Characterization of the isoproturon degrading community : from the field to the genes

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Characterization of the isoproturon degrading community: From the field to the genes

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List of abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
3,4-DCA	3,4-Dichloroaniline
4-IA	4-Isopropylaniline
AFSSA	Agence française de sécurité sanitaire des aliments (French Food Safety Agency)
ANOVA	Analysis of variance
ARDRA	Amplified ribosomal DNA restriction analysis
A-RISA	Automated ribosomal intergenic spacer analysis
CEC	Cation exchange capacity
cfu	Colony forming units
DDIPU	Didemethyl isoproturon
DDT	Dichloro-diphenyl-trichloroethane
DFT	Density functional theory
DGAL	Direction générale de l'alimentation
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dpm	Disintegrations per minute
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EPA	Environment Protection Agency
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
ha	Hactare
HCH	Hexachlorocyclohexane
HPLS	High performance liquid chromatography
IFEN	Institut Français de l'Environnement (French Institute of the Environment)
IGS	Intergenic spacer
INERIS	Institut national de l'environnement industriel et des risques
INRA	Institut National de la Recherche Agronomique (National Institute for Agricultural Research)
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IPU	Isoproturon

kb	Kilo base pair
LD	Lethal dose
MA	Marketing Authorization
MDIPU	Monodemethyl isoproturon
MS	Mineral salt medium
MSA	Mineral salt agar medium
NCBI	National Center for Biotechnology Information
OD	Optical density
ORF	Open reading frame
PAN	Pesticide action network
PBT	Persistent Bioaccumulative and Toxic
PCA	Principal component analysis
PCR	Polymerase chain reaction
POP	Persistent organic pollutants
PSII	Photosystem II
PVPP	Polyvinyl polypyrrolidone
QSAR	Quantitative structure-activity relationship
R & D	Research and development
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAU	Surface Agricole Utile (Utilized agricultural surface)
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SSM	Structure Scientifique Mixte (Joint Scientific Structure)
TE	Tris EDTA
UIPP	l'Union des Industries de la Protection des Plantes (Union of Plant Protection Industries)
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
WFD	Water Framework Directive
WHC	Water holding capacity
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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RESULTS AND DISCUSSION

Chapter 3: SPATIAL VARIABILITY OF ISOPROTURON MINERALIZING ACTIVITY WITHIN A FRENCH AGRICULTURAL FIELD

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GENERAL INTRODUCTION

Agriculture is a key sector in the world economy where countries compete for improving the quality and yield of crop production. In these conditions, pesticides are widely used to (i) increase the crop production quantitatively and qualitatively, (ii) limit the irregularities of crop production linked to the great parasitic catastrophe, (iii) protect the food reserves, (iv) struggle against the vectors of diseases and (v) struggle against the toxins producing parasites such as mycotoxin producing fungi which cause health problems. They are used in a variety of ways including field sprays. Although mainly used for agricultural purposes, they are also used as house hold bug sprays and can be found in most of our everyday environments including workplaces, homes, drinking and recreational waters, soil and air etc.

Pesticides have a long history because since the emergence of agriculture, human beings are facing the development of pests (including weeds, insects and disease agents) causing considerable agricultural losses. If these pests are not controlled, they diminish the quality and quantity of production (Richardson, 1998). In the beginning, either some inorganic chemicals or compounds extracted from plants and animals were used as pesticides. The pyretrine was extracted from chrysanthemum flowers and used to control the pest development during winter storage of crop. This was reported by the Greek civilization and authorization of this compound is still ongoing. However, agricultural revolution in the 19th century has lead to the intensive and diversified use of the pesticides corresponding to compounds derived from minerals and plants. As an example, the development of Bouillie Bordelaise (Bordeaux mixture) in 1880, made of copper sulphate and of lime allowed the better control of cryptogamic diseases in Bordeaux and French vineyard. It is still in use for vineyard and fruit tree protection. Development and application of the pesticides for the control of various types of insectivorous and herbivorous pests is considered as a fundamental contributor to this “Green Revolution”. The use of synthetic organic pesticides began during the early decades of 20th century and it tremendously increased after the World War II, with the introduction of the synthetic organic molecules such as DDT, aldrin (two insecticides) and the herbicide 2,4-D in the agricultural market. Due to their advantages of being effective and cheap, the use of synthetic pesticides carried on increasing in whole of the world including Europe and France. According to a report published by the Union of Plant Protection

Industries (UIPP) in 2004, about 76,000 tonnes of pesticides were sold in France in 2001 which represents in mean approximately 2.0 kg of pesticides applied per hectares.

Although pesticide use ensures the yield in agricultural production, however, when they contaminate the soil and water resources, they could be harmful for the environment and for human beings through the food chain (Barriuso et al., 1996). Due to their intensive and repeated use, and their relative recalcitrance to biodegradation, pesticide residues are persistent in the environment where they have often been detected beyond the permissible limits in different compartments of the environment and in food chain. In many parts of the world, particularly in developing countries, clean drinking water is a limited resource and, in this context, intensive agricultural production is a major environmental and health problem because of which pesticide residues accumulate in surface and ground water (Rasmussen et al., 2005). Contamination with pesticides is restricted not only to developing countries but also in countries in Europe including France where pesticide residues have often been detected in surface and ground water resources. (IFEN, 2003; Giovanni, 1996). As a result, the use of pesticides in conventional agriculture has attracted much attention in recent years due to rising public and governmental concerns about their impact not only on environmental contamination but also on human and animal health.

Phenylurea herbicides are one of the most importantly and world widely used class of pesticides. These substances enter the plant *via* the roots and inhibit the photosynthesis. They are either used for pre- or post-emergence control of broad leaf weeds in cereal production. Some phenylureas, such as diuron, are also used as a non-selective herbicide in vineyards and in urban areas (Sorensen et al., 2003). They are fairly mobile in soil and are often detected as contaminants of rivers, streams, lakes and seawater in European countries as well as in the USA (Field et al., 1997; Thurman et al., 2000).

Isoproturon (IPU) is a phenylurea herbicide used for pre- and post-emergence control of many broadleaf weeds in spring and winter wheat, barley and winter rye (Fournier et al., 1975). It is among the most extensively used herbicides in conventional agriculture in Europe (Nitchke and Schussler, 1998). It is applied to a dose varying from 1500 to 1800 g ha^{-1} on the cereal cultures. However, in October 2003, the French ministry of agriculture restricted the use of IPU to agriculture at 1200 g ha^{-1} in order to limit its dispersion in the environment. As a result of its intensive and repeated use and of its physico-chemical properties, IPU is oftenly detected in surface and ground water in Europe at levels exceeding the European Union

drinking water limit fixed to 0.1 µg l^{-1} (Nitchke and Schusssler, 1998; Spliid and Koppen, 1998; Stangroom et al., 1998). Ecotoxicological data have suggested that IPU, and some of its main metabolites, are carcinogen and harmful to aquatic invertebrates, fresh water algae and microbial activities (Pérés et al., 1996; Mansour et al., 1999). So, keeping the harmful effects of isoproturon in view, there is need of understanding the processes involved in the fate of this herbicide in agricultural soils in order to promote its degradation and to limit its dispersion in the environment.

There are a number of possible abiotic degradation treatments for IPU remediation in the soil environment including chemical treatment, volatilization, photodecomposition and incineration. However, most of them are not applicable for the diffused contamination with low concentration because of being expensive, less efficient and not always environmental friendly. Thus, keeping the environmental concerns associated with isoproturon and other phenylurea herbicides in view, there is need to develop safe, convenient and economically viable methods for its remediation. In this context, several researchers have focused their attention to study the microbial biodegradation which has been reported as a primary mechanism of pesticide dissipation from the soil environment (Cox et al., 1996, Pieuchot et al., 1996). Although bioremediation strategies are more acceptable to the society because of their reduced impact on the natural ecosystem (Zhang and Quiao, 2002), the complexity of the mechanisms responsible for pesticide degradation has made it slow to emerge as an economically viable remediation method (Nerud et al., 2003). It is noteworthy that bioremediation strategies have been developed extensively for taking care of sites heavily contaminated with organic pollutants, however, up to now, the sites diffusely contaminated are only monitored and natural attenuation is the process of interest leading to contaminant abatement. So, the detailed study of the mechanisms and processes involved in microbial biodegradation of pesticides including IPU is required.

Although initially described as relatively low and partial, isoproturon mineralization has been reported in soils repeatedly exposed to IPU. It was hypothesized that following the exposure to IPU, the soil micro flora adapt to its rapid degradation which offers an extra source of carbon, nitrogen and energy promoting the growth of IPU degraders (Sorensen and Aamnad 2001; Bending et al., 2003; Sorensen and Aamand, 2003; El-Sebai et al., 2005). In these soils, called “adapted soils”, up to 60 % of the initially added IPU is mineralized 15 days after its application. Evidence for enhanced IPU degradation has stimulated the research aiming at isolating and characterising pure microbial strains able to mineralize IPU from the

adapted soils. Although several bacterial and fungal strains have been isolated and characterized to be involved in biodegradation of phenylurea herbicides including IPU (Sorensen et al., 2003; El-Sebai et al., 2004; Sorensen et al., 2008; Sun et al., 2009), very little is known about the processes and the genes coding the enzymes responsible for IPU biodegradation.

In this context, my PhD work was carried out with the aim to characterize the processes regulating IPU degradation not only under field conditions by considering geostatistical variability but also in pure cultures with the objective to know more about genetic determinants. The present thesis consists of seven chapters.

The first chapter is a literature review presenting the pesticides, their origin, behaviour in different compartments of the soil as well as the main mechanisms influencing their fate in the soil. This chapter also covers a detailed review of the fates and the ecotoxicological impact of isoproturon. The second chapter presents most of the procedures used for conducting my PhD work.

The third chapter aims at describing the spatial variability of isoproturon mineralization within an agricultural field over a three year winter wheat/ barley / rape seed crop rotation. It reports a three year survey of isoproturon mineralization activity in the field in relation to the physico-chemical properties and periodic application of isoproturon.

The fourth and fifth chapters are focused on the isolation and characterization of a bacterial consortium and of pure strains able to mineralize isoproturon. It describes the degrading capabilities of the bacterial culture and/or isolates as well as their phylogenetic characterization.

As the genes involved in the degradation of isoproturon have not yet been identified, the sixth chapter reports a genomic based approach designed with the aim to identify the genes putatively involved in IPU metabolism.

Finally, the seventh chapter is a general discussion of all the experiments performed during my PhD work aiming at placing its contribution in the light of knowledge about the isoproturon degradation. Conclusions are drawn and future perspecitives for the work are proposed.

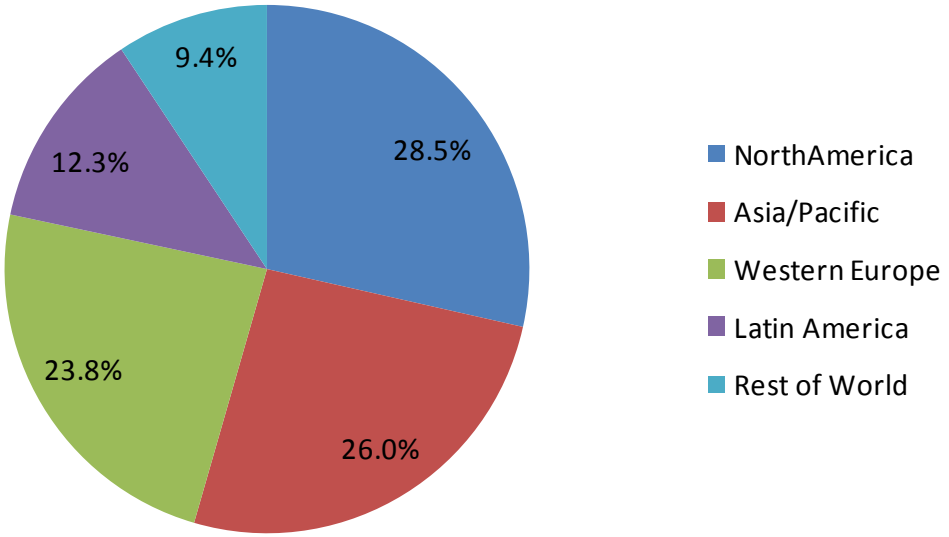


Fig. I-1. World's scenario of agrochemical sales by region (Dewar, 2005)

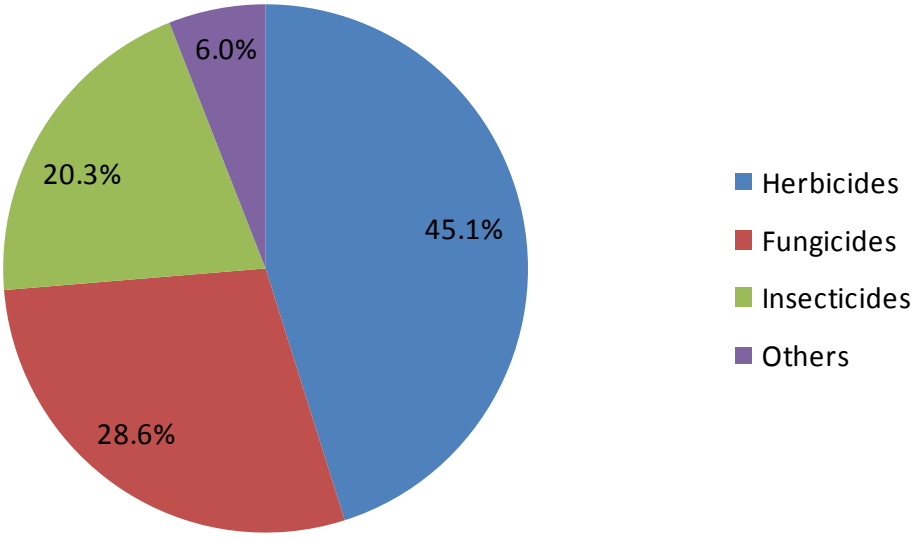


Fig. I-2. World's scenario of agrochemical sales by product category (Dewar, 2005)

REVIEW OF LITERATURE

1. PESTICIDES

The word pesticide is composed of two parts i.e. “pest” and “cide”. Any living organism can be qualified as pest when it occurs where it is not wanted or when it enters in competition with humans or their interest directly or indirectly causing damage to humans, crops or animals. The word “cide” originates from Latin and means the action to kill. As a consequence, “the pesticides are organic or mineral substances prepared and applied by humans to control the development of pests”. FAO (2005) defined a pesticide as “any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies”. From the regulatory point of view, the pesticides used for the protection of crops are also called as phytosanitary products or phyto-pharmaceuticals (directive 91/414/EEC) but in the rest of the manuscript the term of pesticide will preferentially be used.

1.1. Pesticides use and marketing

1.1.1. World’s scenario of pesticide use and marketing

In spite of the relatively poor availability of data detailing pesticide consumption in different countries mostly due to the reluctance of the manufacturers, there are a few reports about the pesticide use worldwide. In 2002, FAO reported that about 2.5 million tons of pesticides per year were applied on crops worldwide (FAO, 2002) and this consumption regularly increased with time. In USA, Environment Protection Agency (EPA) reported the use of 1100 million pounds of pesticides per year (US, EPA, 2002; Gilden et al., 2010). According to a report of Agrow’s Top 20 (Dewar, 2005), global agrochemical market value averaged 26-29 billion US dollars. North America including USA and Canada, represented the largest regional market for agrochemicals with 28.5% sales closely followed by Asia/Pacific and Western Europe with 26% and and 23.8% sales respectively (Fig. I-1)

Herbicides have been reported to account for the largest category of the agrochemical sold in world representing up to 45.1% of the total followed by insecticides and fungicides

Table I-1. Amount of pesticides used in Europe in 2001 (UIPP, 2004)

Country	Fungicides (tons)	Herbicides (tons)	Insecticides (tons)	Others (tons)	Total (tons)	Total (% of Europe) (A)	Cultivable Land (% of Europe) (B)	A/B Ratio
France	54130	32122	2487	10896	99635	34.3	21.0	1.6
Italy	23288	8191	9747	3741	44967	15.5	11.0	1.4
Spain	13790	10374	11631	5099	40894	14.1	21.1	0.7
Germany	8418	13337	868	3601	26224	9.0	12.1	0.7
Portugal	13915	6399	2616	1926	24856	8.5	2.9	2.9
UK	3628	11817	857	3874	20176	6.9	12.0	0.6
Greece	4860	2650	2638	963	11111	3.8	6.0	0.6
Netherlands	3628	2172	227	1840	7867	2.7	1.4	1.9
Belgium	1595	2345	560	566	5066	1.7	1.1	1.5
Austria	1088	1317	94	322	2821	1.0	2.4	0.4
Denmark	511	1925	66	116	2618	0.9	1.9	0.5
Sweden	339	1462	24	40	1865	0.6	2.2	0.3
Finland	192	1120	42	78	1432	0.5	1.6	0.3
Ireland	410	795	84	45	1334	0.5	3.1	0.2

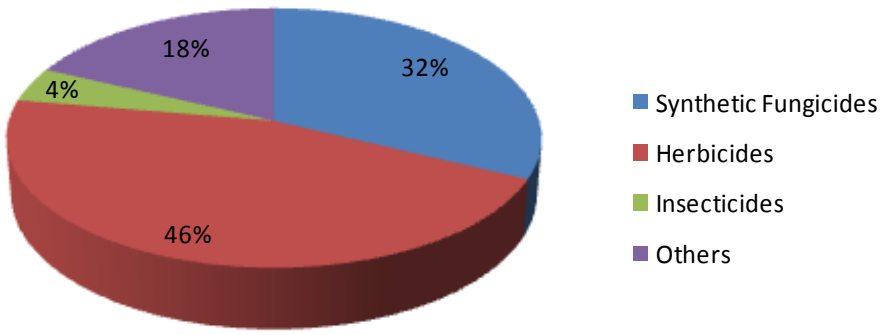


Fig. I-3. Sales of synthetic pesticides by product category in France (UIPP, 2004)

which account for 28.6 and 20.3%, respectively (Fig. I-2). However, it is noteworthy that the amount of each pesticide category used varies from country to country depending upon the pedoclimatic conditions and agricultural practices which deeply influence the crops being planted and the type of pests to treat.

1.1.2. Pesticide marketing and use in France

France, with a utilized agricultural surface (SAU) area of about 30 million hectares is the 3rd largest consumer of pesticides in the world and the 1st in Europe covering about 34.3% of the total consumption of pesticides in EU (Table. I-1).

The Union of Plant Protection Industries (UIPP, 2004) reported that about 76000 tons of pesticides were sold in France in 2004 which is significantly lesser than the amounts sold in 2001 (99600 tons) and 1999 (120500 tons). One could observe that this important decrease is due to two concomitant changes: (i) as a result of EU regulation, the use of heavy metals has been reduced and (ii) the new pesticides applied at low dosage contribute to this decrease. About 75% of the pesticides sold in France corresponded to synthetic products among which herbicides represent the category being the largest accounting for about 46% followed by the fungicides accounting for about 34% (Fig. I-3).

1.2. Registeration of pesticides

Pesticides are resulting from an important effort of R&D carried out nowadays mainly by agro-pharmaceutical firms. In most of the countries, their sale and use are subjected to official authorization which defines the conditions for their use in agreement with the principles of good agricultural practices aiming at preserving production capabilities of the agrosystems. For each active ingredient or commercially prepared formulation, a necessary evaluation is carried out to characterize the intrinsic properties of each substance for indentifying their hazard on the animals including the humans and the environment. These evaluations involve:

- The determination of physical and chemical properties of the active compound such as inflammability, vapour pressure, explosibility, solubility in water and organic solvents etc. It also requires the evaluation of the methods proposed for analyzing the active compound.
- Definition of the conditions for safe use of formulated pesticides such as the packaging and method of application etc.
- Evaluation of the safety for the appliers, farm workers and other people supposed to be exposed to these compounds.

- Estimation of the consumer safety by defining the types of the crops authorized and conditions for their treatment (pesticide applications and pre-harvest interval etc.)
- Prediction of the fate of the pesticides and estimation of their concentrations in the environments including water, soil and air.
- Estimation of the toxicity for flora and fauna including birds, land mammals, aquatic organisms, land arthropods, micro organisms, insects and bees etc.
- Evaluation of the efficiency of the pesticides, effects on yield and quality of the plants, harmful effects on the protected crops and other side effects etc.

In Europe, evaluation of the pesticides is governed by the EU Directive 91/414/EEC which was proposed to harmonize the certification procedures between the different EU members. The European Food Safety Authority (EFSA) is in charge of the evaluation of pesticides at EU level. Each pesticide being used in Europe is evaluated by EFSA on the basis of expertise carried out by a member state. If the opinion of EFSA is favorable, the product is included in Annex-I of the EU directive 91/414/EEC and could be approved by the EU member states. However, this authorization is governed by each EU member state which delivers marketing authorization (MA) on the basis of EU recommendations resulting from EFSA.

In France, the authorizations were delivered until year 2006 by a commission composed of independent experts and coordinated by a joint scientific structure (SSM) involving National Institute for Agricultural Research (INRA) and the Directorate general of food (DGAL). Since September 2006, Plant and Environment Department of the French Food Safety Agency (AFSSA) is in charge of delivering MA. The applications for pesticide authorization contain three complementary sections:

(i) **the scientific dossier** pursuing to the requirements of the EU Directive 91/414/EEC obtained through standard control and test methods

(ii) **the toxicological dossier** providing information on the pesticide characteristics like toxicity to humans, plants, animals, microbes and environment etc.

(iii) **the biological dossier** providing information on the efficiency, possible development of resistance and selectivity of the pesticide.

