates which could be used for bioremediation processes of pesticide wastes.

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REFERENCES

1 Huggenberg F, Jenning EA, Ryan PJ and Burow KW, EL-107, a new selective herbicide for use in cereals. *Proc. Br. Crop Prot. Conf Weeds* **1**, 47-52 (1982).

2 Colbert FO and Ford DH, Isoxaben for broadleaf weed control in ornamentals, turf and nonbearing trees and vines. *Proceedings-Western Society of Weed Science* **40**, 155-163 (1987)

3 Royal Society of Chemistry, *The agrochemicals handbook*, third edition (1991)

4 Mamouni AP, Schmitt P, Mansour M, and Schiavon M, Abiotic degradation pathways of isoxaben in the environment. *Pestic. Sci.* **613**, 13-20 (1992)

5 Rutherford BS, Liquid chromatographic determination of the herbicide isoxaben and its soil metabolite in soil and soil-turf samples. *J. AOAC* **73**, 287-289 (1990)

6 Rouchaud J, Gustin F, Callens D, Van Himme M and Bulcke R, Soil metabolism of the herbicide isoxaben in winter wheat crops. *J. Agric. Food Chem*. **41**, 2142-2148 (1993)

7 Camper ND, Kim JH and Riley MB, Degradation of isoxaben in soils and an aqueous system. *J. Environ. Sci. Health* **B36**, 729-739 (2001)

8 Rouchaud J, Gustin F, Van Himme M, Bulcke R and Sarrazyn R, Soil dissipation of the herbicide isoxaben in cereals and sugar beet. *Weed Res.* **33**, 205-212 (1993)

9 Chandran RS, Derr JF, and Bingham SW, Effect of isoxaben application rate and timing on residual broadleaf weed control in turf. *Weed Technology* **12**, 569-574 (1998)

10 Chandran RS and Derr JF, Duration of broadleaf weed control with isoxaben using soil bioassays. *Weed Technology* **12**, 542-547 (1998)

11 Chandran RS and Derr JF, Isoxaben dissipation in field soils as affected by application timing. *J. Environ. Qual.* **28**, 1760-1764 (1999)

12 Rouchaud J, Neus O, Bulcke R, Callens D and Dekkers T, Isoxaben soil biodegradation in pear tree orchard after repeated high dose application. *Arch. Environ. Contam. Toxicol.* **33**, 247-251 (1997)

13 Fournier J-C, Enumeration of the soil-organisms able to degrade 2,4-D by metabolism or co-metabolism. *Chemosphere* **9**, 169-174 (1980)

14 Desaint S, Hartmann A, Parekh N and Fournier J-C, Genetic diversity of of carbofuran-degrading soil bacteria. *FEMS Microbiol. Ecol*. **34**, 173-180 (2000)

15 Pearson WR and Lipman DJ, Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci*. USA **85**, 2444-2448 (1988)

16 Myers EW and Miller W, Approximate matching of regular expressions. *Bull. Math. Biol.* **51**, 5-37 (1989)

17 Garland JL and Mills AL, Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole carbon-source utilization. *Appl. Environ. Microbiol.* **57**, 2351-2359 (1991)

18 Stoakes L, Schieven BC, Lannigan R, John MA, Reid R and Hussain Z, Evaluation of BIOLOG GP micro plates for their ability to identify clinical Staphylococcal isolates.

Abstract of the 94th General Meeting of the American Society for Microbiology **512** (1994)

19 Manafi M and Kneifel W, Rapid methods for differentiating Gram-positive from Gram-negative aerobic and facultative anaerobic bacteria. *J. Appl. Bacteriol*. **69**, 822-827 (1990)

20 Socrates G, Infrared characteristic group frequencies, tables and charts, Second ed by John Wiley, Chichester (1994)

21 Blecher R, Koch U, Ballhause B and Lingens F,Mikrobieller und abiotischer Abbau des Triazinon-Herbizids Metamitron. *Z. Pfl.Krankh. Pfl.Schutz* **86**, 83-102 (1979)

22 Parekh NR, Walker A, Roberts SJ and Welch SJ, Rapid degradation of the triazinone herbicide metamitron by a *Rhodococcus* sp. isolated from treated soil. *J. Appl. Bacteriol.* **77**,467-475 (1994)

23 Park YH, Suzuki K, Yim DG, Lee KC, Kim E, Yoo YH, Goodfellow M and Komagata K, Supragenic classification of peptidoglycan group B actinomycetes by nucleotide sequencing of 55 ribosomal RNA. *Anton. Leeuw. Int.* J. **64**, 307-313 (1993)

24 Takeuch, M and Hatano K, Union of the genera Microbacterium Orla-Jensen and Aureobacterium Collins et al. in a redefined genus Microbacterium. *Int. J. Syst. Bacteriol*. **48**, 739-747 (1998)

25 Schuman P, Rainey FA, Burghardt J, Stackebrandt E and Weiss N, Reclassification of *Brevibacterium oxydans* (Chatelain and Second 1966) as *Microbacterium oxydans* comb. nov. *Int. J. Syst. Bacteriol*. **49**, 175-177 (1999)

26 Cross T, The occurrence and role of actinomycetes in natural ecosystems. *Proc. IV ISME*, 265-270 (1986)

27 De Schrijver A and De Mot R, Degradation of pesticides by actinomycetes. *Crit. Rev. Microbiol.* **25**, 85-119 (1999)

28 Walker A, Welch SJ and Roberts SJ, Induction and transfer of enhanced biodegradation of the herbicide napropamide in soils. *Pestic. Sci*. **47**, 131-135 (1996)

