



HAL
open science

Isolation and characterization of mesotrione-degrading *Bacillus* sp. from soil

Isabelle Batisson, Olivier Crouzet, Pascale Pascale Besse, P. Besse-Hoggan
Besse-Hoggan, Martine Sancelme, Jean-François Mangot, Clarisse Mallet,
Jacques Bohatier

► **To cite this version:**

Isabelle Batisson, Olivier Crouzet, Pascale Pascale Besse, P. Besse-Hoggan Besse-Hoggan, Martine Sancelme, Jean-François Mangot, et al.. Isolation and characterization of mesotrione-degrading *Bacillus* sp. from soil. *Environmental Pollution*, 2009, 157 (157), pp.1195-1201. 10.1016/j.envpol.2008.12.009 . hal-00365544

HAL Id: hal-00365544

<https://hal.science/hal-00365544>

Submitted on 3 Mar 2009

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Isolation and characterization of mesotrione-degrading *Bacillus* sp. from soil

Isabelle Batisson^{a,*}, Olivier Couzet^a, Pascale Besse-Hoggan^b, Martine Sancelme^b, Jean-François Mangot^a, Clarisse Mallet^a, Jacques Bohatier^a

^aLaboratoire Microorganismes, Génome et Environnement, UMR 6023 CNRS, Université Blaise-Pascal, 63177 Aubière Cedex, France

^bLaboratoire de Synthèse et Étude de Systèmes à Intérêt Biologique, UMR 6504 CNRS, Université Blaise-Pascal, 63177 Aubière Cedex, France

A Bacillus sp. strain isolated from soil was able to completely and rapidly biotransform the triketone herbicide mesotrione.

A B S T R A C T

Dissipation kinetics of mesotrione, a new triketone herbicide, sprayed on soil from Limagne (Puy-de-Dôme, France) showed that the soil microflora were able to biotransform it.

Bacteria from this soil were cultured in mineral salt solution supplemented with mesotrione as sole source of carbon for the isolation of mesotrione-degrading bacteria. The bacterial community structure of the enrichment cultures was analyzed by temporal temperature gradient gel electrophoresis (TTGE). The TTGE fingerprints revealed that mesotrione had an impact on bacterial community structure only at its highest concentrations and showed mesotrione-sensitive and mesotrione-adapted strains. Two adapted strains, identified as *Bacillus* sp. and *Arthrobacter* sp., were isolated by colony hybridization methods.

Biodegradation assays showed that only the *Bacillus* sp. strain was able to completely and rapidly biotransform mesotrione. Among several metabolites formed, 2-amino-4-methylsulfonylbenzoic acid (AMBA) accumulated in the medium. Although sulcotrione has a chemical structure closely resembling that of mesotrione, the isolates were unable to degrade it.

Keywords:

Mesotrione

Bacterial degradation

Soil

Bacillus sp.

2-Amino-4-methylsulfonylbenzoic acid (AMBA)

1. Introduction

Mesotrione {2-(4-methylsulfonyl-2-nitrobenzoyl)1,3-cyclohexanedione}, a member of the triketone family, is a new selective herbicide developed for use in maize culture as a substitute for atrazine (Mitchell et al., 2001). Agricultural application of this compound is steadily increasing. It offers pre- and post-emergence control of all the common broad-leaved weeds together with some of the annual grass weeds. Mesotrione is a herbicide chemically derived from a natural phytotoxin produced by the bottlebrush plant *Callistemon citrinus* (Mitchell et al., 2001). It was developed to minimize adverse effects on the environment. However, it may affect microbial activity and diversity in both soil and aquatic ecosystems (<http://www.dive.afssa.fr/agritox/php/fiches.php>). Indeed, recent work has shown that mesotrione exhibits moderate retention capacity in different soils (Dyson et al., 2002; Chaabane et al., 2008) and so may be leached to surface water.

Mesotrione was also classified as toxicologically dangerous for the environment by the EEC (Agritox data, 29/04/04). However, as it has not been in use for long (marketed in 2001 in Europe), little information about its fate and impact in the environment is

available to date. Two mesotrione biotransformation products, 4-methylsulfonyl-2-nitrobenzoic acid (MNBA) and 2-amino-4-methylsulfonylbenzoic acid (AMBA), have been described (Alferness and Wiebe, 2002). Recently, one mesotrione-degrading bacterial strain was isolated from cloud water (Durand et al., 2006a) and some new mesotrione metabolites have been identified (Durand et al., 2006b). It was shown that the AMBA biodegradation product had a higher toxicity than the parent compound (Bonnet et al., 2008). However, the fate of mesotrione and its biodegradation products in the environment have remained incompletely evaluated. Understanding the impact of mesotrione on the structure, diversity and function of soil microbial communities is thus of major environmental concern.

In this study, we report on the isolation and characterization of a *Bacillus*-degrading strain from an agricultural soil that permitted a rapid transformation of mesotrione. Its metabolic pathway was investigated.

2. Materials and methods

2.1. Sampling site and capacity of soil microbial communities to degrade mesotrione

Soil was collected from 15-year fallow land in Limagne (Puy-de-Dôme, France) that was plowed two years before sampling, planted with maize and never treated with mesotrione. The surface layer (0–5 cm) of the soil, which had a humidity level of 20%, was air-dried, sieved ($\phi = 2$ mm), homogenized and placed in flasks.

* Corresponding author. Tel.: +33 473407870; fax: +33 473407670.
E-mail address: isabelle.batisson@univ-bpclermont.fr (I. Batisson).

Before isolating bacteria, the Limagne soil was tested for its capacity to biodegrade 10 times the agronomic dose of mesotrione (4.5 mg kg⁻¹ dry soil weight). Mesotrione powder (Pestanal, purity 99.9%; Fluka Riedel-de-Haën, Switzerland) was dissolved in distilled water and sprayed on 100 g of dry soil to obtain a final humidity of 20%. The flasks were incubated in the dark at 22 °C. Degradation kinetics was measured for a 42-day incubation period. An abiotic control with autoclaved soil was also carried out under the same conditions. Two samples of each soil (5 g) were taken at regular time intervals and placed in a centrifuge tube with distilled water (20 mL). The tubes were stirred on an orbital shaker at 50 rpm at room temperature for 24 h. After centrifugation at 12,500 × g for 15 min, the supernatant was filtered on PVDF filters and analyzed by HPLC. No adsorption of mesotrione was observed on centrifuge tube and PVDF filters. The mesotrione recovery rate of the extraction procedure after incubation in the Limagne soil was 95.0 ± 2.5% after 7 h of contact (10 replicates).