After reviewing the different dossiers, AFSSA renders its opinion to the approval board of pesticides of Ministry of Agriculture and Fisheries for a decision authorizing or refusing the marketing of a particular pesticide. When a pesticide is authorized for marketing,

Table I-2. Classification of the pesticides based on their targets, mode of action and chemical structure proposed by Arias-Estevez et al. (2008)

By target		By mode or time of action		By chemical structure
Type	Target	Type	Action	
Bactericide (sanitizers or disinfectants)	Bacteria	Contact	Kills by contact with pest	Pesticides can be either organic or inorganic chemicals. Most of today's pesticides are organic
Defoliant[a]	Crop foliage	Eradicant	Effective after infection by pathogen	
Desiccant[a]	Crop foliage	Fumigants	Enters pest as a gas	
Fungicide	Fungi	Nonselective	Toxic to both crop and weed	
Herbicide	Weeds	Post-emergence	Effective when applied after crop or weed emergence	Commonly used inorganic pesticides include copper-based fungicides, lime-sulfur used to control fungi and mites, boric acid used for cockroach control, and ammonium sulfamate herbicides
Insecticide	Insects	Pre-emergence	Effective when applied after planting and before crop or weed emergence	
Miticide (acaricide)	Mites and ticks	Preplant	Effective when applied prior to planting	
Molluscicide	Slugs and snails	Protectants	Effective when applied before pathogen infects plant	
Nematicide	Nematodes	Selective	Toxic only to weed	Organic insecticides can either be natural (usually extracted from plants or bacteria) or synthetic. Most pesticides used today are synthetic organic chemicals. They can be grouped into chemical families based on their structure
Plant growth regulator[a]	Crop growth processes	Soil sterilant	Toxic to all vegetation	
Rodenticide	Rodents	Stomach poison	Kills animal pests after ingestion	
Wood preservative	Wood-destroying organisms	Systemic	Transported through crop or pest following absorption	

Table I-3. Classification of pesticides based on their toxicity proposed by WHO (2005)

Class		**LD_{50} for the rat (mg/kg body weight)**			
		Oral		Dermal	
		Solids	Liquids	Solids	Liquids
Ia	Extremely hazardous	5 or less	20 or less	10 or less	40 or less
Ib	Highly hazardous	5 - 50	20 - 200	10-100	40 – 400
II	Moderately hazardous	50 - 500	200 - 2000	100-1000	400 – 4000
III	Slightly hazardous	Over 500	Over 2000	Over 1000	Over 4000

it is allowed for one or more uses for a specific crop, target parasite and treatment. The MA is re-evaluated every 10 years and can be interrupted at any time on the basis of new information originating from any partner (Firm or Ministry etc).

1.3. General characteristics of pesticides

1.3.1. Nature of pesticides

The pesticides can be of organic or inorganic origin. Inorganic pesticides are mineral elements which are mined from the earth and used as pesticides against selected pests like borates, silicates, copper and sulfur. In France, the use of the Bordeaux mixture (la bouillie bordelaise) prepared with copper sulfate was first reported at the end of nineteenth century in the French vineyard to control *Botrytis* development. On the contrary, organic pesticides can be derived or prepared from living organisms. Of natural origin, they can be derivatives of animal, plant or microbial origin like pyrethrine, nicotine and spinosad etc. Pyrethrins which are the natural insecticidal agents prepared from the flowers of the chrysanthemum plant (*Chrysanthemum cinerariaefolium*) are used since the Greek civilization. Nowadays, most of the organic pesticides under use are synthetic and are forming different classes like carbamates, organophosphates, organochlorines, pyrethroids, triazines and ureas etc.

1.3.2. Classification of pesticides

Under the term pesticide, a wide range of compounds are classified in different groups like insecticides, herbicides, fungicides, acaricides, algicides, rodenticides, avicides, mollusicides and nematacides etc. Several fumigant substances that are used to destroy insects, bacteria and rodenticides are also included in pesticides (Al-Saleh, 1994). More than 500 different pesticide formulations are authorized worldwide to control different types of pests in agriculture sector (Azevedo, 1998; Arias-Estevez et al., 2008).

The pesticides are classified into different categories according to different parameters: they can be classified based on their target, their mode of action, their time of action or their chemical nature (Arias-Estevez et al., 2008). The list of the different pesticide types are given in the Table I-2.

Recently, in response to social pressure highlighting the danger of pesticides for humans and the environment, World Health Organization (WHO, 2005) has proposed to classify the pesticides according to the toxicity of their technical compounds and formulations. The proposed classification is given in the Table I-3. It has to be noticed that this classification was based only on the determination of oral and dermal toxicity of the

pesticides in liquid and solid phases on rats which are considered as standard procedures in toxicology.

LD_{50} is an abbreviation for "Lethal Dose, 50%" or median lethal dose which gives the amount required (usually per body weight) to kill 50% of the test population. In most of the cases, the classification is made on the oral LD_{50} value, however, dermal LD_{50} value is also of prime interest since exposure to pesticides occurs mainly through dermal contact.

Although the pesticides have been categorized into different types, from the agricultural point of view, the term pesticide encompasses mainly the insecticides, herbicides and fungicides which are used to prevent and control the insects, weeds and fungi respectively. We will consider this classification in the rest of the manuscript.

1.3.3. Need or importance of pesticides

It has been reported that about 85 % of the pesticides are used in agriculture (Aspelin, 1997) which, as a result, depends very much on pesticides with a declared dependency on these compounds since firms, farmers and agencies often claimed that crop production will not be economically sustainable without the use of pesticides. Pesticides are used to control the development of pests and diseases. According to the economic report of Knutson et al. (1990), a decrease in the pesticide use in agriculture will lead to an economic impact which will affect not only the farmers, the firms and the suppliers but also the overall economy and consumers.

However, despite this economical challenge, it is vital to judiciously use the pesticides to promote the sustainability of agrosystem and to reduce the potential health hazards of pesticides to consumers or to the environment.

1.3.4. Mode of action of pesticides

The efficacy of the pesticides to kill their target pests depends on the properties of the pesticide and the soil, application technique, environmental conditions, characteristics of the crops, agricultural management and the nature and behavior of the target organisms.

Mode of action of pesticide is the primary mechanism by which the pesticide kills or interacts with the pest organisms. These mechanisms depend upon the pesticides and the target pest organisms. As an example, contact pesticides can kill the target pest organism by weakening or disrupting their cellular membranes causing their death rapidly. Alternatively, systemic pesticides are either absorbed or ingested by the target pest organisms causing physiological or metabolic breakdown. Systemic pesticides are often considered as slow acting pesticides.

Several insecticides have been reported to target the nervous system of the pests. For example, organophosphates disrupt the acetylcholine neurotransmitter in target insects (Colborn, 2006). Uptake of organophosphates in insects mostly takes place through skin, respiratory system or gastrointestinal tract. Carbamates which are used as insecticides, fungicides and herbicides have also been reported to be having the same mode of action but since their inhibition is reversible they are considered as less toxic (Al-Saleh, 1994).

Fungicides like cyazofamid and azoxystrobin inhibit the mycelial growth of the target fungi by inhibiting the complex III activities in their mitochondria. Cyazofamid was found to bind the Q_i centre of the complex III (Mitani et al., 2001). Several other fungicides have been reported to control the targeted fungi either by inhibiting the cell division or by inhibiting the biosynthesis of sterols, proteins and glucides etc (Aubertot et al., 2005).

Most of the herbicides belonging to different families like s-triazines (atrazine), phenylurea herbicides (isoproturon, diuron), uracils (bromacile), triazinones (metribuzine), hyroxybenzonitriles (bromoxynil) and bipyridyls (paraquat) have been reported to target the broadleaf weeds by inhibiting the photosystem I or II. Herbicides may also control the development of target weeds by inhibiting the cell division, synthesis of lipids, amino acids, cellulose and carotenoids etc (Aubertot et al., 2005).

1.3.5. Persistence of pesticides

Persistence can be defined as the ability of a chemical to retain its molecular integrity and hence its physical, chemical, and functional characteristics in different compartments of the environment like soil, air and water. As a consequence, persistence of pesticides may contribute to their transport and distribution over a considerable period of time. Persistence is among the most important factors determining the fate and effects of the pesticides in the environment. This parameter could be used to categorize the pesticides as non-persistence, moderately persistent (few days to 12 weeks), persistent (1 to 18 months) and permanent (many months to several decades). Most of chlorinated hydrocarbons such as DDT, aldrin and dieldrin are persistent originating from agricultural usage. Mercury, lead and arsenic etc are categorized among the pesticides referred as permanent (Al-Saleh, 1994). Persistence is a major parameter conditioning the fate of pesticides in the surface and ground water bodies which can be polluted by pesticides many years after their application (Turgut, 2003, Chen et al., 2005).

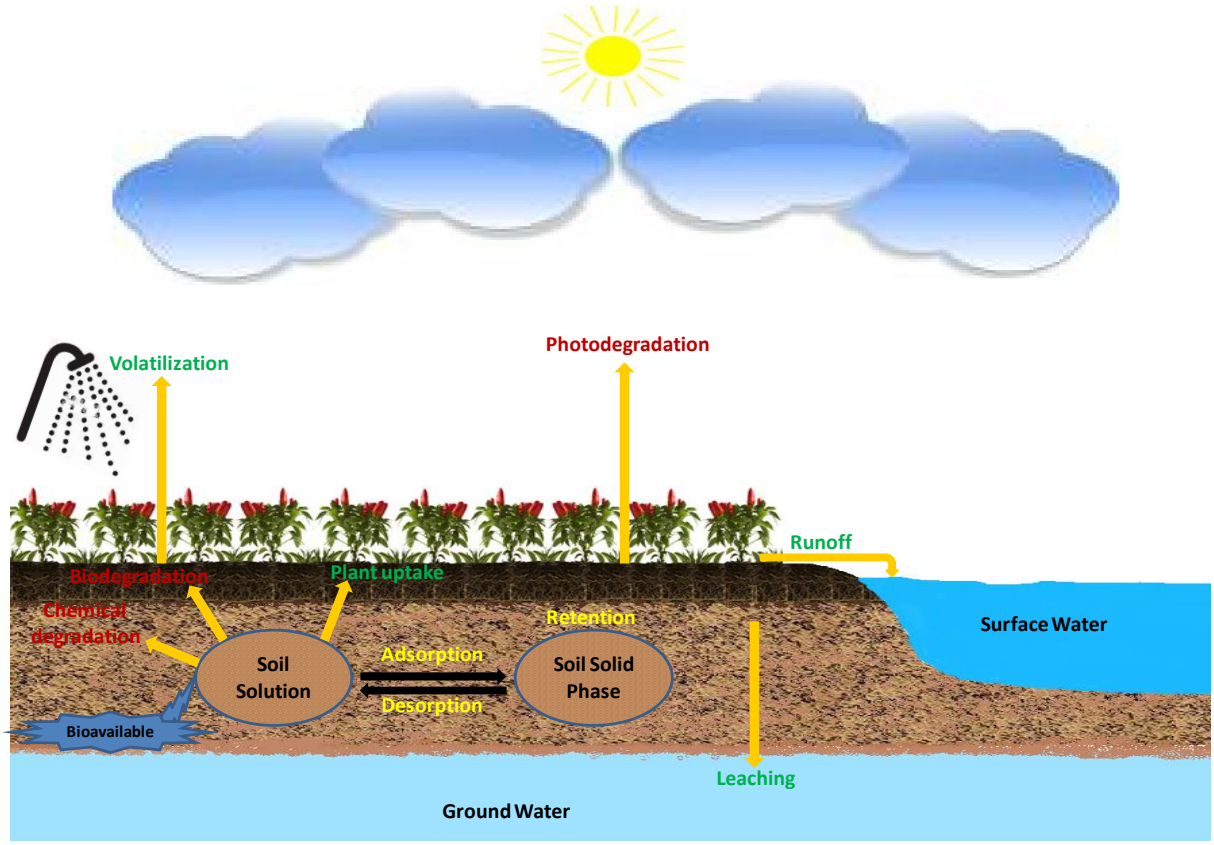


Fig. I-4. Major processes conditioning the fate of pesticides in indifferent compartment of the environment (Modified according to Barriuso et al., 1996)

1.4. Behavior of pesticides in the soil

Since the discovery of the referential occurrence of pesticides in soil and water resources, the research has been stimulated to better understand the parameters affecting the fate of the pesticides after their application. When the pesticides are applied to the soil, their fate is depending on many interacting processes including volatilization, leaching, run off, sorption and degradation etc. A conceptual overview of the processes regulating the fate and transport of pesticides in the soil is shown in the fig. I-4.

Soil is considered not only as a filter and buffer to the storage of the pollutants including pesticides with the help of soil organic carbon (Burauel and Bassmann, 2005) but also as a source from which the pesticides can contaminate the air, water, plants, animals, food and ultimately the human populations through runoff, drainage, interflow and leaching (Abrahams, 2002). After their application, the pesticides are distributed in the solid, liquid and gaseous phases in the vadose zone (Barriuso et al., 1996; Marino et al., 2002) depending upon the constants of adsorption, desorption and volatilization. All these processes are highly influenced by the environmental factors and the physico-chemical properties of the soils and of pesticides. The processes like retention, movement and degradation by which the pesticides, following their application on the soil, are spread in the environment will further be discussed.

1.4.1. Retention of pesticides

The term retention defines all the phenomena contributing to the passage of the different substances including pesticides from the liquid phase to the soild phase (Barriuso et al., 2000). The main processes which are involved in the retention of the pesticides in the soil environment are the adsorption and as a consequence, the formation of the bound residues. Sometimes, precipitation of the pesticides can also contribute to their retention in the soil matrix from where they can leach thereafter.

1.4.1.1. Adsorption

Adsorption is a reversible process of adhesion of the molecules of the gas, liquid or dissolved solids to a surface. In soil environment, adsorption of the pesticide can take place with soil components (clay particules, organic matter). It is well admitted that a large part of the pesticide applied to the field is retained by the soil solid phase due to adsorption. This prevailing phenomenon thereby affects other processes including leaching, volatilization and uptake etc. The adsorbed fraction can vary from a few percent to over 90% of pesticide applied depending on physico-chemical properties of the soil and pesticide (Celis and

Koskinen, 1999; Patakioutas and Albanis, 2002). Adsorbed pesticides are considered as non-available to plants and microbes, however, in their adsorbed form, they can seldomly be decomposed to their respective metabolites as a result of microbial and chemical transformation processes (Grover, 1988). Clay minerals and the organic matter are the soil components playing a key role in the adsorption of the pesticides (Mc Bride, 1994). Adsorption of pesticides to soil components results from a complex of interactions involving Van Der Waals forces, hydrogen bonding, ion exchange or hydrophilic interactions (Grover, 1988; Bollag et al., 1992).

The adsorption properties of the pesticides are reflected by the partition or distribution coefficient (K_d) calculating the partitioning equilibrium of the pesticide between the solid phase (adsorbed) and the liquid phase (available) (Wauchope et al., 2002). As soil organic matter is one of the principal component responsible for the adsorption of the pesticides (Chiou, 1989), a significant positive correlation between the adsorption of pesticides and the organic matter content is often registered (Calvet, 1989; Pignatello, 1998; Lennartz, 1999; Patakioutas and Albanis, 2002; Gawlik et al., 2003; Yu et al., 2006). On this basis, K_{oc} [$K_{oc} = K_d * 100 / C_{org}$, where C_{org} is the percentage of soil organic carbon] is an interesting indicator which takes into account both the K_d and organic matter reflecting the potential adsorption of pesticide in a given soil.

The intensity, rapidity and the reversibility of pesticides adsorption on soil components are variable depending not only upon the physico-chemical properties of the soil and pesticides but also the environment (Koskinen and Harper, 1990; Boesten and Gottesburen, 2000).

1.4.1.1.1. Influence of the nature of the pesticide molecule on adsorption

The physico-chemical properties of the pesticides affect their adsorption in the soils. Interestingly, organic molecules sharing the same skeleton may behave differently in the same type of the soil because of having different radicals (Koshinen and Harper, 1990). Indeed, the presence of radicals plays a role not only in acidic and basic characteristics of the pesticides but also in determining different types of their bonding with the soil components (Bailey and White, 1970). The adsorption of the pesticides in the soil is also influenced by the hydrophilic and hydrophobic properties, types and positions of the substitutions, solubility and delocalization of the charges on the pesticides (Bailey and White, 1970; Grebil, 2000). Recently QSAR based approaches have been developed to estimate the impact of physico-chemical properties on the pesticide fate. QSAR is the process by which chemical structure is

quantitatively correlated with a well defined process, such as biological activity or chemical reactivity.

1.4.1.1.2. Soil factors influencing adsorption of pesticides

Adsorption of the pesticides is influenced by numerous soil factors including nature and amount of organic matter (Kulikova and Perminova, 2002; Ahmad et al., 2006), clay content (Ismail et al., 2002), nature of the clay minerals (Baskaran, et al., 1996; Murphy et al., 1992), presence of oxides hydroxide (Calvet, 1989), organo-mineral associations (Fernandes et al., 2003), soil pH (Goa et al., 1998; Abdullah et al., 2001; Boivin et al., 2005) and presence of dominant cations in the soil solution (Baskaran, et al., 1996; Murphy et al., 1992). Among the soil factors, organic matter and the clay content are considered as the most important factors affecting the adsorption of the pesticides in the soils. Although the pesticide adsorption has been reported to be positively correlated with organic matter content (Patakioutas and Albanis, 2002; Gawlik et al., 2003), the molecular nature or characteristics of the organic matter (like aromatization) have also been proved to be a key factor in determining the sorption of some nonionic pesticides (Ding et al., 2002; Ahmad et al., 2006). In case of the soils having low organic matter, the adsorption of the pesticides is mainly linked to the reactive component of the soil i.e. clay (Spark and Swift, 2002).

1.4.1.1.3. Environmental factors influencing adsorption of pesticides

Adsorption is also affected by the environmental factors like temperature and moisture content etc. However, the effects of these environmental factors on pesticide adsorption are variable. For example, the effect of increase in temperature on the adsorption of *s*-triazines has been found to be contradictory being increased or decreased depending upon the physical or chemical bonds (Calvet et al., 1980; Boesten and Gottesburen, 2000). Furthermore, a decrease in moisture content does not only increase the pesticide adsorption but also promotes the association of the pesticides with other adsorption sites like dissolved organic carbon (Dao and Lavy, 1978; Calvet, 1989).

1.4.1.2. Formation of bound residues

A fraction of the pesticides can also be retained in soil solid phase due to another phenomenon by which the pesticides are irreversibly strongly bound to the soil components and are not immediately desorbed and extracted (Celis and Koskinen, 1999). Non-extractable pesticide residues, also called bound residues, yield in a difference between the isotherms of adsorption and desorption of pesticides in soil. The fraction of the pesticides forming the

bound residues increases over time and can persist in soil for several years. The amount of bound residues in soil varies from a few percent to over 80% of the adsorbed fraction (Patakioutas and Albanis, 2002) depending upon the physico-chemical properties of the pesticides as well the soil components and the aging of the pesticides in the soil (Celis and Koskinen, 1999; Patakioutas and Albanis, 2002). Various hypotheses, which have been proposed to explain the bound residues formation including:

(i) the formation of covalent bonds between the pesticides and soil constituents

(ii) the blocking of the pesticides in the micro-pores of the soil etc (Bollag et al., 1992; Gevao et al., 2000).

However, the mechanisms responsible for their extremely delayed release are still poorly understood although it has been suggested that these residues might be leached following deep physico-chemical changes of the soil or through the degradation of the soil organic matter (Gevao et al., 2000). As the "turn over" of the soil organic matter degradation is very low, some models suggest that pesticide bound residues can persist in soil for several hundreds of years (Barraclough et al., 2005).

1.4.2. Movement and dispersion of pesticides

The second important process regulating the fate of pesticides in the environment is their movement or dispersion. Pesticide movement can sometimes be important especially in cases when the pesticides have to be offensive against their target pests to reach their germinating seeds. However, too much movement of the pesticides can also take them away from their targets and thus compromises not only their efficiency but also the environment by contaminating its compartments. Movement of pesticides can take place by different ways including volatilization, runoff, leaching and uptake or crop removal.

1.4.2.1. Volatilization

Volatilization is a process by which a solid or liquid is converted into gaseous phase. When a pesticide is applied to the field or soil, a fraction of it is volatilized and transferred to the atmosphere (Wauchope et al., 2002). The loss of pesticides through volatilization can sometimes be higher than that of resulting from chemical degradation, runoff or leaching (Taylor and Spencer, 1990). About 90% of the applied amount of different pesticides like trifluraline and lindane can volatilize in a few hours to few days on humid soils (Glotfelty et al., 1984). Volatilization of pesticides contributes to contamination of the air, rain water, fog and ultimately lands where pesticides are not applied (Atlas and Schauffler, 1990). The quantity of pesticides dispersed in the atmosphere due to volatilization depends not only on

soil and climatic conditions such as soil texture, temperature, air velocity, surface littre and soil moisture content (Farmer et al., 1972; Taylor and Spencer, 1990; Gish et al., 1995) but also on the physico-chemical properties of the pesticide (vapor pressure) and its mode of application (size of droplet). Vapor pressure is an important factor for the determination of extent of volatilization of the pesticides. The one having the higher vapor pressure being the more volatile. Volatilization seems to play a minor role for the pesticides harboring a low vapor pressure (Grover, 1988). Volatilization can reduce the efficiency of the pesticide by reducing the amount of pesticide in contact with the pest. Volatilization can considerably be reduced either by (i) incorporating the pesticides in the soils or (ii) avoiding their use under unfavorable conditions like very hot or wet weather conditions.

It has been noticed that a relatively few number of reports suggest that volatilization can occur via the plants, through the process of phytovolatilization, in which soil and water contaminants are uptaken by the plants and are then volatilized into the air through the plant parts (Davis and Erickson, 2002; Doucette et al., 2003).