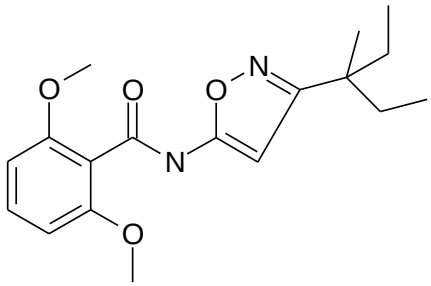
29 Hole SJW and Powles SB, Reduced efficacy and enhanced degradation of carbetamide after repeated applications in Australia. *Weed Res*. **37**, 165-170 (1997)

30 Rouchaud J, Neus O, Bulcke R, Eelen H, Dekkers T, Soil dissipation of diuron, chlorotoluron, simazine, propyzamide, and diflufenican herbicides after repeated applications in fruit tree orchards. *Arch. Environ. Contam. Toxicol*. **39**, 60-65 (2000)

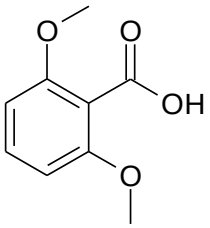
Table 1. Selected characteristics of soils used for this study

Soil	Origin	pH	W.H.C.	Organic matter	Sand	Silt	Clay	Crop and isoxaben history
			% dry weight	(g kg^{-1})	_____%_____			[treatment years]
I	Serignac, France	7.4	24.8	32	10	57	33	Orchard [4]
II	Langon, France	6.8	9.8	13	68	26	6	Vineyard [2]
III	Bristol, England	7.5	17.8	36	57	24	19	Turfgrass [2]
IV	Saint Amand, Belgium	7.4	24.8	24	12	72	16	Cereal [3]
V	Dijon, France	7.3	26.0	23	13	54	33	Turfgrass [0]

Figure 1. Structures of isoxaben (1), 2,6-dimethoxybenzoic acid (2) and 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole (metabolite I; 3).



1



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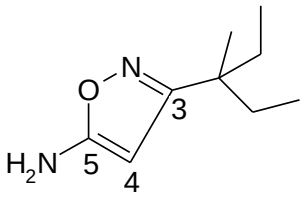

3

Figure 2. Mineralization of labelled isoxaben in BS-nitrogen medium inoculated with the B2b isolate. Means of 4 replicates, S.D. below 5 %.

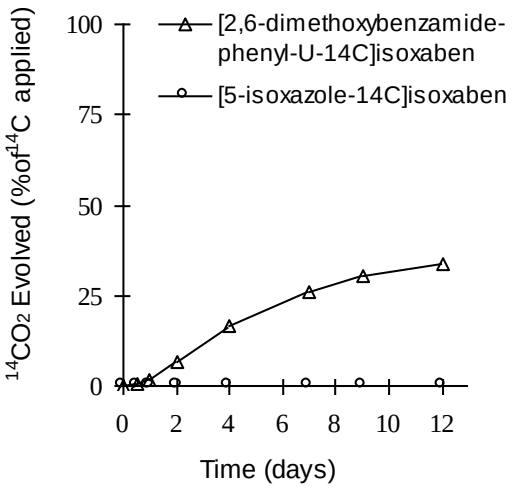

Figure 3. Mineralization of [*isoxazole*-5-^{14}C]isoxaben in natural and autoclaved soil from Dijon and effect of inoculation with the B2b isolate. Means of 4 replicates, S.D. below 3 %.

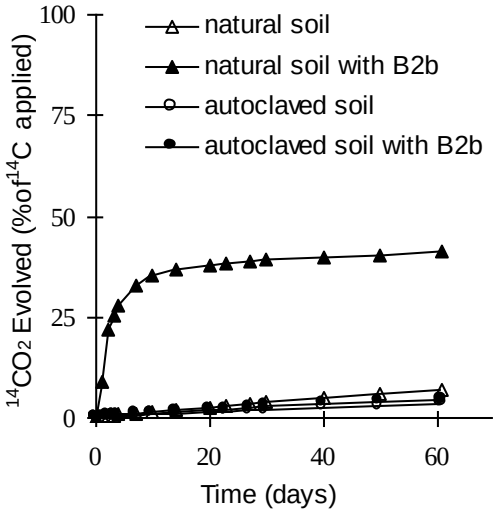

Figure 4. Mineralization of [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben in natural and autoclaved soil from Dijon and effect of inoculation with the B2b isolate. Means of 4 replicates, S.D. below 4 %.

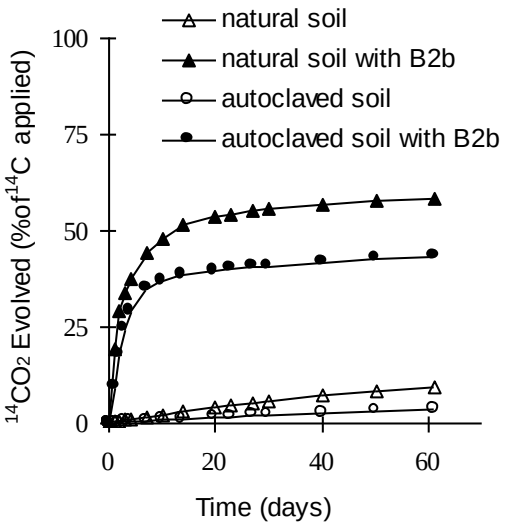

Figure 5. . Mineralization of 5-amino-3-(1-ethtyl-1-methylpropyl)isoxazole (metabolite I) in natural and autoclaved Dijon soil and effect of inoculation with the B2b isolate. Means of 4 replicates, SD below 2 %. There was no distinction between the curves for autoclaved soil with or without inoculum.