2.2. Enrichment cultures in flasks and bacteria isolation

To estimate the degradation capacity of the bacteria to mesotrione, 60 g of soil was added to 500 mL (10% dry w/v) of a mineral salt solution (MS) (Rousseaux et al., 2001). Mesotrione powder, added as the sole source of carbon, was directly dissolved in distilled water before adding the components of the MS medium. The final mesotrione concentration in the enrichment medium was initially 25 mg L⁻¹ and was doubled at three-week intervals in subsequent flasks, for a 77-day enrichment culture, to reach a maximum of 100 mg L⁻¹. To realize enrichment culture, 490 mL of fresh MS was inoculated with 10 mL of the precedent culture. The flasks were incubated at 28 °C on an orbital shaker at 150 rpm in darkness to avoid photolysis of mesotrione. The enrichment cultures were realized in duplicate. Cultures were sampled weekly for molecular analysis and bacteria isolation. A supernatant fraction and a soil particular fraction (pellet) of the cultures were separated by centrifuging (10 min, 8000 × g) before molecular analysis, except for the last enrichment step for which only a supernatant fraction was recovered, no more soil particles being present owing to the successive dilutions. To isolate the strains able to degrade mesotrione, MSA agar (MSA) plates (10 g L⁻¹ agar) were spread with 0.1 mL of a 20 mg mL⁻¹ mesotrione solution in acetone. Other non-seeded MSA plates were used as negative controls. The acetone was then air-evaporated. Ten-fold serial dilutions of the weekly enrichment culture samples were spread onto the agar plates and incubated at 28 °C in the dark for at least eight days.

2.3. PCR amplification and temporal temperature gradient gel electrophoresis (TTGE)

Bacterial genomic DNA was extracted by standard alkaline lysis method from 100 mL of bacterial cultures and was used as template to PCR amplify V6–V8 variable regions of 16S rDNA gene with the bacterium-specific forward primer GC968f and the reverse primer 1401r (MWG, Biotech, Table 1) as described by Pesce et al. (2006).

For cloning (see below), amplification was performed with the 968f/1401r primer as follows: denaturation at 95 °C for 15 min, followed by 30 cycles of 97 °C for 30 s, 62 °C for 30 s and 72 °C for 45 s per cycle, and a final extension step at 72 °C

for 10 min. DNA was quantified using the DNA quantitation kit fluorescence assay (Sigma–Aldrich, France) according to the manufacturer's instructions.

The entire 16S rDNA of isolated bacteria was amplified using the 27f/1492r primers (Table 1). The program consisted of denaturation at 95 °C for 15 min followed by 30 cycles at 94 °C for 45 s, 53 °C for 45 s and 72 °C for 45 s per cycle, and a final extension step for 10 min at 72 °C.

TTGE analyses were performed using a DCode system (Bio-Rad, France) on 300 ng of PCR products applied directly onto 8% (w/v) polyacrylamide gels containing 7 M urea, 1.25X Tris–acetate–EDTA (TAE). Runs were performed in 1.25X TAE at 68 V for 17 h with temperature ranging from 66 °C to 69.7 °C and a ramp rate of 0.2 °C h⁻¹. After completion of electrophoresis, gels were stained for 1 h in the dark in a Gel Star nucleic acid gel stain bath (BMA) and photographed using a Versa Doc™ Imaging System (Bio-Rad) under UV transillumination.

2.4. Clone library construction of single TTGE bands and sequencing

Each DNA band of interest was excised from the polyacrylamide gel and small gel pieces were placed in sterilized vials. The samples underwent passive diffusion (18 h at 37 °C) and the DNA was recovered as described by Batisson et al. (2007). Five µL of eluted DNA was then used as template for a reamplification step using the GC968f/1401r primers, as described above. Purity of the PCR reamplification product was checked by rerunning an aliquot on TTGE gel. The purified DNA band was then amplified with 968f/1401r primers as described above (Table 1) before being cloned into a pGEM-T-Easy vector (Promega, Charbonnières, France) to construct clone libraries. At least four clones were reamplified and rerun on TTGE gel for comparison with the parent band. A minimum of two cloned fragments that co-migrated with the original bands were bidirectionally sequenced (MWG – Biotech). Sequences were submitted to the National Center for Biotechnology Information (NCBI, BLASTn program (Altschul et al., 1997)) for species assignment and to the CHECK-CHIMERA program of the Ribosomal Database Project (RDP (Maidak et al., 1999)) to detect potential chimeric artifacts (Kopczynski et al., 1994).

Entire 16S rDNA sequences of the isolated strains were deposited in GenBank under Accession Nos: EU864320 (Mes11) and EU864321 (Mes16).

2.5. Synthesis of digoxigenin-labeled DNA probe and colony hybridization

Multiple alignments of partial 16S rDNA sequences performed on ClustalW software highlighted V6–V8 variable regions and made it possible to design specific primers for each strain (Table 1). Probes were PCR-labeled with digoxigenin (PCR DIG labeling mix, Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. The PCR program was as follows: 95 °C for 15 min, then 30 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s per cycle, and a final extension step at 72 °C for 7 min, except for the 11F/11R primer pair where the elongation step was carried out at 54 °C for 45 s.

First, to determine the specificity of the probes, the different recombinant pGEM-T plasmids harboring the partial 16S rDNA fragment were denatured at 95 °C for 5 min and spotted onto positively-charged nylon membranes (Hybond-N⁺, Amersham International). DIG-labeled control DNA (Roche Diagnostics) was used as positive control for the dot-blot detection. The DNA was fixed by sandwiching the membrane between two pieces of Whatman 3 MM paper and baking for 2 h in a vacuum at 80 °C. The membrane was then pre-hybridized, hybridized and revealed as described elsewhere (Batisson et al., 2007).