1.4.2.2. Leaching and run off

A large fraction of the pesticides remains dissolved in the soil solution where it constitutes most of the mobile fraction. Depending on soil and climatic conditions including rainfall, the pesticide and its main metabolites in the aqueous phase are either transferred to surface water by runoff i.e. the horizontal transfer over a sloping surface or to the ground water by leaching (i.e. vertical transfer through the soil). The choice of the transport of the pesticides by runoff and leaching normally depends upon the cultural practices like ploughing (Petersen et al., 2001), irrigation technique (Troiano et al., 1993) and the characteristics of the soils like porosity, compaction, texture and structure etc (Flury et al., 1994) and the amount of rainfall. Pesticide runoff will preferentially occur when pesticide application is followed by heavy and sustained rainfall. Pesticide in the soil solution could be leached down into the ground water over a long period contributing to delayed contamination of aquifers (Seta and Karathanasis, 1997). This phenomenon represents a severe problem in Europe and many other parts of the world (Folberth et al., 2009). Leaching of the pesticide takes place either due to (i) the advective transport with water or (ii) percolation in the soil under gravity metric forces. Pesticide leaching and runoff are considerably affected by the pesticide physico-chemical characteristics (i.e. solubility, persistence, stability and adsorption). One could observe that a pesticide is ready to move in water freely only if it is dissolved in water. Similarly, a pesticide which is non-persistent and is rapidly broken down due to some degradation processes is less

likely to move by leaching because its half life in soil is short. Moreover, a pesticide strongly adsorbed to the soil particles is less likely to leach but is more susceptible to erosion processes.

1.4.2.3. Absorption or and uptake (by plants and microorganisms)

Uptake is an active process by which different substances including pesticides enter the plant through the root system typically following the water apoplastic pathway. Only a limited fraction of the applied pesticide is bio-available to the plants and micro organisms via absorption process.

While absorbing the soil solution for their nutrition, the plants also absorb pesticides present in it which are further distributed to almost all other parts through xylem. Once absorbed by the plants, these pesticides either undergo various types and stages of transformation or they remain in the plants until tissue decay or harvest. During the first phase of transformation, the pesticide molecules can undergo different types of reactions such as oxidation, reduction, or hydrolysis. The second phase corresponds to the conjugation of the pesticide or the metabolites, resulting from its transformation in plant tissues, with various compounds such as sugars and glutathione etc. Finally, these conjugated molecules are transferred to the components of the cell wall or stored in vacuoles (Sicbaldi et al., 1997, Dietz and Schnoor, 2001; Singh and Jain, 2003). However, it has to be noticed that pesticide storage is temporary until the harvesting when the plant residues will be incorporated in the soil. This process can yield in crop and food chain contamination. Pesticide uptake by the plants is governed not only by numerous soil and plant characteristics (Susarla et al., 2002; Gao and Zhu, 2003; Bouldin et al., 2006) but also by the physico-chemical properties of the pesticide (i.e. concentration, bioavailability and translocation ability) (Bromilow and Chamberlain, 1995; De Carvalho, 2007).

Soils also offer a number of different niches furnishing diverse habitats inhabiting a wide diversity of microbial populations harboring various metabolic activities allowing their growth in the oligotrophic environment. In this context, the pesticides available in the soil solution are offering an additional nutrient source to these microorganisms for their growth. As a consequence, the ability to degrade this additional nutrient source offers a selection advantage promoting their growth within the overall microbial community of the soil. Numerous soil microbial populations have been found to degrade various pesticides thereby indicating that the soil microorganisms can be considered as a pit of pesticides partly

responsible for natural alteration in soil (Sorensen et al., 2001; Bending et al., 2003; El-Sebai et al., 2004; Sun et al., 2009).

1.4.3. Degradation or breakdown of pesticides

Degradation is the process by which pesticide is transformed to metabolites. When degradation leads to full transformation of xenobiotics to simple residues like carbon dioxide, then it is referred as **mineralization**. Degradation is considered as a beneficial process because it diminishes the presence of pesticides in the soil environment. However, it can be harmful when toxic metabolite are formed and are likely to accumulate in the soil (Mansour et al., 1999). In addition, it can also be harmful when the degradation occurs too rapidly that it can compromise the efficiency of the treatment. Degradation can take place through biotic or abiotic processes and most of the time these two processes work together in the environment. Comprehension of the parameters of the pesticide degradation processes in the soil is a key step in determining the fate of pesticides in the soil.

1.4.3.1. Abiotic degradation

Abiotic degradation is the degradation of a molecule of pesticide through physical or chemical reactions in response to abiotic factors including radiations, chemicals, soil minerals, sailinty, humidity and temperature etc. This type of degradation depends on intrinsic characteristics of the pesticide as well as on soil physico-chemical parameters like pH, temperature, humidity, organic and inorganic minerals etc. Abiotic degradation has been found to be catalyzed by several soil components such as clay, organic matter or metallic oxides (Wolfe, 1990; Senesi, 1993). Chemical degradation and photodegradation of the pesticides are the most common processes contributing towards the abiotic degradation. However, quantitatively this type of degradation is often less important than biodegradation (Beestman and Deming, 1974; Fournier et al., 1975).

Chemical degradation can be important in case of the pesticides sensitive to pH variations and also in the deep horizons characterized by moderate microbial activities. Chemical degradation results from pesticide hydrolysis occurring in aquous solution. However, in case of pesticides like isoproturon, which are stable against the chemical reactions in an aqueous solution under the moderate temperatures and a high range of pH values (Gerecke et al., 2002), chemical degradation is of less consideration.

Photodegradation is caused by the absorption of photons, particularly those wavelengths found in sunlight, such as infrared radiation, visible light, and ultraviolet light. This process occurs only on the soil surface or plants. In most cases, hydrolysis and photolysis

contribute only to a partial degradation of the pesticides leading to the appearance of metabolites that can be possibly recalcitrant to degradation and sometimes more toxic than the parent compound (Mansour et al., 1999; Amoros et al., 2000; Derbalah et al., 2004). Photo degradation process has been used to develop strategies to treat pesticide wastes. Photolysis i.e. breakdown by photons (Durand et al., 1994b; Lacorte and Barcelo, 1994), photocatalysis i.e. acceleration of a photoreaction in the presence of a catalyst (Topalov et al., 2003; Kormali et al., 2004) and Photo-Fenton (Derbalah et al., 2004a) are promising tool to clean pesticide waste that are not easily degraded by conventional methods (Parra et al., 2004; Pizarro et al., 2005; El Madani et al., 2006; Cernigoj et al., 2007; Sharma et al., 2008). This process can operate at room temperature and pressure utilizing natural light without requiring expensive catalysts (Katsumata et al., 2010). It occurs by the generation of the hydroxyl radicals which have the ability to degrade some of the pollutants (Sharma et al., 2010).

1.4.3.2. Biodegradation

Biodegradation is the transformation of complex molecules such as pesticides into simpler one by living organisms like plants and microorganisms. It is quantitatively among the most dominant process occurring in the soil and governing the fate of pesticides in the environment.

1.4.3.2.1. Phytoremediation and Phytodegradation

Phytoremediation is a process which makes use of the plants to remove or neutralize contaminants by their degradation, assimilation, metabolism or detoxification from the polluted soil or water. Phytoremediation encompasses a number of processes such as phytoextraction, phytodegradation, phytovolatilization and rhizoremediation (Hussain et al., 2009a).

Phytodegradation is a chemical modification of environmental substances as a direct result of plant metabolism. In this case, the pesticides are taken up and transformed to metabolites by the catabolic processes of the plants (Macek et al., 2000; Meagher, 2000; Laurent et al., 2006; Peuke and Rennenberg, 2005a, 2005b). Degradation of ethion by water hyacinth (Xia and Ma, 2006), hydrolysis and dealkylation of atrazine by poplar cuttings (Chang et al., 2005), dehalogenation of DDT in aquatic plant elodea (Garrison et al., 2000) and metabolism of metolachlor in *Elodea canadensis* (Rice et al., 1997) are some examples of phytodegradation. During the primary transformation reactions of phytodegradation, common functional groups of the pesticides are attacked by the induced endogenous enzymes through oxidation, reduction or hydrolysis reactions (Sandermann, 1992; Trapp et al., 1994). In a

second transformation reaction, the conjugation of the enzyme with the contaminant takes place by addition of a sugar moiety or glutathione to the contaminant (Hatzios and Penner, 1982; Sandermann, 1992) which plays an important role in the degradation of the pesticide by the plants. These conjugates are often less toxic than their parent compounds and could further be degraded in vacuole at the expense of ATP (Coleman et al., 1997; Edwards et al., 2000). In the last phase, the soluble metabolites are stored in the vacuole or become incorporated in cell wall (Dietz and Schnoor, 2001). Many of the plant enzymes, including peroxidase, cytochrome P450, glutathione-S-transferases, peroxygenases, carboxylesterases, N-, O-glucosyltransferases and N-, O-malonyltransferases, are involved in the phytotransformation of xenobiotics in plant cells (Sandermann, 1994; Macek et al., 2000; Gullner et al., 2001; Wolfe and Hoehamer, 2003; Karavangeli et al., 2005).

Although the plants have the ability to transform several pesticides, they lack the metabolic pathways enabling their complete degradation. Therefore, genetic modification consisting in introducing the degrading genes from the microbes to the plants has been proposed to increase the potential of the plants to degrade pesticides (Eapen et al., 2007). These plants expressing the pesticide degrading enzymes are called transgenic plants. Enhanced potential of xenobiotic degradation has been observed in a number of such transgenic plants including tobacco plants (Sonoki et al., 2005; Wang et al., 2008), leguminous plants (Rodrigues-Lima et al., 2006), poplar (Peuke and Rennenberg, 2005b), potato plants (Inui et al., 2001) and rice plants (Kawahigashi et al., 2003). It is commonly considered that in the coming days when the remediation cost of contaminated lands with conventional approaches like excavation and reburial of contaminated soil will rise many times, phytoremediation can offer a cost-effective solution for *in situ* remediation of contaminated sites as compared to other. Although transgenic plants have a potential for phytoremediation manyfolds more than their parent plants, there are some risks associated with the use of transgenic plants. These risks may include the development of resistant biotypes, creation of super weeds, and horizontal gene transfer among wild types and disruption in genetic makeup of biodiversity (Hussain et al., 2009a). In this context, the use of transgenic plants to clean contaminated land will have to prove innocuity for the environment in particular in terms of gene fluxes.

1.4.3.2.2. Microbial biodegradation

Microbial biodegradation occurs mostly in the soil solution. Pesticide microbial biodegradation is carried out by soil microorganisms like fungi and bacteria possessing a large

set of enzymes susceptible to transform these pesticides. It is the principal mechanism for diminishing the persistence of pesticides in soil environment (Arbeli and Fuentes, 2007). Soil serves as a potential habitat of different types of microorganisms which have the ability to interact not only with other living components but also the physical elements including pesticides for the fulfillment of their energy requirement. When the pesticides are applied to the soils, enzyme-driven biochemical reactions carried out by the indigenous soil microorganisms result in the modification of the structure and toxicological properties of the pesticides leading to their complete conversion into a harmless inorganic end products. (Hussain et al., 2009a). Degradation of the pesticides by the soil microbial communities has been reported by several different researchers (Fenlon et al., 2007; Hussain et al., 2007a; Shi and Bending, 2007; Hussain et al., 2009b; Sun et al., 2009) and it has been described as a primary mechanism of pesticide dissipation from the soils (Fournier et al., 1975; Cox et al., 1996; Pieuchot et al., 1996). The efficiency of pesticide biodegradation varies considerably between the different groups of the microorganisms and even between the different members belonging to the same group of the microorganisms. Although a strong diversity of the microbial species is found in the soil, however, the adaptability of the different degrading microbial species in the contaminated soils assures the continuity of the process of biodegradation (Barriuso et al., 2000). Microbial biodegradation of the pesticides in the soil can be categorized into two principal types based on the mode and pathway of degradation i.e. metabolic and co-metabolic.

1.4.3.2.2.1. Co-metabolic degradation

The co-metabolic degradation corresponds to the non specific degradation of xenobiotic molecule by microorganisms. In most cases, this is a non-inducible phenomenon occurring because of the presence of detoxifying enzymes able to degrade xenobiotics presenting homologies with their substrate. In this case, the target pesticides do not contribute to the growth of the degrading organisms (Dalton and Stirling, 1982; Novick and Alexander, 1985). For this reason, the degradation rate of pesticide in a given environment depends primarily on the size of microbial biomass and on the competitiveness of the degrading microbial population towards sources of energy and nutrients in the soil. In other words, the rate of degradation of a pesticide is related to the size of the biomass and will not increase even after repeated exposure to pesticide treatment (Fournier et al., 1996). In general, the co-metabolism does not yield in extensive degradation of the molecule but rather causes incomplete transformation such as oxidation, hydroxylation, reduction, N-dealkylation or

hydrolysis (Fournier et al. 1996) which may lead to the formation of metabolites that may prove even more toxic and recalcitrant than the parent compound (De Schrijver and De Mot, 1999).

1.4.3.2.2.2. Metabolic degardation

Metabolic microbial biodegradation of the pesticides is carried out by the soil microbial populations harboring specific catabolic enzymes allowing complete mineralization. A large number of pesticide degrading fungal and bacterial populations/strains have been isolated and characterized from the soil environment (Hussain et al., 2009b). Although often complete, metabolic biodegradation can also lead to incomplete degradation resulting in the formation of metabolites (Turnbull et al., 2001; Hangler et al., 2007; Badawi et al., 2009). However, up to now, the full mineralization of the pesticides have been found to be taking place only by the soil bacteria. The enzymes needed for the metabolic degradation of pesticides are either harbored by a single microorganism or scattered in various microbial populations working as a cooperative consortium, jointly involved in the degradation of the pesticides (Fournier et al., 1996)

1.4.3.3. Factors influencing the biodegradation of pesticides

The activity of pesticide biodegradation is influenced by physico-chemical conditions of the soil and climate as well as the physico-chemical properties of pesticides (Aislabie and Lloyd-Jones, 1995; Andrea et al., 2000). Biodegradation is also influenced by proximal factors such as oxygen, pH etc available in the soil environment. In addition, biotic factors such as rhizosphere etc also act on biodegradartion. It has been reported to be affected by the aeration and has been found to be taking place actively under the aerobic environment as compared to that of anaerobic environment (Celis et al., 2008). These physico-chemical conditions affect not only the availability and biodegradability of the pesticides (Walker et al., 1992; Welp and Brummer, 1999) but also the diversity, size and activity of the microbial populations (Smith et al., 1997; Hundt et al., 1998).

1.4.3.3.1. Physico-chemical properties of the pesticides

Pesticides physico-chemical properties are considered as a main factor determining their fate in the environment as well as their toxicity and degradability. A relationship between the structure of the molecules and their degradation has been well explained and it has been found that change in chemical structure of a molecule can have effects on its degradability (De Rose, 1946; Lal, 1983). For example, addition of a third chlorine atom to

the molecule of 2,4-D, can cause a significant reduction in its biodegradability. Based on this knowledge, several QSAR approaches have been developed to predict pesticide fate in the environment. US EPA proposed a tool "PBT Profile" available on internet predicting the fate of pesticide in soil, water and scolenent environment.

1.4.3.3.1.1. Solubility

Among the physico-chemical characteristics, solubility deeply affects the pesticide bioavailability and ultimately their degradation. Indeed, increase in pesticide solubility in the soil solution increases accessibility to the degrading microbial population and thus their degradation rate (Lal, 1983; Wel and Brummer, 1999). The use of surfactants which increases the solubility of pesticides, was proposed to increase the accessibility and biodegradability of the pollutants including pesticides in some contaminated soils (Fu and Alexander, 1985; Aronstein et al., 1991; Iglesias-Jimenez et al., 1997; Golyshin et al., 1999).

1.4.3.3.1.2. Sorption of pesticides

Adsorption and biodegradation are two interacting processes influencing the fate of pesticides in the soil environment. Adsorption of pesticides on soil components reduces their bioavailability and as a consequence their biodegradation is decreased (Lesan and Bhandari, 2003). Thus, we can say that a negative correlation is found between the adsorption of the pesticides and their degradation. Sorption of the pesticides in soils is evaluated by K_{oc} which is the partition coefficient of the contaminant in the organic fraction of the soil. K_{oc} depends on the physico-chemical properties of the contaminant.

1.4.3.3.1.3. Concentration

The concentration of the pesticides in the soils can also affect their biodegradable behavior. Indeed, the same product can be degraded either through metabolic or co-metabolic process depending on its available concentration in the soils solution (Novick and Alexander, 1985).

1.4.3.3.2. Soil factors affecting the degradation

The process of biodegradation is also influenced by the different physico-chemical properties of the soils like pH, clay content, organic matter content, soil moisture, temperature and redox conditions etc. The influence of these parameters on pesticide biodegradation is detailed below.

1.4.3.3.2.1. Soil texture and minerals

The biodegradation of the pesticides is influenced by the soil composition. Amount of clay, oxides, and cation exchange capacity (CEC) influence the pesticide biodegradation because they govern the sorption of the pesticides. Adsorption of the pesticides is related positively with the soil clay content. The sorption affinity of the pesticides is not influenced only by the clay content but also by the type of the clay minerals. For example, montmorillonite works as a better sorbent than kaolinite (Khan, 1980). While investigating the adsorption and biodegradation of a herbicide paraquat in tropical soils of Thailand, it was found that the adsorption was higher in the clayey soils as compared to that of the sandy soils (Amondham et al., 2006). Total iron content and the exchangeable calcium and sodium percentages were also found to be significantly contributing in the sorption of the paraquat. In another study, the sorption of the glyphosphate was reported to increase in the presence of iron and aluminium oxides (Gimsing and Borggaard, 2002).

1.4.3.3.2.2. Soil organic matter

Soil organic matter is one of the very important factors influencing the pesticide degradation activity. It affects the microbial biodegradation activity by not only controlling the pesticide bioavailability and movements but also by providing the nutrients for stimulating the growth of the microbial biomass (Spark and Swift, 2002; Briceno et al., 2007). Addition of the organic matter like compost in the soil is also considered as an inoculum of the exogenous microorganisms and exoenzymes to proliferate the microbial biomass in the soil (Benoit and Barriuso, 1997). Organic matter can also modify the local soil conditions making them more or less favourable for the microbial activities including degradation (Saison et al., 2006). The main source of the organic matter in the soils is the crop residues and their application in the soils has been found to stimulate the microbial growth and degradation of the pesticides (Zhang et al., 2005). Although organic matter plays its role in proliferating the microbial growth and activities, a positive correlation between the soil organic matter content and the sorption of the non-acidic pesticides like isoproturon, atrazine and trifluralin has also been reported (Boivin et al., 2005). However the influence of the organic matter content on the pesticide degradation and sorption cannot be modellized because of some contrasting effects with the change in organic matter contents (Boivin et al., 2005; Fenlon et al., 2007).

1.4.3.3.2.3. Soil pH

Among the physico-chemical factors, soil pH is one of the most important factor which has pronounced effects on the microbial pesticide degradation. The pH is a key factor

having effects not only on the availability of the pesticides in the soil but also on the biotic and abiotic degradation mechanisms. The pH affects the diversity, size and activity of the microbial populations as well as the physico-chemical properties of the soil and of the pesticides. Pesticide biodegradation takes place through the biochemical reactions on secretion of specific enzymes by the microorganisms which have been found to be dependent on pH (Okeke et al., 2002). pH acts on the ionization state of the enzymes modifying their affinity for the substrates and ultimately acting on the microbial activity (Smith et al., 1997; Hundt et al., 1998). Similarly, the size and diversity of the microbial populations are directly affected by the pH. Normally, fungal populations are abundant at low pH, while the bacterial populations are abundant at high pH with 7 to 8.5 as an optimum pH range for the development of bacteria (Smith et al., 1997). The balance of the fungal/bacterial populations, which is of great importance for pesticide biodegradation and accumulation of metabolites, is regulated by the changes in pH. There are a large number of studies showing a relationship between the pH and pesticide biodegradation not only in field conditions but also in pure cultures (Best and Weber, 1974; Houot et al., 2000; Bending et al., 2001; Walker et al., 2001; El sebaï et al., 2004; Rasmussen et al., 2005; Sun et al., 2009).

Although the pH has been found to have direct effects on the diversity and activity of the microorganisms and their biochemical reactions, it has also been reported to influence the adsorption/desorption of the pesticides on the soil constituents and hence the bioavailability and biodegradation. In case of weekly acidic pesticides like bentazone, lowering the pH can increase their adsorption (Boivin et al., 2005) which in turn affects their degradation. Best and Weber (1974) have shown that *s*-triazine herbicides like atrazine were more rapidly degraded at slightly acidic pH and the optimum pH for their degradation was about 6.5 (Houot et al., 2000). Multivariate data analysis for linuron indicated that pH was highly correlated to the initial linuron mineralization rate (Rasmussen et al., 2005).

1.4.3.3.2.4. Soil depth

Soil depth is also considered one of the factors involved in regulating the biodegradation of pesticides. It has been found that in agricultural fields, changes in the soil properties in vertical direction along the soil depth are greater than those occurring on the top soils (Rodriguez-Cruz et al., 2006). The size of the microbial community has been reported to be decreasing along the soil depth (Bending and Rodriguez-Cruz, 2007) due to reduction of organic matter along the soil depth (Vinther et al., 2001). As the degradation rate is determined by a number of interacting biotic and abiotic factors, these changes in the soil

physico-chemical and biological properties along the soil depth profile have been hypothesized to be responsible for the selection of different catabolic communities from top soil to the sub-soil. Bending and Rodriguez-Cruz (2007) studied the degradation rate of isoproturon along the soil depth in a sandy loam soil field and found that the degradation rate declined progressively down the soil profile. This decrease was associated with the lag phase increase as a result of the time length required for the microbial populations to adapt for degradation and growth. Although the degradation rates are often considered to be decreasing along the soil depth, however, the relationship between the top- and sub-soil degradation rates cannot be precised because it can vary for different compounds at a single site or different sites and reversely since each soil showed relatively different profiles. There are also contradictory reports showing higher degradation rate in sub-soils as compared to that of top soils.