For bacteria isolation, colonies from the weekly samples of enrichment cultures spread on MSA plates were streaked in duplicate onto Luria-Bertani agar (LB-agar) plates and incubated at 28 °C for 24–48 h. Colonies of one LB-agar plate were replicated by placing a nylon membrane aseptically on top of the bacteria. The membranes carrying the colony lifts were treated as described by Heikinheimo et al. (2004) before being pre-hybridized, hybridized and visualized, as described above.

2.6. Degradation capacity of mesotrione by the isolated strains

To determine mesotrione degradation capacity, isolates were grown in 100 mL portions of trypticase-soy broth (bioMérieux, Marcy l'Etoile, France) in 500 mL flasks incubated at 28 °C and 200 rpm. The cells (200 mL of the culture) were harvested after 24 h of culture and centrifuged at 8000 × g for 15 min at 4 °C. The bacterial pellet was washed first with an NaCl solution (8 g L⁻¹) and then with Volvic® mineral water. The resting cells were then resuspended in 100 mL of 0.1 mM mesotrione solution and were incubated at 28 °C in the dark on a rotary shaker (200 rpm). Samples (1 mL) were regularly taken directly from the incubation medium and centrifuged at 10,000 × g for 5 min. The supernatants were frozen until analysis by HPLC. The same procedure was followed for the experiment with 1 mM mesotrione, except that the volume of the initial culture was 700 mL. The abiotic controls consisted of preparations incubated under the same conditions with mesotrione alone or with autoclave-killed bacteria. All the degradation test cultures were realized in triplicate.

HPLC analyses were performed at 22 °C using an Agilent 1100 chromatograph equipped with a diode array detector (DAD) and a fluorescent detector (FLD). A reversed-phase column (C-18 Zorbax Eclipse Plus column, 150 mm × 4.6 mm, 3.5 µm) was used at a flow rate of 1 mL min⁻¹. The mobile phase was aqueous H₃PO₄

Table 1
Name and sequence of primers used in this study.

Primer	Sequence (5'–3')	Fragment length (bp)	References
GC-clamp	CGCCCGCCGCGCGCGGGGGCGG GGCGGGGGCAGGGGGGG		
27f	AGAGTTTGATCTGGCTCAG	1484	(Weisburg et al., 1991)
1492r	GGTTACCTTGTACGACTT		(Lane, 1991)
968f	AACGCGAAGAACCCTTAC	490	(Heuer and Smalla, 1997)
1401r	CGGTGTGTACAGACCC		
2F	GGACTAGAATGTGAGG	67	This study
2R	GCACCTGTGTTTGGGT		This study
4F	TACCCCTGACATGGCTG	68	This study
4R	GCACCTGTGTTCTGGT		This study
5F	CAGACTTGACATACAGT	65	This study
5R	GCACCTGTGTCAGCAG		This study
11F	CTAGAGATAGAGCGTTCC	150	This study
11R	GTCACCTTAGAGTGCC		This study
12F	AGCCCTTGACATGCTA	68	This study
12R	GCACCTGTGTTCTAGG		This study
13F	TGCTCTGACATGCAC	69	This study
13R	GCACCTGTGCAAAGC		This study
15F	CACCTTGACATGTACG	68	This study
15R	GCACCTGTGTTACGGC		This study
16F	AAGGCTTGACATGGCC	66	This study
16R	CCACCTGTGAACCGG		This study

The fragment length (bp) obtained after PCR amplification with the forward (F) and reverse (R) primers is also indicated.

(0.01% v/v) (A)/CH₃CN (B) used with a gradient method: 0–3 min 95%A–5%B, 3–11 min from 5% to 85%B, 11–12 min from 85%B to 95%B, 12–13 min from 95 to 5%B, and 13–16 min 95%A–5%B. The injection volume was 10 µL and the detectors were set at $\lambda = 254$ nm (DAD), $\lambda_{exc} = 220$ nm, and $\lambda_{em} = 430$ nm (FLD). The DAD–UV and FLD spectra were compared with those of standards (mesotrione, AMBA, MNBA, sulcotrione) recorded in the mobile phase pH range.

3. Results

3.1. Capacity of microbial communities from Limagne soil to degrade mesotrione

To check that the microflora of the soil were able to degrade mesotrione, dissipation kinetics was measured after a contamination of 10 times the agronomic dose of the molecule. A similar experiment was carried out with autoclaved soil (abiotic control) (Fig. 1). A progressive decrease in mesotrione along the 42-day incubation time was observed, resulting in the dissipation of around 50% of the compound by microbial communities. The dissipation rate in the autoclaved soil was only of 10% for the same period and corresponded to adsorption process. This evidenced the capacity of this soil to biotransform mesotrione. Moreover, AMBA, a known-metabolite of mesotrione, was formed during this experiment and detected in the soil samples (data not shown).

3.2. Enrichment of a bacterial culture that degrades mesotrione

To monitor bacterial population diversity over time and assess the impact of mesotrione on these communities, the V6–V8 16S rDNA amplification products from weekly enrichment culture samples were analyzed by TTGE (Fig. 2). At day 0, numerous bands were detected in both supernatant and pellet fractions on TTGE fingerprints (Fig. 2A). Only temporal modifications were detected during the 21-day experiment with 25 mg L⁻¹ of mesotrione in the supernatant and pellet fraction, but no major differences were observed between the control (C) and mesotrione-treated (M) flasks.

When the cultures were supplemented with 50 mg L⁻¹ of mesotrione (Fig. 2B), the TTGE fingerprints showed a lower bacterial diversity compared with the first enrichment step (25 mg L⁻¹ mesotrione). Although no major variations were observed in the pellet fraction, the control flasks were clearly separated from

mesotrione-treated flasks in the supernatant fractions. A different band pattern was highlighted from day 28 to day 42, with bands found only in the mesotrione-treated flasks compared with controls (e.g. bands 2 and 4, Fig. 2B). Other bands were ubiquitous and were present equally in all flasks irrespective of the culture conditions (e.g. band 11).

These differences between environmental conditions of cultures in flasks (treated versus control) were confirmed when cultures were supplemented with 100 mg L⁻¹ of mesotrione (Fig. 2C). Bands only found or predominant in mesotrione-treated flasks and absent in controls (bands 5, 12, 13, 15, 16) and bands only visible or predominant in untreated flasks (bands 10, 17, 19, 20) became more clearly apparent (Fig. 2C).

3.3. Identification and isolation of mesotrione-degrading strains

Bands of interest (Fig. 2) corresponding to ubiquitous (e.g. band 11), mesotrione sensible (e.g. bands 10, 17, 19, 20) or mesotrione-adapted bands (e.g. bands 2, 4, 5, 12, 13, 15, 16) were excised and sequenced. The nearest neighbor and phylogenetic group of each sequence are listed in Table 2.

To isolate mesotrione-degrading bacterial strains, the enrichment cultures were plated weekly on either mesotrione-supplemented or control MSA. There were no significant differences between plates in cell density and colony morphology. Since it was impossible to distinguish between bacteria in control and contaminated cultures, a colony hybridization method was used. Sequences obtained from the different bands of interest were thus aligned to design primers targeting variable regions of each strain (Table 1) and so generate probes. The cross-reactivity of the probes against the V6–V8 variable regions of 16S rDNA clone library obtained in this study was tested and showed high specificity for each probe. Only two colonies corresponding to bands 11 and 16 (Fig. 2) were successfully isolated. They were named strain Mes11 and Mes16, respectively. Their entire 16S rDNA was then sequenced using the 27f/1492r primer couple (Table 1) and aligned using the BLASTn program. The results matched those obtained with the V6–V8 phylogenetic affiliation of these strains (Table 2) and confirmed that strain Mes11 and Mes16 were 99% similar to *Bacillus* sp. and *Arthrobacter* sp., respectively.

3.4. Capacity of the isolates to degrade mesotrione

We investigated the capacity of each isolate to degrade 0.1–1 mM mesotrione in resting cells. The kinetics of degradation is illustrated in Fig. 3. After 50 h of incubation, the *Arthrobacter* sp. strain (Mes16) did not give rise to 0.1 mM mesotrione degradation (Fig. 3A). Degradation assays with lower concentrations of mesotrione (down to 2 mg L⁻¹ = 6 µM) were also unsuccessful (data not shown). By contrast, the *Bacillus* sp. isolate (Mes11) completely degraded the mesotrione after 50 h and 24 h of incubation with 0.1 mM and 1 mM, respectively (Fig. 3A and B). Furthermore, in the experiment with 1 mM of mesotrione, several metabolites were detected by HPLC during the biodegradation. One was particularly interesting as it accumulated in the medium. This metabolite was identified as AMBA by comparison with the standard. The AMBA metabolite appeared clearly after 3 h of incubation and reached a concentration of about 0.1 mM at the plateau phase that persisted up to 60 h (Fig. 3B). From 60 h to the end of the experiment (120 h), a new increase of AMBA up to a maximum of 0.3 mM was observed. No disappearance of mesotrione was observed with controls containing the pesticide alone (data not shown) or autoclave-killed bacteria (Fig. 3B). Two other metabolites were identified by comparison with metabolites previously described with another *Bacillus* strain (Durand et al., 2006b): the hydroxylamino derivative of mesotrione 1 and the benzisoxazol-3-ol 2 (Fig. 4).

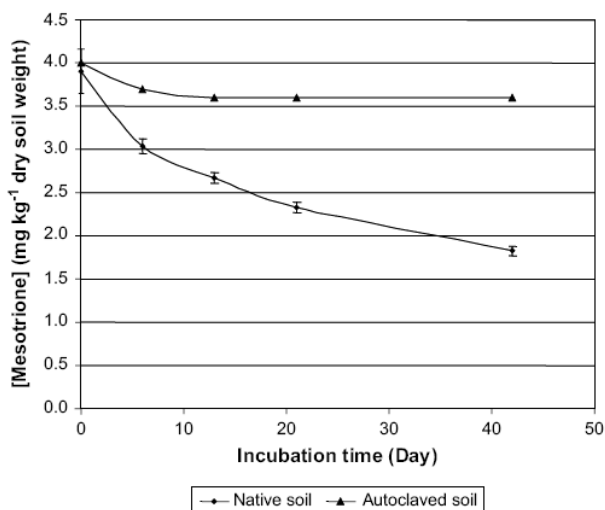


Fig. 1. Mean (\pm s.d.) degradation of the mesotrione by microbial communities of Limagne soil after application of 10 times the agronomic dose (4.5 mg kg⁻¹ dry soil weight). Degradation assays were performed at 28 °C in the dark. Autoclaved soil was used as negative control for biodegradation.