1.4.3.3.2.5. Soil temperature

Temperature is also considered as one of the major factors which have direct effects on the functioning and activity of the microbial communities including biodegrading ability (Arshad et al., 2008). Temperature has been found to affect the cell membrane permeability, physiologically-altering proteins and the rate of enzymatic biochemical reaction occurring in the microorganisms (Mastronicolis et al., 1998; Guillot et al., 2000; Alberty, 2006). Optimum temperature range of 15 to 40°C is favorable for pesticide biodegradation (Hong et al., 2007; Singh et al., 2006). At sub-optimal temperatures (above and below this range), microbial degrading activity is reduced. Incubation temperature of 30°C was found to be optimum for effective degradation of several pesticides including IPU and HCH (Siddique et al., 2002; Sun et al., 2009). Alleto et al. (2006) showed that in three soil profiles, the degradation of IPU was faster at 22°C than at 10°C. Similarly, 2,4-D mineralization was found to significantly decrease in three soils at 10°C as compared to that of at 20°C (Bouseba et al., 2009). However, it did not differ significantly between soil microcosms incubated at 20 and 28 °C.

1.4.3.3.2.6. Soil moisture content

Soil moisture content is a very important pedo-climatic factor influencing both the bioavailability and transport of the pesticides which in turn affect their biodegradation in soil environment (Leistra et al., 2001). Soil water content has pronounced effects on the water availability to the microorganisms as well as on the redox conditions of the soils which results in the variation of various biochemical reactions including degradation (Hussain et al., 2009a). A linear positive correlation was observed between the soil moisture and the

mineralization (Schroll et al., 2006). However, excessive soil moisture content creates anaerobic conditions in the soil which inhibits the biodegradation process (Gibson and Sulfita, 1990: Mohn and Tiedje, 1991). In surface and subsurface soil samples, the isoproturon degradation was found to be more sensitive to soil moisture content than to the temperature (Alletto et al., 2006). For surface layer, the degradation half life was reported to increase significantly when the moisture content was at 50% of WHC as compared to that at 90% of WHC (Alletto et al., 2006). Flooding has also been demonstrated to have influences on the degradation and persistence of different pesticides like linuron, isoproturon and metolachlor in soils (Accinelli et al., 2005).

1.4.3.3.3. Rhizosphere

The rhizosphere is the narrow region of soil influenced by root secretions and associated soil microorganisms. Plant photosynthetic products which can account up to 80% of the pesticides are released in the rhizosphere where they contribute as major source of nutrients in the oligotrophs increasing the size and activity of the soil microbial community. Rhizosphere is often considered as an "external stomach" of the plant forming hot spot. This ecological niche hosts numerous and diverse metabolically active microbial populations (Capdevila et al., 2004). Degradation of the pesticides as well as the diversity of the soil microbial communities and the genes involved in pesticide biodegradation in rhizosphere have extensively been studied by the researchers (Boltner et al., 2005; Leigh et al., 2006; Martin-Laurent et al., 2006; Dams et al., 2007; de Carcer et al., 2007; Ryan et al., 2007). Although a lot of studies have been done, contradictory results are reported. Plant rhizosphere may increase, decrease or have a negligible effect on pesticide degradation pathway depending on the soil and the herbicide considered (Piutti et al., 2002). Genetic structure and diversity of the pesticides degrading microbial communities in rhizosphere of a crop have been reported to vary from those of bulk soil and even from rhizospheres of other crops (Martin-Laurent et al., 2006; Hussain et al., 2009a).

1.4.3.3.4. Repeated exposure to pesticides

Repeated exposure of a soil to pesticide treatment often leads to the adaptation of a part of the soil microbial community for its rapid degradation. This phenomenon is often called accelerated or enhanced degradation (Racke and coats, 1990; El-Sebai et al., 2005). It is one of the practical consequences of the structure and evolution of the microbial populations involved in biodegradation of pesticides (Rouchaud et al., 2000). This mechanism relies on microbial adaptation. Briefly, when a pesticide is applied for the first time on a soil,

Table I-4. Microbial genes and enzymes involved in degradation of different pesticides (Hussain et al., 2009a)

Pesticide	Genus	Gene	Enzyme
Insecticides			
Organochlorine Insecticides			
Hexachlorocyclohexane	*Sphingomonas* *Microbacterium* *Sphingobium*	*lin*, *lin*A, *lin*B, *lin*C, *lin*D, *lin*E, *lin*X	Dehalogenase Dehydrogenase Dehydro-chlorinase Hydrolase
Organophosphorus Insecticides			
Organophosphorus	*Achromobacter* *Flavobacterium* *Clavibacter* *Escherichia* *Delftia* *Pseudomonas*	*mpd*, *opd*, *phn*E, *glp*T, *pde*A	Hydrolase
Fenitrothion	*Burkholderia*	*mpd*, *p*NF1, *d p*NF2	Hydrolase
Methyl parathion	*Plesiomonas*	*mpd*	Hydrolase
Carbamate			
Carbamate	*Arthrobacter* *Methylobacteria* *Methylomonas* *Pseudomonas*	*pcd*	Hydrolase
Carbaryl	*Arthrobacter*	*p*RC1, *p*RC2, *p*RC3	Hydrolase
Carbofuran	*Achromobacter*	*mcd*	Hydrolase
Thiacarbamate	*Rhodococcus*	*thc*F, *thc*G	Haloperoxidases Hydrolase
Herbicides			
Atrazine/Simazine	*Agrobacterium* *Alcaligenes* *Aminobacter* *Arthrobacter* *Burkholderia* *Variovorax* *Pseudomonas* *Pseudaminobacter* *Ralstonia* *Rhizobium* *Rhodococcus* *Sphingomonas* *Stenotrophomonas*	*atz*ABCDEF, *trz*ND, *psb*A1, *trzA*, *smzA*	Hydrolase Urease Cytochrome P-450
Dalapon	*Bradyrhizobium*		Dehalogenase
Phenoxy acid herbicides	*Stenotrophomonas* *Brevundimonas* *Pseudomonas* *Ochrobacterium*	*tfd*A, *tfd*B, *tfd*C, ORF1-ORF6	Dioxygenase Isomerase Reductase
Trifluralin	*Bacteria*	*ndo*B	Dioxygenase
Diuron	*Arthrobacter*	*puh*A, *puh*B	Hydrolase

its biodegradation by the soil micro flora is not observed for a particular period of the time called lag phase. This lag phase corresponds to the enzymatic or genetic adaptation of the soil microbial community until the degradation starts when a minimum significant number of the microorganisms have adapted for degradation of that particular pesticide. It is then followed by an exponential phase during which a rapid degradation of the pesticide takes place. When a pesticide is applied repeatedly on the same soil, the presence of a strong adapted microbial population at the time of pesticide treatment results either in suppression or reduction in the lag phase. The potential of this adaptation of a soil for the degradation of a pesticide can last for a few weeks to few years after the pesticide treatment is stopped (Walker and Welch, 1991). This adaptation is considered as a dominant phenomenon affecting the biodegradation of pesticides in the soils and has been reported for a number of soils repeatedly treated with the pesticides including isoproturon (Sorensen et al., 2001; Sorensen and Aamand, 2003; El-Sebai et al., 2005), atrazine (Yassir et al., 1999) and isoxaben (Arrault, 2001)

1.4.3.4. Genes and enzymes characterized for pesticide biodegradation

Several groups worldwide have conducted researches to isolate and characterize pesticide degrading microbial strains. Further characterization of these microbial strains for the enzymes and genes showed an extensive enzyme system having a great variability and versatility in catabolism (Hussain et al., 2009a). Although a large number of studies report the isolation and characterization of bacterial and fungal strains able to mineralize different pesticides, a few number report the characterization of catabolic genes and related enzymes catalyzing the biodegradation process. Some known pesticide degrading genes are presented in the table I-4. (Sharma et al., 2006; Zhang et al., 2006; Hussain et al., 2009a).

It has been reported that the degradation of organochlorine pesticides like hexachlorocyclohexane (HCH) is most of the times mediated by *lin* genes coding for different types of enzymes including dehalogenase, dehydrogenase and hydrolase etc (Table I-4). Degradation of the different organophosphorus insecticides (like organophosphorus, fenitrothion and methyl parathion) is mostly carried out by hydrolytic enzymes like organophosphorus hydrolase encoded by different types of genes like *mpd* etc. Hydrolase enzymes encoded by different genes like *pcd* and *mcd* etc have also been found to be involved in the degradation of different carbamate pesticides including carbaryl and carbofuron etc. Herbicides have been found to be degraded by the involvement of microbial genes (*atz, trz, psb, tri, tfd, ndo, puh*) encoding different groups of enzymes like hydrolase, dehalogenase, urease, deaminase, dioxygenase and reductase etc (Table I-4).

1.5. Harmful effects of pesticides

Although the use of pesticides ensures the yield and the phytosanitary quality of the crop, they can be harmful for the environment and living organisms including humans when they contaminate the environment and the food chain (Barriuso et al., 1996). Pesticides are created to have a biocidal action on target organism. The action mechanism of the pesticides is not always well described and this lack did not help to predict possible ecotoxicological impact. In addition, available technologies to assess ecotoxicological impact remained scarce. However, they are never completely specific and may impact other organisms including the man when similar targets are existing. Although pesticides are authorized, they are generally low to moderately recalcitrant to degradation by living organisms and could be transferred from the agrosystems to different environmental compartments (air, soil and water) where they are considered as pollutants. As a result of pesticide environmental exposure, impact on the health of the human beings as well as animals have been observed (Azmi et al., 2006; Rothlein et al., 2006; Kiefer and Firestone, 2007). In addition, they contribute to degrade the quality of the environment decreasing the biodiversity (Eskenazi et al., 1999; Benachour et al., 2007; Rosenthal, 2007)

1.5.1. Contamination of the environment with pesticides

1.5.1.1. Soil contamination

Crops grown on the soils are deliberately treated with pesticides to control different types of pests like broadleaf weeds, fungal diseases, insects and nematodes etc. It has been estimated that about 50% of pesticide sprayed on the crops as herbicide misses its targets and falls onto the soil surface (Al-Saleh, 1994). A fraction of pesticides applied to the soils is stabilized by forming the bound residues with soil constituents such as clay and organic matter and thus persists in soil for several years (Barriuso et al., 1996). Thus, the widespread use for pesticides on agricultural soils and their recalcitrant and persistent properties result in the contamination of the soils. At the moment, soil contamination by pesticides is probably underestimated since analytical methods are biased by the extraction, the threshold detection and the possible detection of the metabolites formed. This persistent contamination of the soils with pesticides results not only in direct dermal and ingestion exposures to human beings but also through the food chain due to the translocation of the pollutants from the soil to the plants via root uptake.

Adsorption is a reversible process which under specific conditions, may cause long term delayed (up to several years) contamination. For example, DDT has been reported to

exist in the soils and waters of China, Turkey, Europe and USA although it had been forbidden to use for more than twenty years (Turgut, 2003, Chen et al., 2005).

1.5.1.2. Contamination of surface and subsurface water

Although pesticides are mainly applied in agricultural soils, they can move from the agrosystem to water resources via various types of movements like percolation, leaching, and runoff etc. Contamination of the drinking water with pesticides through direct and diffused sources is considered as a great threat to its quality and suspected to be the main origin of the contamination for human beings. Contamination of the surface and ground water bodies via percolation and runoff etc is considered only a small part as compared to the sources of direct contamination like leakage of spray equipment, spray leftovers, spills of rinsing water from cleaning of spray equipments and spills during the filling operations etc. Pesticides such as organochlorine insecticides have been found to persist in water for a long period of time (20 years) after their use was banned (Larson et al., 1997). As a consequence, several organochlorine insecticides are now classified as POP (persistant organic pollutants) and listed in the Oslow convention. Pesticide residues are among the most toxic chemicals contaminating the water resources (Dupas et al., 1995; Coupe and Blomquist, 2004). In many parts of the world, clean drinking water is a limited resource and, in areas with intensive agricultural production, problem with pesticide residues in surface and ground water has been recognized (Rasmussen et al., 2005). According to a report of The French Institute of Environment (IFEN, 2007) about 97% of surface water and 60% of groundwater in France is being contaminated by pesticides. Regarding the rivers and lakes, 46% of tested waters are of average or poor quality for aquatic life. In the context of the revised water framework directive (WFD, EU), European countries have to consider the quality of not only the drinking water but also the water bodies.

1.5.1.3. Atmospheric pollution

Pesticides are also responsible for the atmospheric contamination. Air has been found to be easily contaminated with pesticides during spray operations due to the evaporation of the droplets which may result in the formation of tiny particles which are carried to great distances with air currents. Due to this undesirable pesticide drift in the air, the pest control efficiency is decreased and the workers as well as the animals near agricultural sites are exposed to pesticides (Al-Saleh, 1994). 2,4-D was the first pesticide to be reported as a pollutant of the air and atmosphere in 1952 (Yao et al., 2008). Since then a lot of work has been done to investigate the occurance and process of the pesticides in air. Pesticide residues

have also been detected in appreciable concentration in rain water and mist even in some urban communities in France (Bedos et al., 2002; Sauret-Szczepanski et al., 2006).

In order to monitor the air quality, different commissions have been activated to estimate the quantities of the pesticides in atmosphere in various countries including Canada and France (Yao et al., 2008). Since 2000, the French network for monitoring air quality has been undertaken to measure the quantities of pesticides in the air. A report of this network published on central areas in 2002 indicated that out of 19 pesticides analyzed, 18 were found present in air at concentrations ranging from 0.11 to 23 ng m^{-3}. Some pesticides such as trifluralin and lindane were even detected in all samples.

1.5.2. Ecotoxicological impact

Although the pesticides are created and applied for controlling the selected targeted living organisms, however, they may also have pronounced harmful effects on other non-target organisms including plants, animals, microorganisms and human beings.

1.5.2.1. Harmful effects on human beings

The wide use of pesticides has been found to affect not only the workers who are handling them but also to the rest of population who are not using them. Exposure of the pesticides to the human beings could occur through:

- inhalation of pesticide contaminated air
- drinking of the contaminated water
- ingestion of the pesticide contaminated food
- dermal contact
- across the placenta

The exposure of the pesticides to human beings can be described in several ways like accidental or incidental, acute or chronic, intentional or unintentional and occupational or non-occupational. It has also been reported that about 3 million people worldwide are poisoned and about 200,000 die because of this poisoning (WHO, 1990; FAO, 2000). This dilemma is predominantly happening in the developing countries where some pesticides banned by developed countries are still being used (Ecobichon, 2001; Wilson and Tisdell, 2001) and where the development of distribution channels remains poorly organized. A study conducted by the "Directorate General for Health and Consumer Protection" of the European Union showed that 49.5% of samples of food plants of French origin tested contained pesticide residues with 8.3 % at rates higher than the maximum allowed (Tron et al., 2001). This alarming fact is confirmed by measuring levels of pesticides found in the human body.

Various studies from people from different countries showed that 99% of people tested stocked DDT in their fat tissues (Turusov et al., 2002). Moreover, the presence of many pesticides has been detected in breast milk, sometimes at concentrations exceeding the acceptable daily intake for infants (Jensen, 1983; Stevens et al., 1993; Romero et al., 2000).

The exposure of the pesticides to the human beings may result in different types of the harmful symptoms starting from the carcinogenic effects to the effects on the digestive, reproductive and respiratory systems in human beings. Some of the harmful effects of the pesticides on the human beings are described as under:

- Reproductive and endocrine system

Pesticides may affect the reproductive and endocrine systems in human beings. A negative correlation was found to exist between the size of the newborn baby and the level of contamination of their with insecticides i.e. chlorpyrifos and diazinon (Perera et al., 2005). Various pesticides such as 2,4-D or atrazine, found at considerable concentration in some human beings, resulted in an increase in the mortality rate of mouse embryos (Greenlee et al., 2004).

Several pesticides have been found to be responsible for altered thyroid function and decreased testosterone and estradiol functions leading to the reduced fertility later in life (Meeker et al., 2006; Meeker et al., 2008). They also cause gestational diabetes (Saldana et al., 2007), menstrual irregularities (Farr et al., 2004) and fetal death related to congenital birth defects (Bell et al., 2001).

- Neurodevelopmental

It has been found that the exposure of pesticides to the children and prenatals can lead to social behavioural problems (Ribas-Fito et al., 2007) and neurodevelopmental delays (Eskenazi et al., 2007; Handal et al., 2007). Even a single exposure of a child before or after the birth with a pesticide like DDT can make him sensitive to the health effects on exposure in later stages (Colborn, 2006)

- Immune system

There are evidences that high-dose exposures of the pesticides may affect the immune system (Caress and Steinemann, 2003; Weselak et al., 2007), increased risk of allergy (Weselak et al., 2007) and multiple chemical sensitivity (Caress and Steinemann, 2003)

- Cancer

Cancer is considered as one of the most common end point while studying the harmful effects of different chemicals on the health. Many of the previous research have indicated the possible carcinogenic effects of the pesticides which can cause cancer in brain, kidney,

prostate, pancreas, liver, breast, lung and skin (Zahm and Ward, 1998; Clark and Snedeker, 2005; Dharmani and Jaga, 2005; Infante-Rivard and Weichenthal, 2007)

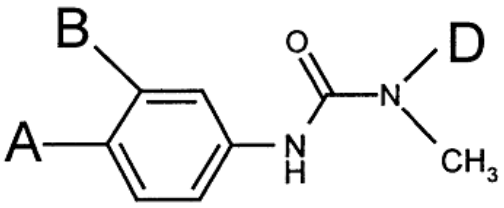
1.5.2.2. Harmful effects on plants and animals

Most of the pesticides have also been reported to have effects on the growth and metabolism of non-target living organisms including plants and animals. Plants are exposed to pesticides either by uptake from the contaminated soils. These pesticides on exposure to the plants, can have adverse effects on their photosynthetic activity and growth. While studying the effects on some aquatic submerged macrophytes, several pesticides like isoproturon, atrazine and diuron were found to affect not only their photosynthetic efficiency but also their growth (Knauert et al., 2010). Watanabe et al. (2001) demonstrated the changes in fatty acid composition of neutral lipid in mung bean cotyledons due to peroxidation induced by a peroxidizing herbicide, oxyfluorfen.

Animals can be exposed to pesticides either by direct contact from the soils and water or by ingestion of the pesticide contaminated fodders and water. Pesticides have also been reported to have various types of hazardous effects on terrestrial and aquatic animals including fish, birds and mammals etc. It has been demonstrated that exposure of some mammals to the pesticides such as aldrin, atrazine, chlordane and dieldrin disrupt sexual differentiation (LeBlanc et al., 1997). Similarly, frogs and tadpoles have been reported to be demasculinized transforming them to hermaphrodites on exposure to even low concentrations of atrazine (Hayes et al., 2002; Hayes et al., 2003). Pesticides can also cause toxicological effects on oxidative stress enzymes such as catalase, superoxide, dismutase, glutathione reductase and glutathione S-transferase in fish (Peixoto et al., 2006) and some of the pesticides have been identified as a factor causing death in fish and reproductive failure in birds (Rao et al., 1993)

1.5.2.3. Effects on microorganisms

Microbial community is very important for the soil and aquatic environments where they play a critical role in the primary productivity, nutrient cycling and decomposition of different compounds. Exposure of the microorganisms to pesticides can bring out a number of acute and chronic toxicity effects either on their growth and activity or their distribution in the environment. Several pesticides like endosulfan have been reported to inhibit the population of the heterotrophic bacteria ranging from 30.99% at concentration 0.002 mg L^{-1} to 70.58% at 2 mg L^{-1} (Rajendran et al., 1990; De-Lorenzo et al., 2001). Pesticide treatments have been found to induce the changes in microbial community composition, the functional responses



	A	B	D
Isoproturon 3-(4-isopropylphenyl)-N,N-dimethylurea		H	CH_3
Diuron N-(3,4-dichlorophenyl)-N,N-dimethylurea	Cl	Cl	CH_3
Monuron N-(4-chlorophenyl)-N,N-dimethylurea	Cl	H	CH_3
Chlorotoluron 3-(3-chloro-p-tolyl)-N,N-dimethylurea	CH_3	Cl	CH_3
Fenuron 3-phenyl-N,N-dimethylurea	H	H	CH_3
Fluometuron N-(3-trifluoromethylphenyl)-N,N-dimethylurea	H	CF_3	CH_3
Clorobromuron N-(4-bromo-3-chlorophenyl)-N-methoxy-N-methylurea	Br	Cl	OCH_3
Linuron 3-(3,4-dichlorophenyl)-N-methoxy-N-methylurea	Cl	Cl	OCH_3
Metabromuron N-(4-bromophenyl)-N-methoxy-N-methylurea	Br	H	OCH_3

Fig. I-5. General structure of commonly used phenylurea herbicides (modified from Sorensen et al., 2003).

related to the microbial activity, microbial respiration and many other multidirectional shifts (Widenfalk et al., 2008). It also leads to the adaptation of soil microflora to pesticide biodegradation (appearance of a new function).

2. PHENYLUREA HERBICIDES

2.1. Structure, properties and ecotoxicology of phenylurea herbicides

A herbicide is defined as a pesticide used to selectively kill the specific targeted broadleaf weeds while leaving the crop unharmed. Herbicides are used to avoid competition for nutrients of the crop with weeds and to ensure the definite quantity and quality of the crop. The phenylurea herbicide family, commercialized for more than 50 years, is one of the most important class of herbicides used worldwide. Most oftenly, they are used for pre- or post-emergence control of broad leaf weeds in cotton, fruit or cereal production. Diuron is also used as a non-selective herbicide in vineyards and in urban areas, as an algicide in antifouling paints (Sorensen et al., 2003) and also as total herbicides on non-crop areas such as roads and railway lines (Giacomazzi and Cochet, 2004). Phenylureas are systemic herbicides inhibiting the photosynthesis by disrupting the electron transfer in the photosystem II (Ducruet, 1991). Photosynthetic microorganisms such as green algae are also sensitive to phenylurea herbicides.