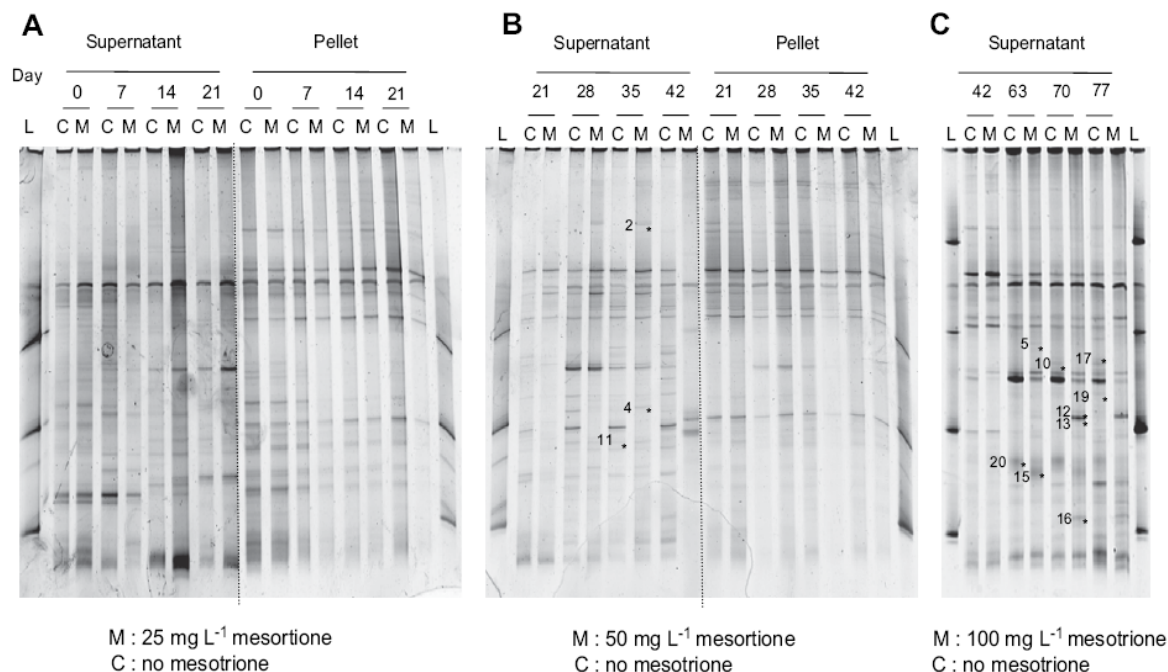


Fig. 2. Study of bacterial community evolution by TTE analysis of V6-V8 16S rDNA fragments in control (C; without mesotrione) and mesotrione-treated (M) enrichment cultures inoculated with Limagne soil from day 0 to day 77. Two enrichment steps of 21 days (for 25 mg L⁻¹ (A) and 50 mg L⁻¹ (B) mesotrione) and one of 35 days (for 100 mg L⁻¹ (C) mesotrione) were carried out. Numbers represent the bands of interest that were excised from the gel, purified and sequenced. L: Ladder composed of *Alcaligenes faecalis*, *Kurthia zopfii*, *Stenotrophomonas maltophilia* and *Flavobacterium aquatile*.

The capacity of *Bacillus* sp. and *Arthrobacter* sp. to degrade 0.1 mM of sulcotrione, another triketone family member, was also tested under the same conditions, but no degradation was observed (data not shown).

4. Discussion

4.1. Enrichment cultures with mesotrione

Although the MS medium used for enrichment cultures did not contain a carbon source (Rousseaux et al., 2001), bacterial

growth was observed in control flasks similar to that of the mesotrione-treated flasks (data not shown). The same observation has already been made for the isolation of other pesticide-degradation bacteria (Turnbull et al., 2001; Batisson et al., 2007). In our study, the bacterial growth in the control flask could be explained by the supply of some organic matter and nutrient sources in the initial soil inocula and could also explain the bacterial background observed on MSA plates. Carbon sources could also be supplied by impurities in the agar (Cullington and Walker, 1999; El Fantroussi, 2000; Tumbull et al., 2001; Dejonghe et al., 2003).

Table 2

Similarity to nearest neighbors and closest phylogenetic affiliation of TTE band sequences detected in enrichment cultures based on BLASTn comparison with the GenBank database.

Band	Nearest neighbor		Accession number	Phylogenetic group, Class - Family	References
	Similarity (%)	Species			
2	99	[Uncultured soil bacterium clone DS-249]	AY289362.1	Acidobacteria - Acidobacteriaceae	Zhou et al. (2003)
4	97	[<i>Herbaspirillum</i> sp. YM23]	EU418729	β -Proteobacteria - Oxalobacteraceae	Sun, Unpublished
5	91	[Uncultured bacterium clone OTU289]	DQ684283.1	Chlamydia - Parachlamydiaceae	LeBlanc et al. (2007)
11	99	[<i>Bacillus</i> sp. HNL20]	EU373361.1	Bacilli - Bacillaceae	Cho et al., Unpublished
	99	{ <i>Bacillus</i> sp. G3DM-48}	EU037283.1	Bacilli - Bacillaceae	Desai et al., Unpublished
12	99	[Uncultured eubacterium OCG9]	AB047120.1	α -Proteobacteria - unclassified Rhizobiales	Watanabe et al. (2001)
13	96	[Uncultured <i>Verrucomicrobia</i> bacterium clone AKYH1486]	AY922120	Verrucomicrobiae - Verrucomicrobiaceae	Tringe et al. (2005)
15	99	[<i>Acidovorax</i> sp. NO-1]	EU521706.1	β -Proteobacteria - Comamonadaceae	Fan et al., Unpublished
16	100	[Uncultured bacterium clone OTU506]	DQ684500.1	Actinobacteria - Micrococcaceae	LeBlanc et al. (2007)
	99	{ <i>Arthrobacter</i> sp.}	DQ158001.1	Actinobacteria - Micrococcaceae	Nakatsu and Beasley, Unpublished
10	99	[Unidentified bacterium wb1_A01]	AF317740	γ Proteobacteria - Pseudomonadaceae	Holmes et al. (2001)
17	97	[Uncultured bacterium clone OTU001]	DQ683995.1	Chlamydiae - Parachlamydiaceae	LeBlanc et al. (2007)
19	94	[Uncultured bacterium clone L3B_344]	EF551894.1	α -Proteobacteria - Phyllobacteriaceae	Hiibel et al., Unpublished
20	99	[<i>Acidovorax facilis</i> strain LMG 2193]	EU024133.1	β -Proteobacteria - Comamonadaceae	Ait Tayeb et al. (2008)

[]: Alignment of V6-V8 16S rDNA sequences; { } : alignment of entire 16S rDNA sequences.