Phenylurea herbicides are constituted of two types of the substitutes on the phenyl ring. Several of them are made of N,N-dimethyl substitutes e.g. isoproturon, chlorotoluron, monuron, and diuron etc, while, several others are constituted of N-methoxy-N-methyl substitutes e.g. linuron, monolinuron, chlorobromuron etc (Sorensen et al., 2003). General structure of commonly used phenylurea herbicides is given in the fig. I-5.

At room temperature, all the phenylureas are present in solid state. When observed in pure form, they are characterized as odorless, white or colorless crystalline compounds. They are available in formulations (i.e. emulsifiable and suspension concentrates). Because of having low vapor pressures and Henry's law constants (Table I-5), most of the phenylureas are found to be having very low volatilization from the soil and plant surfaces under the normal atmospheric conditions. Most phenylureas are known to have relatively high water solubilities and low tendencies to sorb to soil components. That's why, they are mobile in the soil environment. Phenyl urea herbicides have high hydrolytic stability at neutral pH, however, their stability can decreases when the pH of the medium switches towards the acidic

Table I-5. Physico-chemical properties of the phenylurea herbicides used in this study and corresponding degrading isolates. K_{OC} is the organic carbon normalized distribution coefficient. Data were obtained from FOOTPRINT (www.eu-footprint.org) and AGRITOX (http://www.dive.afssa.fr/agritox/php/fiches.php).

ISO name IUPAC name	**Solubility in water** **(mg L^{-1})**	**Vapour Pressure** **(mPa)**	**Henry's constant (K_H)** **(Pa m^3 mol^{-1})**	**K_{OC}** **(L kg^{-1})**	**Biodegrading microbial isolates**	**References**
Linuron 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea	64	5.1	2×10^{-4}	620	*Bacillus sphaericus* ATCC12123 *Streptomyces* sp. PS 1/5 *Arthrobacter globiformis* D47 *Variovorax* sp. WDL1 *Variovorax* sp. SRS16 *Mortierella* sp. Gr4 *Sphingobium* sp. YBL1	Wallnöfer (1969) Shelton et al. (1996) Cullington and Walker (1999) Dejonghe et al. (2003) Sorensen et al. (2005) Badawi et al. (2009) Sun et al. (2009)
Monolinuron 3-(4-chlorophenyl)-1-methoxy-1-methylurea	735	6.4	3.8×10^{-4}	200	*Bacillus sphaericus* ATCC12123 *Arthrobacter globiformis* D47	Wallnöfer (1969) Cullington and Walker (1999)
Isoproturon 3-(4-isopropylphenyl)-1,1-dimethylurea	70	3.3×10^{-3}	9.7×10^{-6}	1039	*Arthrobacter globiformis* D47 *Bjerkandera adusta* *Oxysporus* sp. *Sphingomonas* sp. SRS2 *Sphingomonas* sp. F35 *Methylopila* sp. TES *Mortierella* sp. Gr4 *Sphingobium* sp. YBL1, YBL2, YBL3	Cullington and Walker (1999) Khadrani et al. (1999) Khadrani et al. (1999) Sorensen et al. (2001) Bending et al. (2003) El-Sebai et al. (2004) Badawi et al. (2009) Sun et al. (2009)
Diuron 3-(3,4-dichlorophenyl)-1,1-dimethylurea	34	1.2	2.0×10^{-6}	1067	*Streptomyces* sp. PS 1/5 *Actinomyces* sp. CCT4916 *Arthrobacter globiformis* D47 *Bjerkandera adusta* *Oxysporus* sp. *Pseusomonas* sp. Bk8 *Arthrobacter* sp. N2 *Pseudomonas* sp. 1B78 *Variovorax* sp. SRS16 *Mortierella* sp. Gr4 *Sphingobium* sp. YBL1, YBL2, YBL3	Shelton et al. (1996) Esposito et al. (1998) Cullington and Walker (1999) Khadrani et al. (1999) Khadrani et al. (1999) El-Deeb et al. (2000) Tixier et al. (2002) Batisson et al. (2007) Sorensen et al. (2008) Badawi et al. (2009) Sun et al. (2009)
Chlorotoluron 3-(3-chloro-p-tolyl)-1,1-dimethylurea	74	5.0	1.44×10^{-5}	205	*Arthrobacter globiformis* D47 *Mortierella* sp. Gr4	Cullington and Walker (1999) Badawi et al. (2009)

or basic conditions. They are generally considered to be having a moderate persistence in the environment.

Phenylurea herbicides have been found to have several harmful effects not only on the environment but also on the living organisms. Many of the phenylurea herbicides have been found to be endocrine disrupters and to have genotoxic or ecotoxic effects (Caux et al., 1998; Tixier et al., 2001; Lintelmann et al., 2003; Giacomazzi and Cochet, 2004; Badawi et al., 2009). Phenylurea herbicides such as diuron can also be responsible for carcinogenic, mutagenic, reproductive and teratogenic effects at high doses not only for animals but also for human beings (Giacomazzi and Cochet, 2004; Oturan et al., 2008). It is also toxic to birds, fish, worms, snails, and many other aquatic as well as terrestrial living organisms (Giacomazzi and Cochet, 2004). Phenylurea herbicides have also been reported to have effects on the bacterial community structure and their activities (Sorensen et al., 2003; Giacomazzi and Cochet, 2004). Chlorotoluron and isoproturon have also been reported to inhibit wheat seed germination and growth (Song et al., 2006). Linuron acts as an antagonist against the androgen receptor thereby affecting male fertility (McIntyre et al., 2002). Phenylurea aniline metabolites (i.e. 4-isopropylaniline and 3,4-DCA) formed during biotic and abiotic degradation processes, have also been reported to have adverse ecotoxicological effects (Tixier et al., 2001).

2.2. Environmental fates of phenylurea herbicides

Phenylurea herbicides can contaminate the environment by several different ways. Most of the phenylurea herbicides enter the environment as diffuse contamination from agricultural sources as a result of normal spraying practices (Johnson et al., 2001, Gooddy et al., 2002). They could also be released directly from antifouling painting in the water resources. In addition, their application on railways, highways and green parks also leads to the contamination of the water resources. The dispersion of herbicides from the field to the environment under natural conditions is affected by plant uptake, dynamics of water flow, degradation, sorption to solid phases and dissolved or colloidal phase migration etc as described before (Gooddy et al., 2007).

2.3. Movement and dispersion of phenylurea herbicides

Following their application on agricultural field, as a result of heavy precipitation the phenylurea herbicides can undergo surface runoff and leaching to the ground water bodies (Johnson et al., 2001; Gooddy et al., 2002). As a result they are often detected as

contaminants of surface and ground water bodies in European countries as well as in the USA (Field et al., 1997; Thurman et al., 2000; Gerecke et al., 2001; Johnson et al., 2001; Gooddy et al., 2002; Sorensen et al., 2003; Claver et al., 2006; Batisson et al., 2007). Therefore, several of phenylurea herbicides have been listed as potential groundwater contaminants (EPA, USA) and hazardous substances (EU WFD 2000/60/EC).

2.4. Degradation of phenylurea herbicides

Phenylurea herbicides are stable against the chemical degradation within a wide pH range of 4 to 10 (Hill et al., 1955; Gerecke et al., 2001; Salvestrini et al., 2002). They undergo photochemical degradation when they are exposed to sunlight. This led to the formation of some demethylated or formylated derivative products which are more toxic than their mother compound (Tixier et al., 2000; Amine-Khodja et al., 2004). Some of the phenylurea herbicides, like diuron, are degraded through hydrolysis leading to the formation of different types of the degradation products (Giacomazzi and Cochet, 2004).

Although the abiotic processes mainly photochemical and hydrolysis, account for the degradation of phenylurea herbicides, numerous reports indicate that biodegradation resulting from microbial activities plays an important role in the environmental fates of various phenylurea herbicides including isoproturon, fluometuron, diuron, metabromuron and linuron etc (Bozarth et al., 1971; Cullington and Walker, 1999; El-Fantroussi, 2000; Bending et al., 2003; Breugelmans et al., 2007; El-Sebai et al., 2007). Although initial studies were reporting a slow natural biodegradation rate of phenylurea herbicides (Pieuchot et al., 1996; Johnson et al., 1998; Berger, 1999; Zablotowicz et al., 2000), their rapid and extensive degradation has been reported suggesting an adaptation of the soil microbial (Bending et al., 2001; Sorensen and Aamand, 2003; El-Sebai et al., 2007).

Isolation of phenylurea degrading microorganisms from the soils adapted to phenylurea degradation was performed by enrichment cultures using mineral salt medium, containing a phenylurea herbicide as a sole source of carbon and nitrogen. Several bacterial and fungal isolates able to degrade the different phenylurea herbicides have been isolated and characterized for the extents and degrees of their degrading abilities. However, up to now, most of the studies have been focused on the bacterial strains. Some of the known bacterial and fungal strains degrading different phenylurea herbicides presented in Table I-5.

Although a wide variety of fungal and bacterial isolates had the ability to partially degrade different phenylurea herbicides (Cullington and Walker, 1999; Khadrani et al., 1999; Turnbull et al., 2001; Tixier et al., 2002; Badawi et al., 2009), only few bacterial isolates have

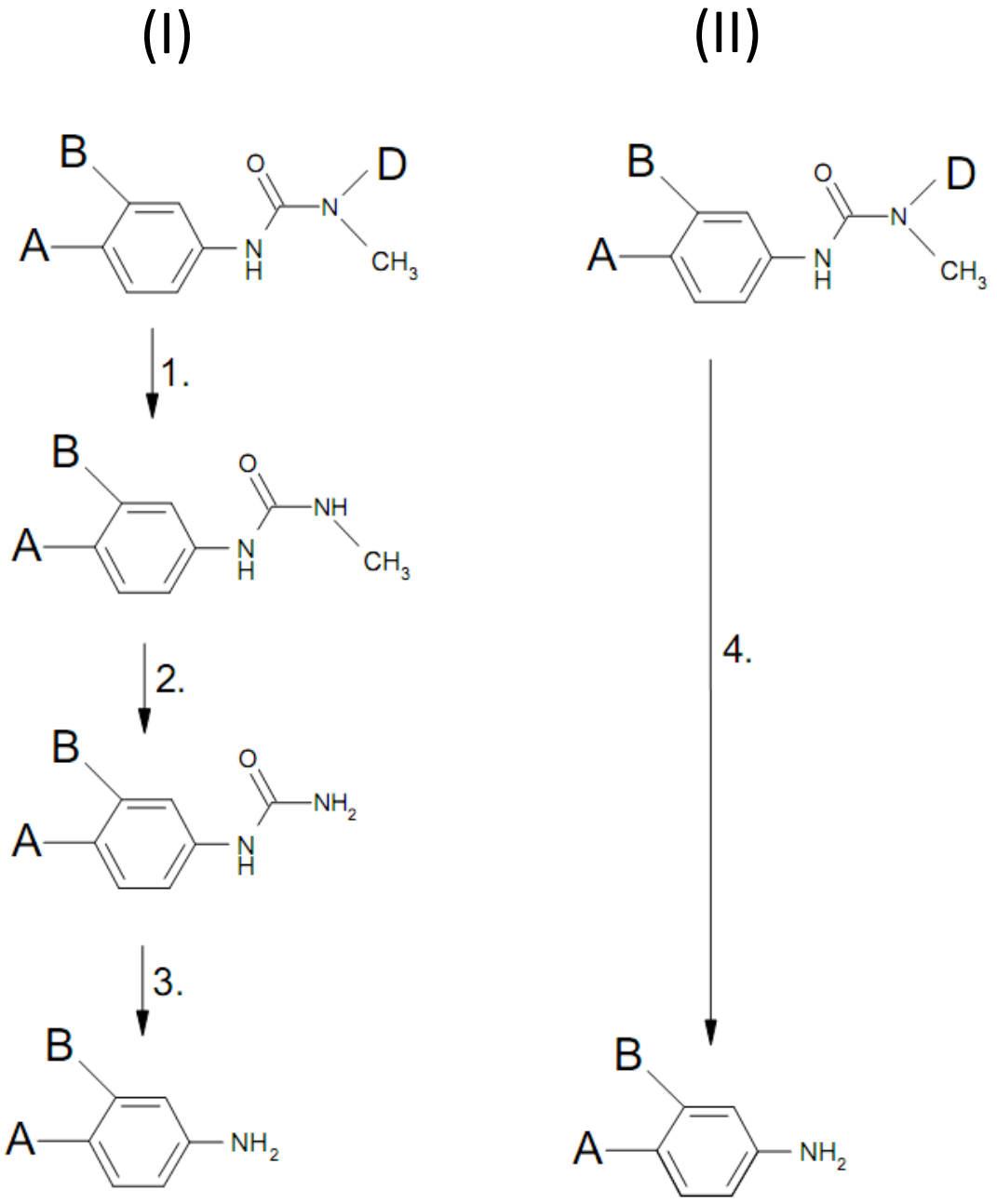


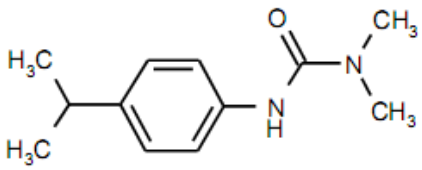
Fig. I-6. Metabolic pathways proposed for different types of phenylurea herbicides (Sorensen et al., 2003). Pathway (I): involves the N-dealkylation (steps 1 and 2) of the urea side chain and hydrolysis of the urea side chain (step 3) to produce aniline derivative. Pathway (II): Direct hydrolysis of the phenylurea herbicides to their aniline derivatives (step 4).

the ability to completely mineralize these herbicides (Sorensen et al., 2001; Dejonghe et al., 2003; El-Sebai et al., 2004; Sorensen et al., 2005; Sorensen et al., 2008; Sun et al., 2009).

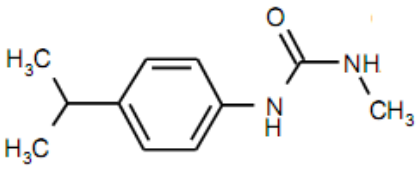
Besides the degradation of phenylurea herbicides by pure microbial isolates, several studies also underlined the importance of bacterial consortia operating in synergistic cooperation for the phenylurea herbicide degradation including metobromuron (El-Fantroussi et al., 2001), linuron (Breugelmans et al., 2007), isoproturon (Sorensen et al., 2002) and diuron (Sorensen et al., 2008).

2.5. Metabolic pathways involved in phenylurea herbicide degradation

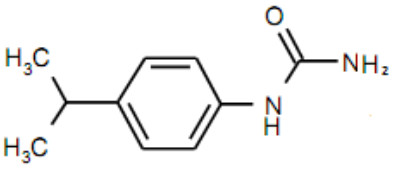
A metabolic pathway is defined as a sequential series of enzymatic reactions connected by their intermediary metabolites leading to the transformation of a compound. The phenylurea herbicides metabolic pathway has been proposed on the basis of the identification of intermediary metabolites of phenylurea herbicides like diuron, fluometuron, metobromuron and isoproturon (Tweedy et al., 1970; Bozarth and Funderburk, 1971; Field et al., 1997; Sorensen et al., 2001; Sorensen et al., 2003; Sun et al., 2009). In most of the cases, the metabolic pathways of the different phenylurea herbicides share similarities (Fig I-6). For N,N-dimethylurea substituted phenylurea herbicides, metabolism generally occurs through successive N-demethylation reactions, while, for N-methoxy-N-methylurea substituted phenylureas it occurs either through N-demethylation or N-demethoxylation reactions. In almost all studied phenylurea herbicides, these initial degradation steps are followed by the hydrolysis of the urea side chain resulting in the appearance of the corresponding aniline metabolites (Fig. I-6, Pathway-I) which might further be metabolized (Sorensen et al., 2003). However, some of the bacterial strains like *Arthrobacter* strains D47, *Arthrobacter* sp. N2 and *Bacillus sphaericus* strain ATCC12123 have the ability to directly hydrolyze a wide range of phenylurea herbicides including IPU to their aniline derivatives (Fig. I-6, Pathway-II) (Engelhardt et al., 1973; Turnbull et al., 2001; Tixier et al., 2002). A generalized metabolic pathway for known phenylurea herbicides up to the accumulation of their aniline derivatives is shown in the fig. I-6. The lower degradation pathway for phenylurea herbicides beyond the aniline derivatives is not well-known.



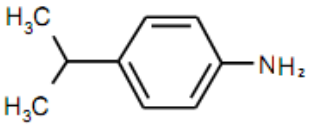
Isoproturon



Monodemethyl isoproturon



Didemethyl isoproturon



4-isopropylaniline

Fig. I-7. Structural formulas of isoproturon and its metabolites

3. ISOPROTURON (IPU)

3.1. Use and mode of action of IPU

Isoproturon is an organic selective phenylurea herbicide used for pre- and post-emergence control of many broadleaf weeds in spring and winter wheat, barley and winter rye (Fournier et al., 1975). It mainly targets the annual grasses such as field foxtail, bentgrass, annual bluegrass and ryegrass, and to a lesser extent wild oats and several annual broadleaf weeds (Tomlin, 1994; Roberts, 1998), including feverfew, chickweed and the lady's mantle (Rigaud and Lebreton, 2004). It is among the most extensively used herbicides in conventional agriculture in Europe (Nitchke and Schussler, 1998). According to a report of "National Institute of Industrial Environment and their Risks (INERIS)", annual consumption of isoproturon was estimated to be more than 1000 tons, ranking it amongst the 15 most used phytosanitary products in France (INERIS, 2007). It is applied to a dose varying from 1500 to 1800 g ha^{-1} on the cereal crops. However, in October 2003 in France, the use of IPU in agriculture was restricted to 1200 g ha^{-1} in order to limit its dispersion in the environment especially in the water resources. It was also recommended that the maximuim dose of IPU should be adjusted between 800 to 1200 g ha^{-1} according to the soil conditions and the growth stage of the weeds, with lower doses for (i) soils with a large internal drainage like sandy soils (ii) soils with low clay content (< 20%) and (iii) the weed at a very early stage (1 to 3 leaves). Intermediate doses should be used for the soils having 20 to 30% of the clay content and the weeds with intermediatery stage of tillering.

Isoproturon is preferentially absorbed by the weed through its root system and leaves. Following the root penetration, isoproturon moves to the aerial parts through xylem where it causes symptoms of toxicity. IPU inhibits the transfer of electrons to the photosystem II (PSII) and induces oxidative stress resulting in lipids, proteins and other cellular components damaging. As a result, it causes the necrosis and clorosis of the weeds (Caux et al., 1998). It does not only inhibit the growth but also produces some toxic compounds leading to the death of the cells and ultimately the weed plant death (Tomlin, 1994; Gauvrit and Hebrard, 1997; Rutherford and Krieger-Liszkay, 2001).

3.2. Molecular structure and physico-chemical properties of IPU

Isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea] is constituted of a phenyl ring (C_6H_4) with a methylurea [(NH_2)-CO-NH-CH_3)] branched on C1 and a dimethyl branched on C4 (Fig. I-7). Vrielynck et al. (2006) carried out the structural and vibrational study of

Table I-6. Structure refinement and crystal data of isoproturon (Vrielynck et al., 2006)

Identification code	Isoproturon
Empirical formula	$C_12H_{18}N_2O$
Formula weight	206.28
Temperature	293(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	*Pbca*
Unit cell dimensions	$a=10.1862(17)$ Å, $\alpha=90°$ $b=11.0301(19)$ Å, $\beta=90°$ $c=20.981(4)$ Å, $\gamma=90°$
Volume	2357.4(7) $Å^3$
Z	8
Density (calculated)	1.162 mg/m^3
Absorption coefficient	0.075 mm^{-1}
$F(000)$	896
Crystal size	$0.30\times0.25\times0.04$ mm^3
Theta range for data collection	1.94–31.29°.
Index ranges	$-14\leq h\leq 14$, $-15\leq k\leq 15$, $-29\leq l\leq 30$
Reflections collected	12,860
Independent reflections	3056 [R(int) = 0.0803]
Completeness to theta = 31.29°	79.4%
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	3056/0/191
Goodness-of-fit on F2	0.805
Final R indices [$I>2\sigma(I)$]	$R1=0.0508$, $\omega R2=0.1247$
R indices (all data)	$R1=0.2779$, $\omega R2=0.1619$
Extinction coefficient	0.0011(6)
Largest diff. peak and hole	0.155 and −0.146 e $Å^{-3}$

Table I-7. Formulas and nomenclature of isoproturon and its known metabolites

Current name	IUPAC name	Molecular formula	Molecular weight	Abbreviation
Isoproturon	3-(4-isopropylphenyl)-1,1-dimethylurea	$C_{12}H_{18}N_2O$	206.29 $gmol^{-1}$	IPU
Monodemethy lisoproturon	3-(4-isopropylphenyl)-1-methylurea	$C_{11}H_{16}N_2O$	192 $gmol^{-1}$	MDIPU
Didemethyl isoproturon	3-(4-isopropylphenyl)-urea	$C_{10}H_{14}N_2O$	178 $gmol^{-1}$	DDIPU
4-isopropylaniline	4-isopropyl-aniline	$C_9H_{13}N$	135 $gmol^{-1}$	4-IA

isoproturon and further refined the structure of isoproturon molecule using the crystallographic and geometrical parameters for the optimized structure of isoprotuon obtained by density functional theory (DFT) as given in the table I-6. In this study, conformational changes occurring in isoproturon crystals from solid state to liquid state were also studied and the crystal molecular packing was reported to be stabilized by hydrogen boding between the NH group and carbonyl group of the neighboring IPU molecules in soild state.

The principal route for isoproturon biodegradation is through the demethylation at the nitrogen atom leading to the formation of the two main metabolites monodemethyl isoproturon and didemethyl isoproturon. Phenylurea side chain is then hydrolyzed to reach another known metabolite i.e. 4-Isopropylaniline (Tomlin, 1994; Sorensen et al., 2001). The structural formulas, molecular weights and IUPAC names of IPU and its known metabolites are shown in Fig. I-7 and table I-7.