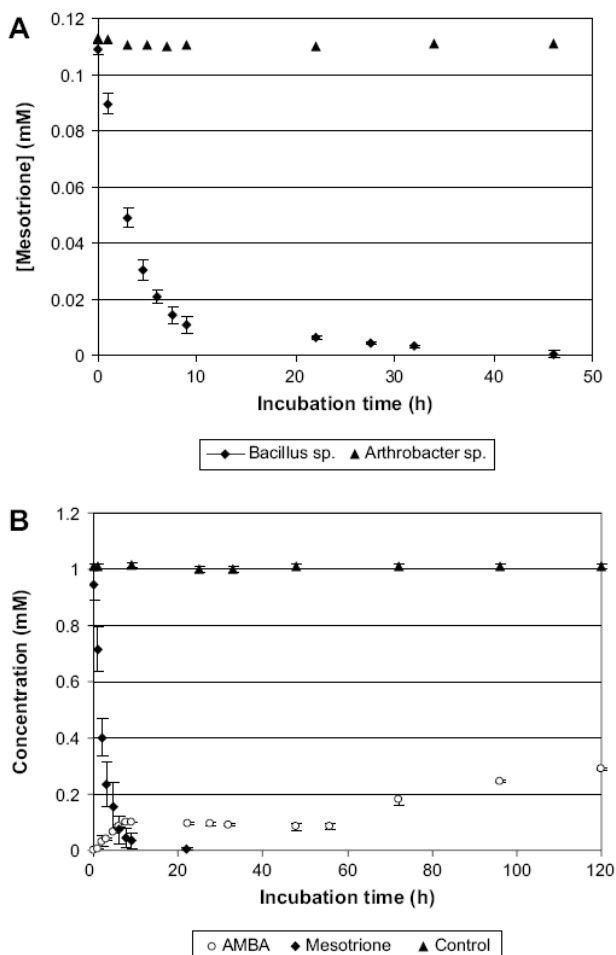


Fig. 3. Degradation assays with resting cells. (A) Degradation of 0.1 mM mesotrione by *Bacillus* sp. and *Arthrobacter* sp. (B) Time courses of mesotrione (1 mM) and AMBA during biodegradation by *Bacillus* sp. Abiotic control of mesotrione corresponds to the dissipation observed with autoclaved bacteria. Assays were performed at 28 °C in the dark. Errors bars represent the mean (\pm s.d.) of three independent experiments.

4.2. Bacterial culture response to mesotrione exposure

Unlike the spreading of bacteria on agar plates, the TTGE approach highlighted some differences in bacterial populations of control and mesotrione-treated cultures. We were able to reveal shifts in the dominant bacterial community with regard to natural populations (day 0) and between control and mesotrione-treated flasks (Fig. 2).

After only seven days of enrichment culture, the shift of the bacterial community observed in both treated and control flasks compared with the initial bacterial community was probably due to the phase of acclimatization to the experimental conditions and/or the unculturability of some bacteria. These observations were confirmed after the second week of enrichment culture (Fig. 2). Effects of mesotrione on the bacterial community were only detected seven days after treatment with 50 mg L⁻¹ of herbicide (day 28), suggesting that a latency phase was required to allow induction of specific enzyme systems in response to the organic pollution pressure (Četkauskaitė et al., 1998). Thereafter and for higher concentrations of mesotrione, bands that were maintained, changed in intensity or appeared only in the mesotrione exposure

flasks were interpreted as corresponding to bacterial species potentially able to use this molecule for their growth as observed in previous studies (El Fantroussi et al., 1999; El Fantroussi, 2000; Crecchio et al., 2001; Bending et al., 2003; Pesce et al., 2006; Batisson et al., 2007).

4.3. Identification and isolation of bacteria adapted to mesotrione

TTGE bands of interest were sequenced and affiliated to diverse phylogenetic groups (Table 2). Strains corresponding to bands 2 and 13 were affiliated to bacteria isolated from natural soil or water environmental samples (Zhou et al., 2003; Tringe et al., 2005). However, band 13 also showed 95% similarity with an uncultured *Verrucomicrobia* bacterium encountered in diuron-contaminated microcosms (Pesce et al., 2008) suggesting that the *Verrucomicrobia* bacterial group might play an important role in the ecosystem after chemical pollutant exposure. Nearest neighbors of strains corresponding to bands 5 and 12 were uncultured bacteria found in soil rhizosphere communities altered by the culture of genetically modified plants (LeBlanc et al., 2007) and oil-contaminated water (Watanabe et al., 2001), respectively, emphasizing the capacity of *Chlamydia* and α -*Proteobacteria* to adapt to modifications of their environment. Furthermore, 16S rDNA corresponding to band 5 was also 90% similar to that of *Chlamydia* found in phenylurea herbicide-contaminated water (Pesce et al., 2008), likewise showing the capacity of these strains to acclimatize to organic compound pollution. Bands 4 and 15 sequences showed high similarity with bacteria isolated from a copper mine (Sun, unpublished data; Accession No. EU418729) and from a gold mine contaminated with arsenite (Fan et al., unpublished data; Accession No. EU521706). Two strains, showing 99% similarities to *Arthrobacter* sp. (band 16) and *Bacillus* sp. (band 11), were successfully isolated from our enrichment cultures with mesotrione. They were also encountered in soil microcosms contaminated with xylene and chromate (Nakatsu and Beasley, unpublished data; Accession No. DQ158001) and in long-term chromium-contaminated sediments (Desai et al., unpublished data; Accession No. EU037283), respectively. *Arthrobacter* sp. and *Bacillus* sp. were also isolated from a microcosm-enrichment cultures supplemented with chloropicrin and metam sodium (Ibekwe et al., 2004), suggesting a resistance and/or degradation capacity of these strains for numerous xenobiotics.

Despite many attempts, no other strains adapted to mesotrione were isolated from the MSA plates. This could be due to a loss of degradation capacity on solid media (El Fantroussi, 2000).

4.4. Biodegradation of mesotrione

Pure-culture mesotrione degradation experiments in resting cells indicated that the strain Mes16, although responsible for a clearly visible band specific to treated flasks (Fig. 2C), was not able to degrade mesotrione (Fig. 3A). This result denotes that mesotrione had no toxic effect on this strain and suggests that the Mes16 isolate may require a synergistic interaction with other bacteria to degrade mesotrione. When isolate Mes11 was tested, we observed that within the first 24 h of incubation, 1 mM mesotrione was completely degraded (Fig. 3B). It was not surprising to find mesotrione-degrading bacteria in our soil since the presence of specific microflora able to degrade triketone had been already found in soil free of mesotrione treatment (Chaabane et al., 2008). However, a difference in percentage and rate of mesotrione degradation was observed between native soil and liquid culture that reflects the different environmental conditions of the bacteria (no mesotrione-adapted microbial communities in soil with complex organic matter available versus mesotrione-adapted bacteria in pure liquid culture with only mesotrione as the sole carbon source).

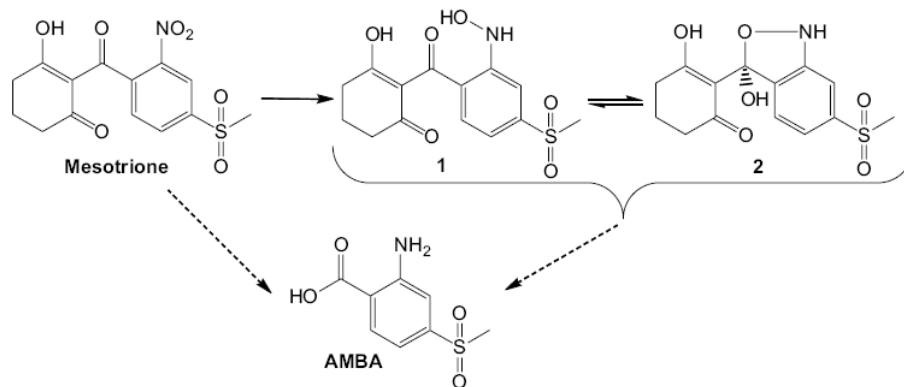


Fig. 4. Proposed scheme of mesotrione biodegradation by *Bacillus* sp.

This biodegradation of mesotrione with Mes11 led to the formation of several detected metabolites. The concentration of some of these reached a maximum and then decreased. Two of them have already been identified during mesotrione degradation by the *Bacillus* sp. 3B6 strain (Durand et al., 2006b) and were produced in the same order of magnitude by the Mes11 strain: an hydroxylamino derivative **1** obtained by reduction of the nitro group of mesotrione and a cyclized compound **2** (isoxazolol), formed by an intramolecular cyclization of the hydroxyl moiety of the hydroxylamine on the carbonyl group (Fig. 4). Another metabolite, which accumulated in the medium, was identified as AMBA by comparison of its retention time and UV spectrum with those of a standard. Contrary to what was observed by Alferness and Wiebe (2002), no MNBA degradation product could be detected after mesotrione degradation by the Mes11 strain. This result is in accordance with those obtained with the *Bacillus* sp. strain 3B6 (Durand et al., 2006a). This 3B6 strain was also able to completely and rapidly degrade mesotrione and led to the formation of five metabolites, three of them (AMBA, **1** and **2**) being also produced by the Mes11 strain. This result highlights the important role of these *Bacillus* species in triketone degradation. Nevertheless, with both these *Bacillus* strains, AMBA accumulated in the medium, indicating that mesotrione was not completely mineralized under the conditions used. The kinetics of AMBA appearance displayed two steps: a first increase of AMBA concentration to a plateau within the first hours of incubation followed by a second higher increase after 60 h (Fig. 3B). This could be the result of a two-pathway degradation mechanism as suggested by the mesotrione degradation model proposed by Durand et al. (2006b): (i) a minor pathway leading almost directly to AMBA by an oxidative cleavage of mesotrione followed by a reduction of the nitro group. This could explain the almost immediate formation of AMBA; (ii) a major pathway leading to AMBA via a reductive pathway of the amino group. Several metabolites were formed before being transformed into AMBA. The formation of these metabolites and their degradation will delay the appearance of AMBA. This may explain the increase in AMBA concentration after 60 h of incubation, this time corresponding to the disappearance of the two identified metabolites **1** and **2** (data not shown). Therefore a similar biodegradation pathway of mesotrione was observed with the two isolated *Bacillus* strains: 3B6 and Mes11. Nevertheless, the identification of the last two metabolites detected with Mes11 is under investigation and would allow understanding in more details the fate of mesotrione.

As sulcotrione, another member of the triketone family had a chemical structure closely resembling that of mesotrione, the capacity of the isolates to degrade this molecule was also tested. No degradation of sulcotrione was observed with either strain,

underlining the substance-specificity of the *Bacillus* sp. isolated in our study. This suggests that the Mes11 strain could attack the mesotrione at the level of the benzoyl 4-substituent for degradation, and highlights the importance of this position on the molecule not only for its herbicidal activity (Lee et al., 1998; Mitchell et al., 2001) but also for its remanence.

Studies to obtain further data on the mesotrione degradation pathway with Mes11 and identify the degrading gene involved in mesotrione biotransformation are in progress in our laboratory.

Acknowledgments

The authors thank M. Debote of INRA of Clermont-Ferrand (Crouel) for use of the experimental field. They also thank Tiffany Charbouillot for her assistance in HPLC analyses.

References

- Ait Tayeb, L., Lefevre, M., Passet, V., Diancourt, L., Brisse, S., Grimont, P.A.D., 2008. Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organisms derived from *rpoB*, *gyrB* and *rrs* gene sequences. *Research Microbiology* 159, 169–177.
- Alferness, P., Wiebe, L., 2002. Determination of mesotrione residues and metabolites in crops, soil, and water by liquid chromatography with fluorescence detection. *Journal of Agricultural and Food Chemistry* 50, 3926–3934.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.
- Batissou, I., Pesce, S., Besse-Hoggan, P., Sancelme, M., Bohatier, J., 2007. Isolation and characterization of diuron-degrading bacteria from lotic surface water. *Microbial Ecology* 54, 761–770.
- Bending, G.D., Lincoln, S.D., Sørensen, S.R., Morgan, J.A.W., Aamand, J., Walker, A., 2003. In-field spatial variability in the degradation of the phenyl-urea herbicide isoproturon is the result of interactions between degradative *Sphingomonas* spp. and soil pH. *Applied and Environmental Microbiology* 69, 827–834.
- Bonnet, J.-L., Bonnemoy, F., Dusser, M., Bohatier, J., 2008. Toxicity assessment of the herbicides sulcotrione and mesotrione toward two reference environmental microorganisms: *Tetrahymena pyriformis* and *Vibrio fischeri*. *Archives of Environmental Contamination and Toxicology* 55, 576–583.
- Četkauskaitė, A., Grigonis, U., Berzinskienė, J., 1998. Biodegradation: selection of suitable model. *Ecotoxicology and Environmental Safety* 40, 19–28.
- Chaabane, H., Vulliet, E., Calvayrac, C., Coste, C.-M., Cooper, J.-F., 2008. Behaviour of sulcotrione and mesotrione in two soils. *Pest Management Science* 64, 86–93.
- Crecchio, C., Curci, M., Pizzigallo, M.D.R., Ricciuti, P., Ruggiero, P., 2001. Molecular approaches to investigate herbicide-induced bacterial community changes in soil microcosms. *Biology and Fertility of Soils* 33, 460–466.
- Cullington, J.E., Walker, A., 1999. Rapid biodegradation of diuron and other phenylurea herbicides by a soil bacterium. *Soil Biology and Biochemistry* 31, 677–686.
- Dejonghe, W., Berteloot, E., Goris, J., Boon, N., Crul, K., Maertens, S., Höfte, M., De Vos, P., Verstraete, W., Top, E.M., 2003. Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovorax* strain. *Applied and Environmental Microbiology* 69, 1532–1541.
- Durand, S., Amato, P., Sancelme, M., Delort, A.-M., Combourieu, B., Besse-Hoggan, P., 2006a. First isolation and characterization of a bacterial strain that biotransforms the herbicide mesotrione. *Letters in Applied Microbiology* 43, 222–228.