Isoproturon ($C_{12}H_{18}N_2O$, MW 206.29 g mol^{-1}) is found as a colorless solid crystalline powder at room temperature (Table. I-6). It is not an ionic molecule and hence does not dissociate in water. It is slightly volatile having low vapor pressure (3.3 µPa) and Henry's Law constant (14.6 e^{-6} Pa. $m^3.mol^{-1}$) values at 20°C (AgriTox, AFSSA-DIVE, 2007). Its solubility in water is about 70 mg l^{-1} at 20°C. It is readily soluble in most of the organic solvents like acetone, dichloroethane and xylene etc (AgriTox, AFSSA-DIVE, 2007). It is highly stable on a wide range of pH from 4 to 13, with the degradation half life of at least more than 200 days to 1560 day. It shows a variable stability against the photolysis in water with a DT50 ranging from 4.5 days to 88 days (AgriTox, AFSSA-DIVE, 2007).

3.3. Presence of isoproturon in the environment

The commercial substances containing isoproturon exist in liquid and solid formulations. Pollution of the environment with isoproturon may originate from industrial release during the manufacturing processes (point contamination). It can also result from agricultural use (diffused contamination) and as a result of point contamination in farmyards. However, point contamination was reported to represent less than 2% of the overall contamination (INERIS, 2007), thus, indicating that agricultural use represents the main IPU source into the environment.

Due to low vapor pressure, isoproturon is very rarely found in the air (Tissier et al., 2005). Isoproturon has a very low tendency to adsorb on the soils and sediments, and is considered as a moderately mobile substance (INERIS, 2007). This is confirmed by the PBT

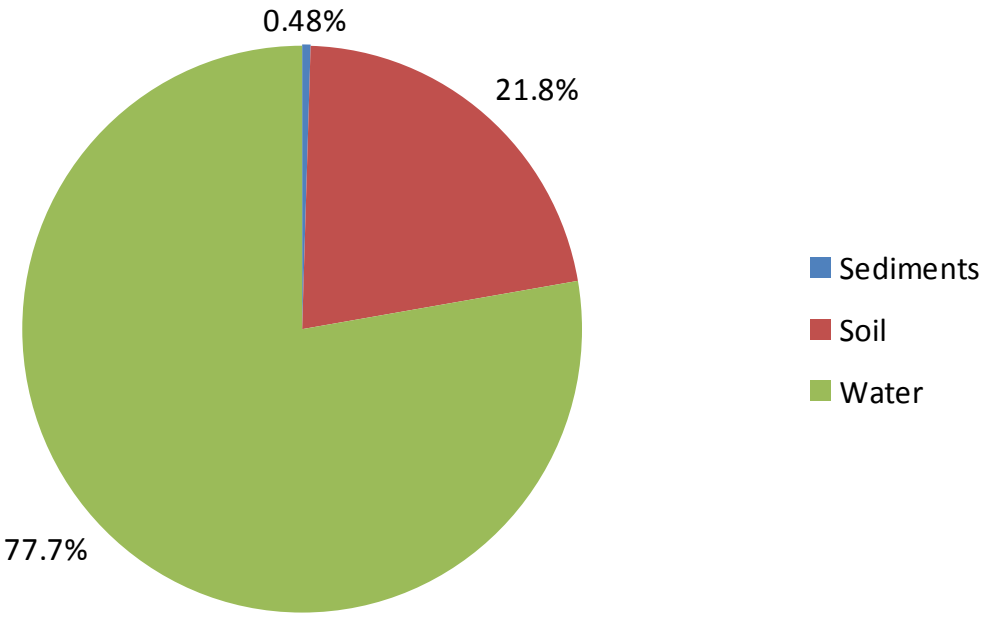


Fig. I-8. Distribution of isoproturon in different compartments of environment (Tissier et al., 2005)

profiler (www.pbtprofiler.net) which has been recommended by the EPA (USA) to estimate the fate of pesticides in different compartments of the atmosphere. PBT profiler suggests that IPU is mainly found in the soil (86%) and water (13%) but negligible in air and sediments. However, several studies suggest that it is predominantly found in the water compartment (Fig. I-8). Isoproturon is often detected in surface and ground waters in Europe at levels exceeding the European Union drinking water limit fixed to 0.1 µg l^{-1} (Nitchke and Schussler, 1998; Spliid and Koppen, 1998; Stangroom et al., 1998). According to a report of IFEN it was found in 87% of the surface and 86% of the ground water samples tested in France (IFEN, 2007). The importance of the contamination of water resources with IPU depends on environmental factors such as rainfall (intensity and timing) and on physico-chemical properties. High solubility of isoproturon in water and its stability at high range of pH values contribute to its persistence in the soil environment and favours its transfer to water resources. As a consequence, IPU is relatively recalcitrant in the environment being degraded up to 40% three months after its application (Nicholls et al., 1993; Harris et al., 1994). It has also been included in a list of hazardous substances compiled by European Commission in 2001 (European Commission, 2001).

3.4. Ecotoxicology of isoproturon

Ecotoxicological data have suggested that repeated exposure to acute concentrations of IPU and some of its main metabolites notably 4-isopropylaniline (4-IA) are harmful not only for human beings but also for plants, animals and microorganisms. 4-isopropylaniline, the aniline metabolite of IPU, was shown to be 600 times more toxic than isoproturon by applying the Microtox $^{(R)}$ test (bioluminescence inhibition of *Vibrio fischeri*) (Tixier et al., 2002). Isoproturon was found to have significant dose-responsive mutagenic effect resulting in chromosomal aberration and sperm shape abnormality (Behera and Bhunya, 1990). Similarly, it was found to cause potential hepatocarcinogenicity in rats resulting in tumor-promoting activity (Hoshiya et al., 1993).

Pérés et al. (1996) reported that isoproturon applied on soil microcosms exerts a selection pressure on the soil algal community causing the elimination of sensitive diatom species and favouring the development of more resistant periphytons. A test of inhibition of the algal growth in response to isoproturon exposure revealed an EC_{50} (72h) of 0.077 mgL^{-1} for the species *Scenedesmus subspicatus* and an EC_{50} (7 days) of 0.06 mgL^{-1} for *Lemna minor* (Nitschke et al., 1999). Further studies indicated that isoproturon adversely affects the algal growth and metabolism. It has been shown that in response to IPU exposure (10 µg L^{-1} for 4

days), *Scenedesmus obliquus* and *Scenedesmus quadricauda* growth rate and photosynthetic activity was considerably reduced (Dosnon-Olette et al., 2010). Growth and effective quantum yields of the algal strain *Scenedesmus vacuolatus* resulting in cumulative decreased biomass production were found to be reversibly inhibited by the varying concentration and exposure times of isoproturon (Vallotton et al., 2009). Although the growth was affected by the exposure of this algal species to isoproturon, a slight increase in IPU tolerance was also observed. Similarly, the toxic effects of isoproturon on alga *Scenedesmus obliquus* were studied and a linear correlation was observed between the changes in cell density and changes in photosynthetic-fluorescence parameters in response to isoproturon application (Dewez et al., 2008). Schmitt-Jansen and Altenburger (2005) studied the toxic effects of isoproturon on periphyton communities in a microcosm environment and found that the biomass production of the algal community was decreased in response to higher concentration of isoproturon. It was also concluded that IPU could modify the structure of the algal community even at sub-acute concentration toxicity level.

Isoproturon has also been reported to have pronounced effects on plants besides weeds which are targetted including macrophytes and agricultural crops. Indeed, the growth of the wheat seedlings (*Triticum aestivum*) was reported to be inhibited at varying concentrations of isoproturon. Wheat seedlings showed reduced chlorophyll content even at low concentration (2 mg kg^{-1}) of isoproturon on a short time exposure. Isoproturon was also found to modify the antioxidant enzymes activity (Yin et al., 2008). Isoproturon was also found to have effects on the growth of two macrophytes *Elodea densa* and *Ludwigia natans* (Grollier et al., 1997). It also causes toxic effects on phytoplanktons when it was applied in mixture with other herbicides (Knauert et al., 2008). Photosynthetic efficiency of three submerged macrophytes *Elodea canadensis*, *Myriophyllum spicatum* and *Potamogeton lucens* was found to be affected following their exposure to environmentally relevant concentration of isoproturon (Knauert et al., 2010). Isoproturon can also enter the food web via its chain by the process of bioaccumulation in non target plants. Such non-target bioaccumulation was observed in a macrophyte *Lemna minor* while studying the fate of isoproturon in a fresh water microcosm (Bottcher and Schroll, 2007).

Moreover, the effects of isoproturon on animals are also not negligible: daily injections of 675 mg kg^{-1} of isoproturon in rats considerably increase the peroxidation reactions in their cerebral and tissue level. These peroxidations generate the appearance of reactive forms of oxygen species which alter the cellular membrane and to some extent can trigger the cell death (Hazarika and Sarkar, 2001).

Isoproturon was shown to accumulate in the water earthworm *Tubifex tubifex*. By increasing IPU concentration (0.1 to 10 mg L^{-1}) and exposure time (4 to 7 days) an increase in the mortality of *Tubifex tubifex* from 5 to 37% was observed (Paris-Palacios et al., 2010). Although isoproturon has lethal effects on earthworms including Tubifex but sublethal effects like reduction in growth rate, decrease of protein contents, and increase in antioxidative defence enzyme activities are more important being induced within 2 days of exposure even with low concentration ranging from 0.1 to 10 mg L^{-1} (Mosleh et al., 2003; Mosleh et al., 2005; Mosleh, 2009). The use of herbicides containing isoproturon in agricultural soil was found to have adverse effects on the development of early larval stages of spawn and tadpoles of amphibians by modifying the the activity of microsomal and soluble glutathione-S-transferase towards different model substrates (Greulich et al., 2002).

3.5. Adaptation of the regulation of IPU use

The active substance isoproturon was licensed in European Union according to (i) the Annex-1 of the Directive 91/414/EEC (Official Journal of European Communities, 1991) authorizing plant-protection products and (ii) the Directive 2002/18/EC (Official Journal of European Communities, 2002). This last directive applicable from January 1, 2003 was proposed on the basis of the report of December 7, 2001 of Directorate General "Consumer Health and Protection" (SANCO; SANCO/3045/99-final 12 March 2002). It mainly stresses on the following points:

- The member states must pay particular attention towards the protection of the ground water when isoproturon is applied on vulnerable soils, climatic conditions or doses higher than those recommended in the evaluation report [1,5 $kg.ha^{-1}$ used alone] and must apply risk mitigation measures, where appropriate.
- The member states must also pay attention to the protection of aquatic organisms and must ensure that permission is accompanied by mitigation measures.

This Directive has been implemented in France in 2002 as mentioned by "Journal official" Number 87 of 13 April 2002. In France, according to a notice to the users and distributers of the pesticides in accordance with the provisions of Articles L. 253-1 to L. 253-17 of the rural code on the marketing of pesticides for agricultural use and the proposal of the committee for approval of pesticides for agricultural use, the maximum dose of isoproturon was fixed to 1200 grams per hectare when it is used alone (Journal official, France, 18 February 2004).

Because of its harmful effects on the aquatic life, isoproturon is not registered in many countries including United States, Canada and Australia, but it is registered in United Kingdom, India and New Zealand (PAN Pesticide Database, 2010).

Similarly, it has not been registered in pacific Africa countries including South Africa, Madagascar, Uganda and Mauritania (PAN Pesticide Database, 2010).

The molecule has been banned in several European countries including Denmark, Greece, Finland, Portugal, Hungary, Finland and Cyprus (PAN Pesticide Database, 2010). In Denmark this decision was made with reference to the Danish EPA Statutory order no. 189 of March 22, 1999 (Danish Environmental Protection Agency, Ministry of the Environment, Denmark).

3.6. Fate of isoproturon in soils

Following its application, adsorption and desorption on the soil components determine the fate and dynamics of IPU. The partition coefficient (K_d) of the pesticides between the soil solid phase and the soil solution is often used to estimate the dynamics of pesticide in the soil environment (Walker et al., 1999; Zhang et al., 2007).

3.6.1. Adsorption and desorption of IPU

As compared to other herbicides, isoproturon adsorption is relatively weak being of the same same order of magnitude than that of atrazine and much inferior to that of other phenylurea herbicides (Perrin-Ganier et al., 1995). Isoproturon showed a K_d value below 10 L kg^{-1} suggesting that isoproturon is only poorly retained in some soils (Sorensen et al., 2003; Tian et al., 2010).

The adsorption of isoproturon is dependent on a large number of interacting factors including the physico-chemical properties of both soil and isoproturon. However, two of the most important factors are soil organic matter and clay contents because the K_d of isoproturon was found to be dependent (i) on organic carbon content in surface soil layers (Coquet and Barriuso, 2002; Ertli et al., 2004) and (ii) on clay content in subsurface soil depths (Coquet et al., 2004). Adsorption of isoproturon is proportional to the soil organic matter content (Hance, 1965; Stevensen, 1972; Kozak and Weber, 1983; Barthelemy, 1987; Madhum and Freed, 1987). Indeed, the adsorption of isoproturon as well as trifluralin on five UK soils was reported to be related to the organic carbon fraction in the soils (Cooke et al., 2004). IPU adsorption on the organic fraction of soil is susceptible to be of physical nature involving low energy interactions (Gaillardon et al., 1980). In addition, adsorption of isoproturon was also

shown to be correlated with the clay content of the soil (Coquet, 2003). Not only the quantity but also the clay type influences the adsorption of isoproturon in soils. Isoproturon is not adsorbed on quartz, calcite or iron oxides while it is strongly adsorbed to kaolinites with a K_d of 1.15 L kg^{-1}. Similarly, IPU partition coefficient (K_d) was shown to vary from 0.5 L kg^{-1} to 5.8 L kg^{-1} for sandy loam and clay loam soil respectively containing about 3% of organic matter (Moss, 1985; Blair et al., 1990). pH is also an important factor conditioning the extent of adsorption with isoproturon being more adsorbed in a solution at pH 5 than at pH 8 (Ertli et al., 2004). It has also been suggested that pH could be considered as a second most important factor after organic matter content influencing IPU adsorption (Coquet and Barriuso, 2002). Isoproturon adsorption is more important in soils showing high moisture content (62%) than in low moisture content (35%). While determining the variation of pesticide sorption isotherm in soil at a catchment scale, the K_d of isoproturon was found to be ranging from 0.47 L kg^{-1} to 1.81 L kg^{-1} with an average value of 0.85 L kg^{-1} (Coquet, 2002).

The pesticide adsorption has also been found to be affected by extrinsic factors including agricultural practices such as soil amendments with organic matter which ultimately affect IPU dynamics in the environment (Si et al., 2006). Tian et al. (2010) demonstrated that the adsorption of isoproturon on soils is significantly increased by charcoal amendment. Charcoal amendment was shown not only to increase adsorption of IPU on soil but also to enhance the hysteresis of isoproturon desorption from the soil.

IPU adsorption is rapid immediately after its application which then carries on decreasing with the course of time. However, desorption is a continuous gradual process working directly in relationto time. It seems that the adsorption process evolving during the course of time also modify its bioavailability and stabilization in the soil.

3.6.2. Isoproturon transfer or movement

Although isoproturon showed a low solubility in water due to its weak adsorption but it can be transferred to water resources. However, it has been found that isoproturon is not detected beyond the 10 cm depth (Blair et al., 1990). IPU was shown to be more mobile in soils than other phenylurea herbicides (Tian et al., 2010). Indeed, following application on clay soil in spring season, even low precipitations can cause about 0.04% exportation of IPU towards the drainage water (Schiavon, 1992). Concentration of isoproturon in the drainage water can reach up to 20 µg L^{-1} (Traub-Eberhard et al., 1994). The quantity of isoproturon detected in the drainage water varied from 3.43 to 549.62 µg L^{-1} and 2.5 to 325.89 µg L^{-1} in clayey and brown leached soils respectively (Schiavon et al., 1997). In winter season,

isoproturon exported to the drainage water varied from 11 to 130.2 µg L^{-1} and 5.9 to 160 µg L^{-1} in in clayey and brown leached soils respectively (Schiavon et al., 1992; Schiavon et al., 1994). Experiments conducted on soil mini columns treated with ^{14}C labeled isoproturon showed that about 10% of the initially applied isoproturon was transferred highlighting the importance of the phenomenon. Isoproturon transfer seems to be limited three months after its application (Perrin-Ganier et al., 1995). On the contrary, a field study showed that IPU can leach at least up to three years after its application (Johnson et al., 2001).

3.6.3. Degradation and mineralization of isoproturon

In the soil environment, isoproturon was shown to be degraded into simple harmless or less harmful compounds through both abiotic and biotic processes.

3.6.3.1. Abiotic degradation

IPU is stable against chemical degradation in aqueous solution at moderate temperatures and at pH values varying between 4 and 10 (Gerecke et al., 2002). As a consequence, chemical degradation of IPU is often considered very low in most of the agricultural soils. However, under the action of UV radiation, IPU can be photochemically decomposed in the water as well as in the first millimeters of soil leading to its transformation to monodemethyl isoproturon (MDIPU) and then to didemethyl isoproturon (DDIPU) (Kulshrestha and Mukerjee, 1986). The photodecomposition of IPU in aqueous medium can reach up to 50% of the initially added product in about 70 days. This reaction can also be realized and detected on soil surface (Kulshrestha, 1982). The solarization by polyethylene mulching was shown to promote IPU dissipation in agricultural soils (Navarro et al., 2009). Recent studies described the enhanced photocatalytic degradation of isoproturon and of its main metabolites by solar light in the aqueous systems in the presence of some catalysts (Sharma et al., 2008a, b). In addition, IPU is degraded by ozonation in aqueous solution forming different types of intermediate and final by-products (Mascolo et al., 2001). Ozonation provokes the hydroxylation of the aromatic ring or side chain and may break the isopropyl alkyl chain.

3.6.3.2. Biodegradation of isoproturon

Biodegradation of isoproturon is considered to be the main phenomenon responsible for its natural attenuation from the environment. It has been found that microalgae belonging to *Scenedesmus* genus removed about 58% of the initially added isoproturon in liquid culture (Dosnon-Olette et al., 2010). Similarly, IPU metabolism resulting in accumulation of its

metabolites MDIPU, DDIPU and 4-IA has also been observed in some earthworms like *Tubifex* (Paris-Palacios et al., 2010) and even in some amphibians like spawn and tadpoles (Greulich et al., 2002). However, microbial biodegradation is the primary mechanism of IPU degradation and dissipation not only in the soil environment (Fournier et al., 1975; Gaillardon and Sabar, 1994; Cox et al., 1996; Pieuchot et al., 1996). The microbial communities are responsible for the purification of the soil because of their capability to reduce the fate of pesticides in the environment.

3.6.3.2.1. Biodegradation of isoproturon in agricultural soils

Isoproturon degradation in the soil and sediments mainly take place through the microbiological processes (Fournier et al., 1975; Mudd et al., 1983; Cox et al., 1996). Initially several studies reported that IPU was biodegraded through N-dealkylation transormation at low rate in various soils and subsurface environments (Johnson et al., 1998; Berger, 1999; Larsen et al., 2000; Kristensen et al., 2001; Sorensen et al., 2001). Moreover, later on agricultural soils regularly exposed to IPU and having different physico-chemical properties, showed enhanced isoproturon degradation ability being degraded in only 5 days after its third treatment (Cox et al., 1996). The authors hypothesized that in response to IPU exposure, the soil micro flora might adapted to rapid IPU degradation. The enhanced biodegradation of IPU in response to repeated application over a longer period has been reported in several studies (Bending et al., 2003; Sorensen and Aamnad, 2001; Walker et al., 2001). This phenomenon was first observed in an agricultural field (Deep Slade, UK) (Cullington and Walker, 1999; Bending et al., 2001). It was then reported in a Danish agricultural field where occurrence of IPU mineralization was found to be related to previous exposure (Sorensen and Aamand, 2003). More recently, El Sebai et al. (2005) reported enhanced IPU biodegradation in a French agricultural soil regularly exposed to IPU. Reid et al. (2005) studied intrinsic and induced isoproturon catabolic activity in different soils. It was shown that IPU catabolic activity was significantly higher in soils treated with this herbicide and that organic amendment promotes this activity.

Isoproturon biodegradation seems not to be evenly distributed across agricultural fields (Beck et al., 1996; Bending et al., 2001; Walker et al., 2001; Walker et al., 2002; El-Sebai et al., 2007). As an example, Beck et al. (1996) showed that the time for 50% reduction in isoproturon ranged from 31 to 483 days in 25 different samples collected within an agricultural field. This observation highlights the importance of spatial heterogeneity of IPU degrading activity at the field scale. Spatial heterogeneity of microbial activity responsible for

degradation of isoproturon in agricultural fields stimulated the further studies for identifying the underlying explaining processes and mechanisms in the different regions of the world including China, Denmark, United Kingdom and France. Several researchers studied the spatial and in-field spatial heterogeneity of isoproturon degradation. Spatial heterogeneity in IPU degradation was associated with variations in soil factors (like pH, soil moisture, temperature etc) and with microbiological factors such as microbial growth and biomass.

Walker et al. (2001) found that IPU degrading spatial variability within a British agricultural field at Deep Slade was correlated with the soil pH and the microbial biomass. Later on, Bending et al. (2003) confirmed these results by indicating that rapid degradation of isoproturon in an agricultural field of Deep Slade (UK) was associated with the proliferation of isoproturon degrading micro organisms as a result of the repeated exposure to this herbicide and the spatial variability was also found to be associated with the pH. These two factors (i.e. pH and bacterial biomass) were also found to be responsible for the spatial variability of isoproturon mineralization activity determined for 50 soil samples within an agricultural field in France (El-Sebai et al., 2005; El-Sebai et al., 2007). The negative effect of pH on IPU degrading activity was also shown in a study conducted on 30 samples taken from different locations at the Brimstone farm experimental site, Oxfordshire,UK (Walker et al., 2002). In addition, the degradation or mineralization rate was also associated with the growth-linked metabolism of IPU by the soil microorganisms (Sorensen et al., 2003). This was already suggested by Bending et al. (2001) who reported that heterogeneity of isoproturon metabolic activity along the transect of the soil was most likely due to variation in the dynamics of the isoproturon degrading microbial communites and the significance of growth linked and cometabolic degradation. Similarly, the spatial heterogeneity of isoproturon mineralizing activity within an agricultural field was also found to be stimulated by the presence of compost (Vieublé-Gonod et al., 2009). This stimulation of the mineralization activity was supposed to be encouraged by the microbial characteristics of the compost, physical properties of the soils and physico-chemical conditions in the area with high mineralization activity.

3.6.3.2.2. Pure culture studies on IPU biodegradation

Enhanced degradation of IPU has stimulated research aiming at isolating and characterizing microbial strains able to entirely mineralize IPU from these adapted soils. Several studies have been carried out with this goal by using enrichment cultures (Cox et al., 1996; Sorensen and Aamand, 2003; El-Sebai et al., 2004; Sun et al., 2009) and, up to now,

several microbial strains including bacteria and fungi have been isolated and characterized for isoproturon degradation.

3.6.3.2.2.1. Bacterial IPU degradation

Several different bacterial strains harboring IPU degrading abilities have been isolated from different countries including United Kingdom, China, Denmark and France. Most of the isolated bacterial strains had the ability to only partially degrade isoproturon while only few were able to entirely mineralize IPU.

Starting from soils exposed to isoproturon in laboratory, Roberts et al. (1998) used three different isolation routes (flask enrichment/flask degradation assay, flask enrichment/microplate degradation assay, MPN assay/microplate degradation assay) to obtain pure bacterial cultures able to degrade isoproturon and use it as a sole source of carbon and nitrogen. In this study 36 bacterial strains belonging to 16 distinct phenotypes were characterized for IPU degradation. Some of these strains were able to degrade IPU metabolites monodemethylisoproturon (MDIPU) and didemethylisoproturon (DDIPU) as well as some other related phenylurea herbicides like diuron and linuron. Transient accumulation of IPU metabolites MDIPU and DDIPU were also observed during IPU degradation by some of the bacterial strains. Cullington and Walker (1999) isolated a bacterial strain *Arthrobacter globiformis* able to degrade diuron as sole source of carbon and nitrogen. *A. globiformis* also had the ability to slowly transform other phenylurea herbicides including isoproturon to their aniline derivatives by hydrolysis of the urea side chain. They showed the degrading performance of *A. globiformis* could be improved by providing a carbon and energy source. Further characterization of this bacterial strain, led to identify a catabolic plasmid harboring a phenylurea hydrolase gene *puhA* (Turnbull et al., 2001). Similar IPU catabolic activity was reported later on in *Arthrobacter* sp. N2 able to degrade different phenylurea herbicides including isoproturon to their aniline derivatives (Tixier et al., 2002). A bacterial culture having the ability to degrade IPU metabolites (i.e. MDIPU and 4-IA) was isolated from a suspension of soil treated with isoproturon (Sorensen and Aamand, 2001).

Sphingomonas sp. SRS2, the first IPU mineralizing bacterial strain was isolated from the Deep Slade agricultural soil of UK (Sorensen et al., 2001). This bacterial strain had the ability to degrade not only the known IPU metabolites (i.e. MDIPU, DDIPU and 4-IA) but also some closely related phenylurea herbicides like diuron and chlorotoluron. Based on the transient accumulation of metabolites during IPU metabolism, the initial steps of IPU metabolic pathway were proposed for SRS2 (Sorensen et al., 2001; Sorensen et al., 2003).

Further research indicated that IPU mineralizing activity of the strain SRS2 is significantly enhanced when it is co-cultured with an unidentified bacterial strain SRS1 or in the presence of certain amino acids in the liquid medium (Sorensen et al., 2002). Another IPU mineralizing bacterial strain *Methylopila* sp. TES was isolated and characterized from an IPU treated agricultural field of France (El-Sebai et al., 2004). This bacterial strain had the ability to degrade IPU and its known metabolites, however, it could not degrade other structurally related phenylurea herbicides. Based on this finding, it was hypothesized that *Methylopila* sp. TES might harbor a catabolic pathway highly specific for isoproturon. More recently, Sun et al. (2009) described the isolation and characterization of three IPU mineralizing *Sphingobium* strains YBL1, YBL2 and YBL3 from the soils of two herbicide plants. The optimum conditions for IPU degradation by these strains were pH 7.0 and temperature 30°C. Isoproturon degradation by these bacterial strains was found to be initiated by N-demethylation of the urea side chain resulting in accumulation of first metabolite MDIPU. These three strains had the ability to degrade related dimethylurea substituted herbicides like chlorotoluron, diuron and fluometuron. YBL1 was also capable of degrading methoxymethylphenyl-urea herbicides such as linuron. Furthermore, accumulation of catechol and the first metabolite after phenyl ring cleavage i.e. cis,cis-muconic acid was also observed during the mineralization of aniline by the *Sphingobium* sp. strain YBL2 suggesting that the lower pathway could take the route of β-ketoadipate pathway. This hypothesis was further reinforced by the amplification of *catA* gene coding for catechol 1,2-dioxygenase from YBL2.

3.6.3.2.2.2. Fungal IPU degradation

Although most of the studies focused on bacterial biodegradation of isoproturon, a number of studies indicate the involvement of fungi in isoproturon degradation.

Among 90 fungal strains tested for degradation, Vroumsia et al. (1996) reported that 10 strains belonging to *Zygomycetes* and *Agonomycetes* have the ability to degrade more than 50% of the initially added isoproturon in the presence of a carbon source. Among them, a fungal strain *Rhizoctonia solani* was found to be more efficient being able to degrade 84%, 86 % and 73% of the initially added isoproturon, diuron and chlorotoluron, respectively, only in two days. No accumulation of the metabolites of any of these phenylurea herbicides was reported. These results also confirm the results of Steiman et al. (1994) who reported that *Rhizoctonia* sp. has the ability to rapidly degrade isoproturon. Certain species of Basidiomycetes such as *Bjerkandera adusta* have been reported to have degraded 80% of the initially added isoproturon in the presence of glucose after only 5 days of incubation

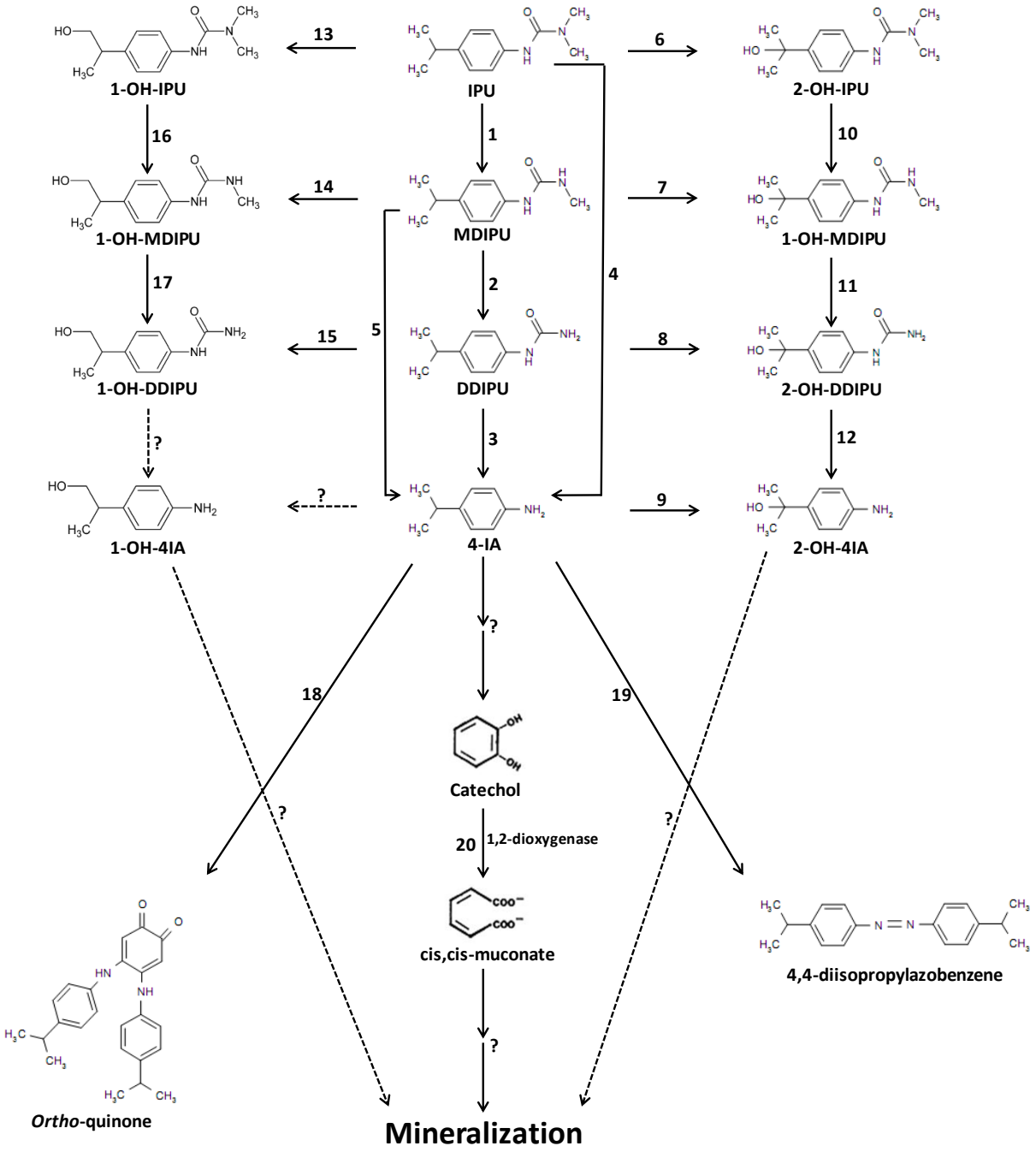


Fig. I-9. Proposed IPU metabolic pathways

(Khadrani et al., 1999). It was also found that some fungal strains belonging to *Basidiomycetes*, *Ascomycetes* and *Zygomycetes* have the ability to transform isoproturon to hydroxylated or demethylated products. Hydroxylation was found to be dominant mode of isoproturon transformation by these fungi (Ronhede et al., 2005). Interestingly, the mineralization of isoproturon in the soil was reported to be stimulated by this hydroxylation (Ronhede et al., 2007). Similarly, Hangler et al. (2007) screened 27 fungal strains for isoproturon degradation, out of which 15 were found to be able to convert isoproturon to some polar metabolites. *Cunninghamella elegans* JS/2 was found to convert isoproturon into several metabolites. It was found that the degradation capability of this strain was inducible in response to isoproturon exposure and that several inducible enzymes are involved in degradation of isoproturon. Most recently, Badawi et al. (2009) reported the degradation of of different phenylurea herbicides including isoproturon by a soil fungus *Mortierella* sp. Gr4. This fungal strain had the ability to degrade isoproturon by two successive processes i.e. N-demethylation of the urea side chain and hydroxylation of the isopropyl ring constituents. This study also suggested that the fungal pathways for the degradation of herbicides differ from the pathways followed by the bacterial strains.

3.6.3.3. IPU metabolic pathway

As already described above, it is well known that the degradation of isoproturon in soil environment primarily takes place through microbial processes, although abiotic processes including photodegradation were shown to occur (Ronhede et al., 2005). Both bacterial and fungal populations have been shown to be able to degrade IPU. However, up to now, microbial degrading pathway was mostly derived from bacterial species. Based on the characterization of IPU metabolites detected in the environment and resulting from bacterial and fungal IPU degrading ability, a number of synthetic IPU metabolic pathways were recently proposed.

Although a complete IPU metabolic pathway still remains to be elucidated, the initial steps of IPU transformation were proposed (Sorensen et al., 2001; Sorensen et al., 2003; Badawi et al., 2009; Sun et al., 2009). It has been suggested that demethylation of the dimethylurea side chain of IPU yielded in formation of MDIPU (3-(4-isopropylphenyl)-1-methylurea) (Fig I-9, Step 1). This initial step is often recognized as the limiting step of IPU degradation (Sorensen et al., 2001; Sorensen et al., 2003; Sun et al., 2009). MDIPU has been detected in high concentrations in agricultural soils following IPU degradation (Berger, 1999;

Perrin-Ganier et al., 2001) and also in bacterial and fungal cultures (Sorensen et al., 2003; Badawi et al., 2009; Sun et al., 2009).

Based on the identification of the metabolites formed during the transformation of IPU by *Sphingomonas* sp. SRS2, a metabolic pathway has been proposed: it is initiated by two successive N-demethylations of IPU to MDIPU and DDIPU, followed by the cleavage of the urea side chain to 4-IA (Fig. I-9, Steps 1-3) and finally by the mineralization of the phenyl structure (Sorensen et al., 2001; Sorensen et al., 2003). Successive demethylations of IPU resulting in the formation of MDIPU and DDIPU have also been reported in a fungal strain *Mortierella* sp. Gr4 (Badawi et al., 2009). In addition, *Arthrobacter globiformis* D47 (Turnbull et al., 2001) and *Arthrobacter globiformis* N2 (Tixier et al., 2002) degrade IPU directly into 4-IA by hydrolytic cleavage of the urea side chain without passing through MDIPU and DDIPU (Fig. I-9. Step 4). Another metabolic pathway based on the cleavage of the methylurea group of MDIPU resulting in the direct accumulation of 4-IA (Fig. I-9. Step 5) has also been reported in a mixed bacterial culture (Sorensen and Aamand, 2001). Two alternative metabolic pathways based on the hydroxylation of the urea side chain of IPU resulting in 1-OH-IPU or 2-OH-IPU (Fig. I-9. Steps 6,13), have also been proposed in fungal strains, mixed bacterial cultures and in agricultural soils (Sorensen et al., 2003, Badawi et al., 2009). Although 1-OH-IPU has been reported as a dead-end metabolite produced by mixed bacterial cultures derived from soil (Sorensen et al., 2003), the proceeding steps (Fig. I-9. Steps 13-17) of this first alternative pathway involving its related low stream metabolites like 1-OH-MDIPU and 1-OH-DDIPU have been proposed for IPU degradation by a fungal strain *Mortierella* sp. Gr4 (Badawi et al., 2009). The second alternative pathway (Fig. I-9. Steps 6-12) derived from hydroxylation of IPU to 2-OH-IPU was proposed by Sorensen et al. (2003) based on the detection of its different downstream metabolites like 2-OH-MDIPU, 2-OH-DDIPU and 2-OH-4IA during the degradation of IPU in different soils. Very little information is available on these hydroxylated metabolites and no organism having the ability to produce them has yet been isolated.

Although some initial steps of IPU metabolic pathway have been proposed, the degradation beyond the aniline derivative (4-IA) has not yet been well described in most of the studies related to IPU mineralization. Although not well reported in phenylurea herbicides, the aniline compounds are generally metabolized through formation of a key intermediate metabolite catechol by their oxidative deamination (Sorensen et al., 2003). Catechol is further degraded by entering the different ring cleavage pathways like *ortho*-cleavage and *meta*-cleavage (Sun et al., 2009). Catechol is a key intermediate of the β-

ketoadipate pathway responsible for degradation of many aromatic compounds (Borraccino et al., 2001; Alva and Peyton, 2003; Nayak et al., 2009) to corresponding aliphatic metabolite i.e. cis,cis-muconic acid (Nayak et al., 2009). This key intermediate was also detected during the mineralization of aniline by the IPU degrading bacterial strains YBL1, YBL2 and YBL3 (Sun et al., 2009). In addition, *catA* gene coding for catechol 1,2-dioxygenase was also detected in these isolates suggesting that phenyl ring of IPU might be degraded through *ortho*-cleavege of the catechol (Fig. I-9. Step 20). However, this finding requires further elucidation. Indeed, aniline compounds, including 4-IA, have also been reported to undergo various chemical reactions in soils to form various types of products (Pieuchot et al., 1996; Scheunert and Reuter, 2000; Perrin-Ganier et al., 2001). 4-IA has also been reported to form dead-end products disubstituted *ortho*-quinone (Fig. I-9. Step 18) and 4,4-diisopropylazobenzene (Fig. I-9. Step 19) (Scheunert and Reuter, 2000; Perrin-Ganier et al., 2001).

4. OBJECTIVES

Although several researches have already been carried out to explore the isoproturon degradation in agricultural fields and the potential for isoproturon degradation has been reported in a number of bacterial and fungal strains, very little is known about the processes and the genes involved in the regulation of this metabolic activity. In this context, the present study was carried out from the field to the gene level with the following objectives;

- To estimate the spatial variability of isoproturon mineralization activity within an agricultural field periodically treated with IPU not only in relation to the soil physico-chemical and microbiological parameters but also in relation to the pesticide application plan over a three year crop rotation (winter wheat / barley / rape seed).
- To isolate and carry out the molecular and physiological characterization of the bacterial community and related bacterial strains involved in isoproturon mineralization.
- To develop a genomic strategy aiming at identifying the genes coding the enzymes involved in isoproturon mineralization pathway.

Table II-1. Crop and herbicide application history of the field over three years

	2008	2009	2010
Crop	Barley	Colza	Winter wheat
Herbicides used	Isoproturon (1.2 kg ha^{-1}) Prowl	Treflan Typhon Glyphosate Colzor trio	Archipel (0.25 kg ha^{-1}) Mesosulfuron methyl(30 g kg^{-1}) Iodosulfuron methyl(30 g kg^{-1}) Mefenpyrdiethyl(90 g kg^{-1}) Actirob Radar SURF
Date of application	12-10-2007	29-08-2008	04-03-2010

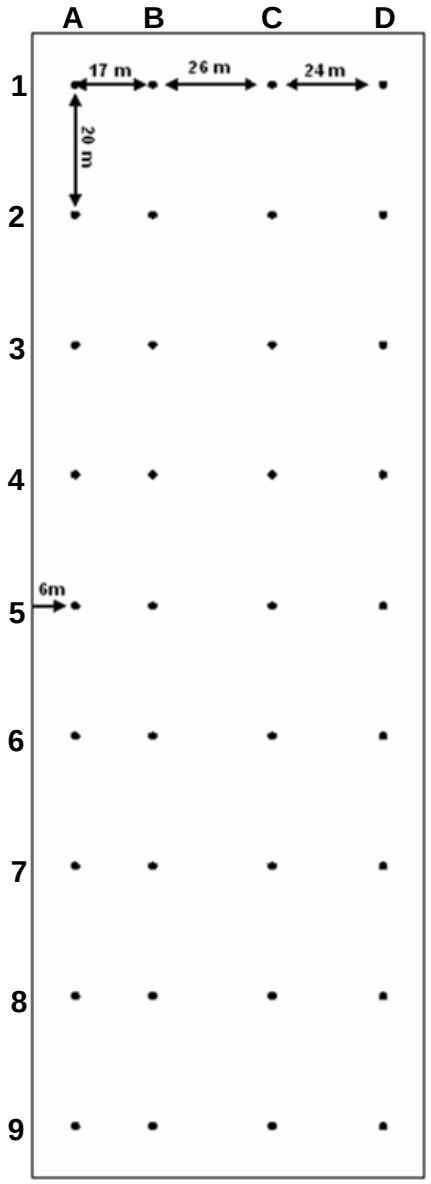


Fig . II-1. Schematic representation of the Epoisses field indicating the sampling grid used to collect the 36 soil samples.

MATERIALS AND METHODS

1. Soil

1.1. History of the field

The soil used in this study was collected from a field at the experimental farm of the Dijon center of the National Institute of Agronomical Research (INRA) (Breteniere, France). This field was cultivated under rapeseed / winter wheat / barley crop rotation and had been periodically treated with phenylurea herbicide isoproturon (IPU). History of the crop and pesticide application of the field over three years is presented in table II-1.

1.2. Soil sampling

Soil sampling was carried out in april 2008, 2009 and 2010. During three years (2008-2010), this field has only been treated with isoproturon before 2008 sampling. According to the sampling plan presented in the fig. II-1, 36 soil samples were collected from the depth of 0-15 cm using a soil sampler. The soil samples were sieved at 5 mm and the samples were subdivided into two aliquots. First aliquot was air dried for physicochemical analysis, whereas, the second was conserved at 4°C for biological analysis. From the second aliquot, sub-samples were stored at -20°C for the molecular analysis based on soil DNA extraction.

2. Herbicides and their metabolites used in this study

The principal herbicide used in this study was analytical grade isoproturon (IPU) and its known metabolites i.e. monodemethyl isoprotuon (MDIPU), didemethyl isoproturon (DDIPU) and 4-isopropylaniline (4-IA). Other structurally related phenylurea herbicides like diuron, chlorotoluron, linuron and monolinuron as well as aniline derivatives of different phenylurea herbicides like 3-chloroaniline, 4-chloroaniline, 4-bromoaniline, 3-chloro-4-methylaniline, 3,4-dichloroaniline (3,4-DCA) were also used to estimate the degrading abilities of isoproturon degrading isolates. All the herbicides and metabolites had more than 99% purity and were purchased from different chemical companies including Riedel-de-Haen (Germany), Dr. Ehrenstorfer-Schafers (Augsburg, Germany), Sigma-Aldrich (Germany). ^{14}C ring-labelled IPU (specific activity 18 mCi $mmol^{-1}$; 99% radiochemical purity) was purchased from International Isotopes (Munich, Germany).