- Durand, S., Légeret, B., Martin, A.-S., Sancelme, M., Delort, A.-M., Besse-Hoggan, P., Combourieu, B., 2006b. Biotransformation of a triketone herbicide mesotrione by a *Bacillus* strain. Metabolite profiling using liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 20, 2603–2613.
- Dyson, J.S., Beulke, S., Brown, C.D., Lane, M.C.G., 2002. Adsorption and degradation of the weak acid mesotrione in soil and environmental fate implications. *Organic compounds in the environment. Journal of Environmental Quality* 31, 613–618.
- El Fantroussi, S., 2000. Enrichment and molecular characterization of a bacterial culture that degrades methoxy-methyl urea herbicides and their aniline derivatives. *Applied and Environmental Microbiology* 66, 5110–5115.
- El Fantroussi, S., Verschuere, L., Verstraete, W., Top, E.M., 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16SrRNA gene fingerprints and community-level physiological profiles. *Applied and Environmental Microbiology* 65, 982–988.
- Heikinheimo, A., Lindström, M., Korkeala, H., 2004. Enumeration and isolation of *cpe*-positive *Clostridium perfringens* spores from feces. *Journal of Clinical Microbiology* 42, 3992–3997.
- Heuer, H., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In: van Elsas, J.D. (Ed.), *Modern Soil Microbiology*. Marcel Dekker, New York, pp. 353–373.
- Holmes, A.J., Tujula, N.A., Holley, M., Contos, A., James, J.M., Rogers, P., Gillings, M.R., 2001. Phylogenetic structure of unusual aquatic microbial formations in Nularbor caves, Australia. *Environmental Microbiology* 3, 256–264.
- Ibekwe, A.M., Papiernik, S.K., Yang, C.-H., 2004. Enrichment and molecular characterization of chloropicrin- and metam-sodium-degrading microbial communities. *Applied and Environmental Microbiology* 66, 325–332.
- Kopczynski, E.D., Bateson, M.M., Ward, D.M., 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Applied and Environmental Microbiology* 60, 746–748.
- Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons, Inc., New York, NY, pp. 115–148.
- LeBlanc, P.M., Hamelin, R.C., Filion, M., 2007. Alteration of soil rhizosphere communities following genetic transformation of white spruce. *Applied and Environmental Microbiology* 73, 4128–4134.
- Lee, D.L., Knudsen, C.G., Michaelay, W.J., Chin, H., Nguyen, N.H., Carter, C.G., Cromartie, T.H., Lake, B.H., Shribbs, J.M., Fraser, T., 1998. The structure-activity relationships of the triketone class of HPPD herbicides. *Pesticide Science* 54, 377–384.
- Maidak, B.L., Cole, J.R., Parker Jr., C.T., Garrity, G.M., Larsen, N., Li, B., Lilburn, T.G., McCaughey, M.J., Olsen, G.J., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M., Woese, C.R., 1999. A new version of the RDP (ribosomal database project). *Nucleic Acids Research* 27, 171–173.
- Mitchell, G., Bartlett, D.W., Fraser, T.E.M., Hawkes, T.R., Holt, D.C., Townson, J.K., Wichert, R.A., 2001. Mesotrione: a new selective herbicide for use in maize. *Pest Management Science* 57, 120–128.
- Pesce, S., Fajon, C., Bardot, C., Bonnemoy, F., Portelli, C., Bohatier, J., 2006. Effects of the phenylurea herbicide diuron on natural riverine microbial communities in an experimental study. *Aquatic Toxicology* 78, 303–314.
- Pesce, S., Bardot, C., Lehours, A.-C., Batisson, I., Bohatier, J., Fajon, C., 2008. Effects of diuron in microcosms on natural riverine bacterial community composition: new insight into phylogenetic approach using PCR-TTGE analysis. *Aquatic Sciences* 70, 410–418.
- Rousseaux, S., Hartmann, A., Soulas, G., 2001. Isolation and characterisation of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils. *FEMS Microbiology Ecology* 36, 211–222.
- Tringe, S.G., von Mering, C., Kobayashi, A., Salamov, A.A., Chen, K., Chang, H.W., Podar, M., Short, J.M., Mathur, E.J., Detter, J.C., Bork, P., Hugenholtz, P., Rubin, E.M., 2005. Comparative metagenomics of microbial communities. *Science* 22, 554–557.
- Turnbull, G.A., Ousley, M., Walker, A., Shaw, E., Morgan, J.A.W., 2001. Degradation of substituted phenylurea herbicides by *Arthrobacter globiformis* strain D47 and characterization of a plasmid-associated hydrolase gene, *puhA*. *Applied and Environmental Microbiology* 67, 2270–2275.
- Watanabe, K., Kodama, Y., Harayama, S., 2001. Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *Journal of Microbiological Methods* 44, 253–262.
- Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173, 697–703.
- Zhou, J., Xia, B., Huang, H., Treves, D.S., Hauser, L.J., Mural, R.J., Palumbo, A.V., Tiedje, J.M., 2003. Bacterial phylogenetic diversity and a novel candidate division of two humid region, sandy surface soils. *Soil Biology and Biochemistry* 35, 915–924.