A

B

Photo II-1. System used for the determination of the soil microbial C biomass. **A**: vacuum incubator along with vacuum pump. **B**: Total carbon analyzer (Dohrman DC80) used to determine the concentration of organic carbon in solution

3. Estimation of physico-chemical properties of the soil samples

Soil physico-chemical analyses were carried out by the Laboratory of Soil Analysis (INRA, Arras). The physico-chemical paramaters determined were equivalent humidity (EH), organic matter (OM) content, organic carbon (C) content, total nitrogen (N) content, carbon to nitrogen ratio (C/N), cation exchange capacity (CEC) and the soil pH.

4. Estimation of biological parameters of the soil samples

4.1. Enumeration of culturable bacteria

The culturable bacteria were enumerated on ten times diluted nutrient agar medium (NA/10) (Appendix-IC) supplemented with 100 mg L^{-1} of cycloheximide to inhibit the growth of fungi. Briefly, 10 g (equivalent dry weight) of soil were suspended in 90 ml of the distilled sterilised water, mixed well in waring blender and 10 times serially diluted. 100 µl of the dilutions 10^{-4}, 10^{-5} and 10^{-6} were inoculated on NA/10 medium plates (n=3 per sample) and incubated at 20°C for 15 days. Bacterial colonies were enumerated on each box after 10 and 20 days.

4.2. Estimation of microbial C biomass

The soil microbial C biomass was estimated using the fumigation/extraction technique as previously described (Vance et al., 1987). Extractable C was measured by automated UV-persulphate oxidation method in a Dohrman DC80 analyser (Wu et al., 1996) (Photo II-1). The microbial C biomass was determined using the formula:

Microbial C biomass = $(C_{\text{fumigated extract}} - C_{\text{unfumigated extract}})/K_c$.

The calculated K_c factor of 0.37 was used to convert extractable C into microbial C biomass (Nicolardot et al., 1984).

4.3. Estimation of IPU mineralization and sorption

4.3.1. IPU Mineralization kinetics

IPU mineralization was determined by radiorespirometery using ^{14}C ring-labelled isoproturon. For each soil sample, 40 g equivalent dry weight of soil was placed in a glass container. The humidity of the soil was adjusted to 80% of the water holding capacity of the soil keeping in view the quantity of the water to be needed for adding IPU. The soil samples were treated with 1.5 mg IPU kg^{-1} of soil and about 2 kBq of ^{14}C ring-labelled IPU in 0.8 ml of water. The soil samples were incubated in an airtight radiorespirometer jars containing:

Photo II-2. Packard (Tri-Corb 2100 Tr) liquid scintillation counter for the determination of radioactivity

Photo II-3. Biological oxidizer-500 for the measurement of non-extractable radioactivity

- a glass container containing the soil sample,
- a plastic bottle containing 5 ml of 0.2 M NaOH which serves as CO_2 trap,
- a plastic bottle containing 10 ml of water to maintain humidity within the respirometer jar.

The samples were incubated in the dark at 20°C. The NaOH traps were regularly changed over the incubation period and analysed for radioactivity content by liquid scintillation counting (Packard 1900TR-Tricarb) using 10 ml of scintillation fluid (ACSII, Amersham) (Photo II-2).

4.3.2. Extraction of pesticide residues

4.3.2.1. Estimation of extractable IPU residues

At the end of the IPU mineralization study (at the plateau of the IPU mineralization curve), the ^{14}C ring-labelled IPU remaining in the sample was extracted with methanol as followed: 20 g of soil (equivalent dry weight) was added with 40 mL of methanol (chemical grade) in a centrifugation tube (250 mL) and agitated at 150 rpm for 16 hours at 20 °C. The samples were then centrifuged for 10 minutes at 6000 rpm. The supernatant was recovered and a 2 mL aliquot was analysed for radioactivity content by liquid scintillation counting. Soil pellets were also recovered and air dried for further analysis of bound IPU residues.

4.3.2.2. Estimation of bound IPU residues (non-extractable)

Determination of the non-extractable IPU residues (corresponding to the bound residues) was performed from the dried pellet of soil obtained after methanol extraction. Air-dried soil pellets were crushed and sieved under a chemical hood. Quantification of the ^{14}C ring-labelled IPU bound residues was performed by combustion of 500 mg of soil samples under O_2 flow at 900 °C for 4 min using Biological Oxydiser OX-500 (EG&G Instruments, France) (Photo II-3). The ^{14}C-CO_2 was trapped in 15 mL of mixture (Oxysolve C – 400 scintillate) and the radioactivity was determined by liquid scintillation counting. The percentage of IPU bound residues was calculated and expressed in relation to the quantity of the initially added ^{14}C ring-labelled IPU.

4.4. Molecular analysis of the global structure of soil microbial community

4.4.1. Extraction of DNA directly from soil

The DNA was extracted directly from the soil samples according to the ISO standard ISO (CD11063) derived from Martin-Laurent et al. (2001). Briefly, 500 mg of glass beads of

Photo II-4. Mini-bead beater disrupter (Mikro-Dismembrator S, Sortorius)

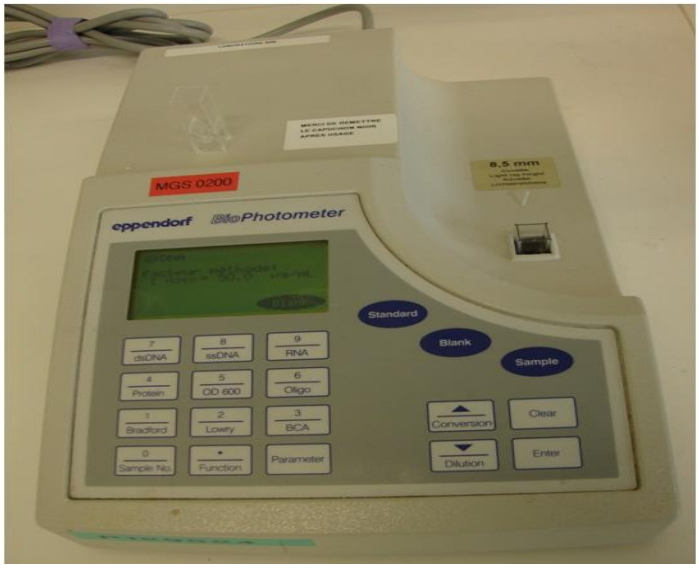


Photo II-5. Spectrophotometer (Biophotometer, Eppendorff) for the estimation of DNA amount

Photo II-6. PTC 200 gradient thermocycler (MJ Research)

106 µm (Sigma), 2 beads of 1 mm diameter (Sigma) and 1 ml of extraction buffer (Appendix-IIA) were added to the soil sample (250 mg) in a 2 ml screw tube. The samples were shaken at 1600 rpm for 30 seconds in a mini-bead beater cell disrupter (Mikro-Dismembrator S; Sartorius AG, Germany) (Photo II-4). The tubes were incubated at 70°C for 10 min, centrifuged at 14000 g for 1 min at 4°C and the supernatant was carefully recovered. The proteins were precipitated by adding 1/10th volume of 3 M sodium acetate (pH 5.5) and by incubation for 10 min on ice. After centrifugation at 14000 x g for 5 min (4°C), the supernatant was recovered and added with one volume of cold isopropanol. The samples were incubated at -20°C for 30 minutes and then centrifuged (13000 rpm) for 30 min at 4°C. The supernatant was discarded and the DNA pellet was washed with 70 % cold (-20°C) ethanol. Traces of ethanol were eliminated by incubation for 15 min at 37°C. Soil DNA was then resuspended in 50 µl of ultra pure water. Soil DNA was then purified by combining an affinity column for humic acid (PVPP) with an exclusion column (Sepharose 4B) (Appendix-IIB,C).

4.4.2. Quality control and quantification of soil DNA extract

Soil DNA extract was quantified at 260 nm by spectrophotometry using a biophotometer (Eppendorf, germany) (Photo II-5). Its quality was verified by calculating the A260/A280 ratio and by electrophoretic separation on 1 % agarose gel. Each DNA sample was diluted to obtain a concentration of 1 ng μl^{-1}. Quantification of the soil DNA was also performed on agarose gel by using the DNA standards as previously described (Ranjard et al., 2003)

4.4.3. Analysis of the global structure of the bacterial community (A-RISA)

The global structure of the bacterial community was determined by Automated Ribosomal Intergenic Spacer Analysis (A-RISA). The intergenic spacer found between the 16S and 23S rDNA of ribosomal operon was amplified with the primers 1522IRD800 (5'-TCG GGC TGG ATC ACC TCC TT-3') and 132R (5'-CCG GGT TTC CCC ATT CGG-3'). The reactional mix was prepared in 25 µl containing 2.5 µl 10X incubation buffer, 0.2 mM of each dNTP, 0.5 µM of each primer, 3.75U of Taq polymerase, 500 ng of T4 gen 32 and 5 ng of the extracted DNA. The PCR reaction was carried out in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Mass) (Photo II-6) with the following programme: 1 cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 30 sec at 55°C, 1 min at 72°C and one final cycle of 5 min at 72°C. The PCR was verified by migrating 3 µL of the amplicon on 2% agarose gel. For A-RISA fingerprinting, PCR products were loaded on a 3.7% solidified gel

Photo II-7. LiCor (IR2, Bioscience) DNA sequencer for A-RISA fingerprinting

matrix (KB Plus, Li-COR, Bioscience) and run under denaturing conditions for 15 h at 1500V/80W on a LiCor DNA sequencer (Science Tec) (Photo II-7). The data were analyzed using the 1D-Scan software (Science Tec) and data obtained was analyzed by Prep RISA software. The data obtained from Prep RISA were further analyzed by ADE4 software allowing a principal component analysis (PCA) of the RISA fingerprint which helps in studying the global structure of the bacterial communities. The coordinate of each sample on the first four axes of the PCA were recovered for further geostatistical analysis.

5. Isolation of IPU degrading consortium or pure strains

5.1. Enrichment cultures

Isolation of the IPU degrading bacterial consortium or strains was carried out by conducting the selective enrichment cultures starting from a composite soil sample as previously described by El Sebai et al. (2004). Briefly, 10 g of the soil (equivalent dry weight) was added to 90 ml of the mineral salt (MSIPU) medium containing IPU as a sole source of carbon and nitrogen, and incubated at 20°C with 150 rpm. Aliquots were regularly taken and IPU remaining in the medium was quantified by high performance liquid chromatography (HPLC) as described below. When about 50% of the initially added IPU was degraded, 10 ml of the soil slurry was transferred to 90 mL of a new MSIPU medium and incubated under the same conditions. 12 enrichment cycles were realized and 1 mL aliquot from each enrichment culture was preserved at -20°C for further molecular analysis based on DNA extraction. The complexity of the structure of the bacterial community during the enrichment procedure was estimated by performing amplified 16S rDNA restriction fragment analysis (ARDRA) as described below.

5.2. Isolation of IPU degrading bacterial isolates

After 12 enrichments, the suspensions were diluted in a series of 10^{-1} to 10^{-6} in sterile water. 100 µl of 10^{-2} to 10^{-4} dilutions were inoculated on different types of agar media plates: MSIPU (Appendix-1A), NA (Appendix-1C), LB (Appendix-1E) and MSIPU added with a defined mix of amino acids (Appendix-1A). The plates were incubated at 20°C and observed regularly. The isolated bacterial colonies were tested for IPU degradation by HPLC analysis after incubating in MSIPU liquid media. Aliquots were collected, added with equal volume of 50% glycerol solution, mixed thoroughly, frozen in liquid nitrogen and preserved at -80°C.

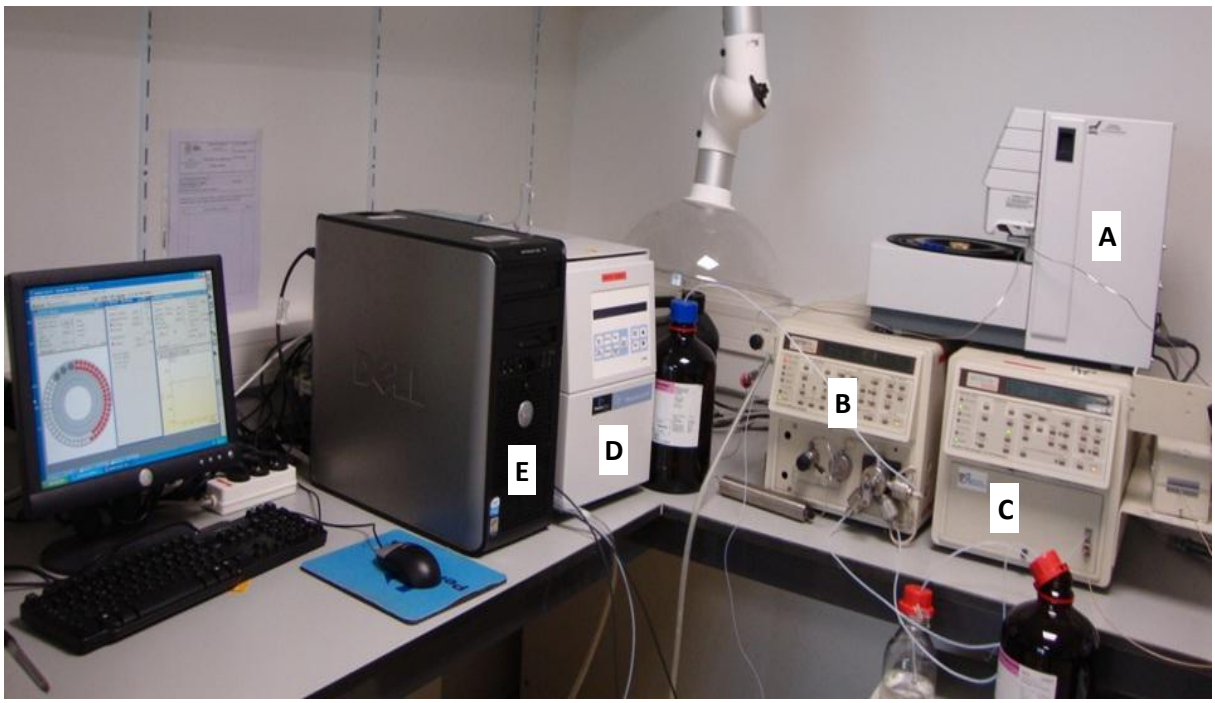


Photo II-8. System of HPLC analysis (Varian)

A: Autosampler

B: Pump for delivering the mobile phase

C: UV detector

D: Radioactivity detector

E: Processing computer

5.3. IPU quantification using HPLC analysis

IPU degradation in the bacterial culture was estimated by HPLC analysis by quantifying the remaining IPU. To do so, 800 µl aliquot taken regularly from the bacterial culture was centrifuged to obtain a clear supernatant. The supernatant (approximately 650 µl) was then analyzed on HPLC chain constituted of an automatic injector (Star-410), a Microsorb-MV C18 column (25 cm long with 4.6 mm internal diameter, Varian) and a UV detector (Wavelength 243 nm) (Photo II-8). The HPLC analysis was realized at room temperature under isocratic conditions with a mobile phase composed of acetonitrile and water (75/25, v/v) at 1.0 ml min^{-1} flow. The following formula was used to calculate the percentage of IPU degradation.

$$\% \text{ degradation} = (A_c - A_s)/A_c * 100$$

Where, A_c is the area of peak of the control and A_s is the area of peak of the sample.

6. Characterization of IPU degrading bacterial consortium or isolates

6.1. Physiological characterization

6.1.1. Estimation of degradation capabilities by HPLC

The capability of IPU degrading bacterial consortium and strain to degrade IPU as well as its metabolites (MDIPU, DDIPU, 4-IA), different phenylurea herbicides (i.e. diuron, linuron, monolinuron and chlorotoluron) and aniline derivative of different phenylurea herbicides (3-Chloroaniline, 4-Chloroaniline, 4-Bromoaniline, 3-Chloro-4-methylaniline, 3,4-Dichloroaniline) was estimated by HPLC. The bacterial cells were produced in MSIPU liquid medium, recovered by centrifugation (6000 rpm, 10 min at 4°C), washed twice with KNAPP buffer (KB) (Appendix-IB) and then resuspended in 10 ml of the KB. Cells (0.5 AU) were added with phenylurea herbicide or metabolite (50 mg L^{-1}) in KB. The samples were incubated at 28°C under agitation (125 rpm). Aliquots were regularly taken and analyzed by HPLC as described above.

6.1.2. IPU mineralization kinetics

IPU mineralization kinetics of IPU degrading bacterial consortium and pure strains were determined by radiorespirometery using ^{14}C ring-labelled IPU. Briefly, 10 ml of the culture (OD=1.0) was added with 50 mg L^{-1} of IPU and 1 kBq ^{14}C ring-labelled IPU. Microbial cultures (n = 3) were incubated at 28°C under agitation (125 rpm) in close respirometer jars. $^{14}CO_2$ evolved from ^{14}C ring-labelled IPU was trapped in 5 ml of 0.2 M

NaOH. NaOH traps were regularly changed over the incubation period. The $^{14}CO_2$ in the NaOH traps was estimated by liquid scintillation counter (Packard 1900TR-Tricarb) using 10 ml of scintillation fluid (ACSII, Amersham). The radioactivity remaining in the culture media as well as the one incorporated in the bacterial biomass was estimated. Briefly, cell suspension was centrifuged at 6000 rpm for 5 min and radioactivity was estimated from 2 mL aliquot of the supernatant as well as the whole of the cell pellet in the same way as above.

6.1.3. Estimation of effect of pH on IPU degradation kinetics

The effect of pH on IPU degrading capability of the bacterial consortium/strain was estimated at 5.5, 6.5, 7.5 or 8.5 pH values. Bacterial cells grown in liquid MSIPU were recovered by centrifugation. Bacterial pellet was washed twice with KB. Cells were then resuspended (0.5 optical density) in KB added either with herbicides or metabolites. They were incubated at 28°C under agitation (125 rpm) and samples were regularly taken for HPLC analysis. At the end of the incubation period, cells were enumerated on NA plates. Briefly, ten-fold dilution from 10^{-4} to 10^{-6} were plated on NA medium. The plates were incubated at 28°C and the colony forming unites (cfu) were enumerated after 10 days of incubation.

6.2. Molecular characterization based on DNA study

6.2.1. Extraction of genomic DNA

To perform genomic DNA extraction, the bacterial consortium or the pure strain cells were grown in liquid MSIPU medium for one week at 28°C under agitation (150 rpm). Genomic DNA was extracted from 100 ml of cell suspension (OD 1.0) using the QIAGEN Blood and Cell culture DNA Midi Kit according to the manufacturer's instructions (QIAGEN, France). Before starting the extraction, 7 µl of RNase A solution (100 mg ml^{-1}) were added to 3.5 ml of the aliquot of buffer B1 (50 mM Tris.Cl, pH 8.0; 50 mM EDTA). 75 ml of the bacterial culture was centrifuged at 6000 rpm for 10 min and the supernatant was discarded thoroughly. The bacterial pellet was resuspended in 3.5 ml of the buffer B1 (with RNase A) by vortexing at top speed. 80 µl of the lysozyme stock solution (100mg ml^{-1}) and 100 µl of proteinase K stock solution (20 mg ml^{-1}) were added to the suspension, mixed and incubated at 37°C for 45 min. Then 1.2 ml of buffer B2 (3 M guanidine HCl; 20% Tween-20) were added, mixed by inverting the tube several times and incubated at 50°C for 30 min. QIAGEN Genomic-tip 100/G was equilibrated with 4 ml of QBT buffer and removed by gravity flow. The sample was vortexed for 10 sec. at maximum speed and applied to the equilibrated QIAGEN Genomic-tip where it enters the resin by gravity flow. QIAGEN Genomic-tip was

washed 2 times with 7.5 ml of the wash buffer QC. The genomic DNA was eluted with 5 ml of the elution buffer QF. DNA was then precipitated by adding 3.5 ml of room-temperature isopropanol and inverting the tube 10-20 times. The precipitated DNA was spooled by using a glass rod. The spooled DNA was transferred to a microcentrifuge tube containing 700 µl of the EB buffer. DNA concentration was measured by spectrophotometery (A260/280).

6.2.2. ARDRA, cloning and sequencing of 16S rDNA

6.2.2.1. 16S rDNA amplification

16S rDNA sequence was amplified from the bacterial DNA template with universal primers 27f (5'-AGA GTT TGA TCH TGG CTC AG-3') and 1492r (5'-TAC GGH TAC CTT GTT ACG ACT T-3') (Gürtler and Stanisich, 1996). The amplification reaction was carried out in 25 µl volume containing 2.5 µl of 10X Taq polymerase buffer, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 µM of each primer and 0.625 U of Taq DNA polymerase. Bacterial genomic DNA (10 ng μL^{-1}) was used as template. PCR reaction was realized in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Mass) using the following programme: 1 cycle of 4 min at 94°C; 39 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and one final cycle of 15 min at 72°C. The quality of the 16S rDNA amplicon was verified by loading 5 µl PCR product along with 1kb size marker (Invitrogen, France) on 1 % agarose gel. After the electrophoresis conducted at 100 volts for 30 minutes in 1X TBE buffer the gel was stained with ethidium bromide (200 µg/l), revealed under UV and captured using image capture devices.

6.2.2.2. Purification of 16S rDNA

16S rDNA PCR products were purified using the MinElute PCR Purification Kit according to the manufacturer's recommendations (QIAGEN, France). 5 volumes of PBI buffer were added to 1 volume of the PCR product and mixed gently. The samples was applied to a MinElute column placed in a 2 ml collection tube. After centrifugation for 1 min at 10,000 × g, the flow was discarded and the MinElute column was washed with 750 µl of the PE buffer. After removal of washing buffer by centrifugation, the purified 16S rDNA amplicon was eluted with 20 µl of EB buffer (10 mM Tris-Cl, pH 8.5).

6.2.2.3. ARDRA Fingerprinting

For studying the structure of the bacterial community involved in IPU mineralization as well as distinguished isolates, 16S rDNA restriction polymorphism was estimated. The PCR product was digested with restriction enzyme *AluI* (Fermentas, France). Briefly, a 10 µl

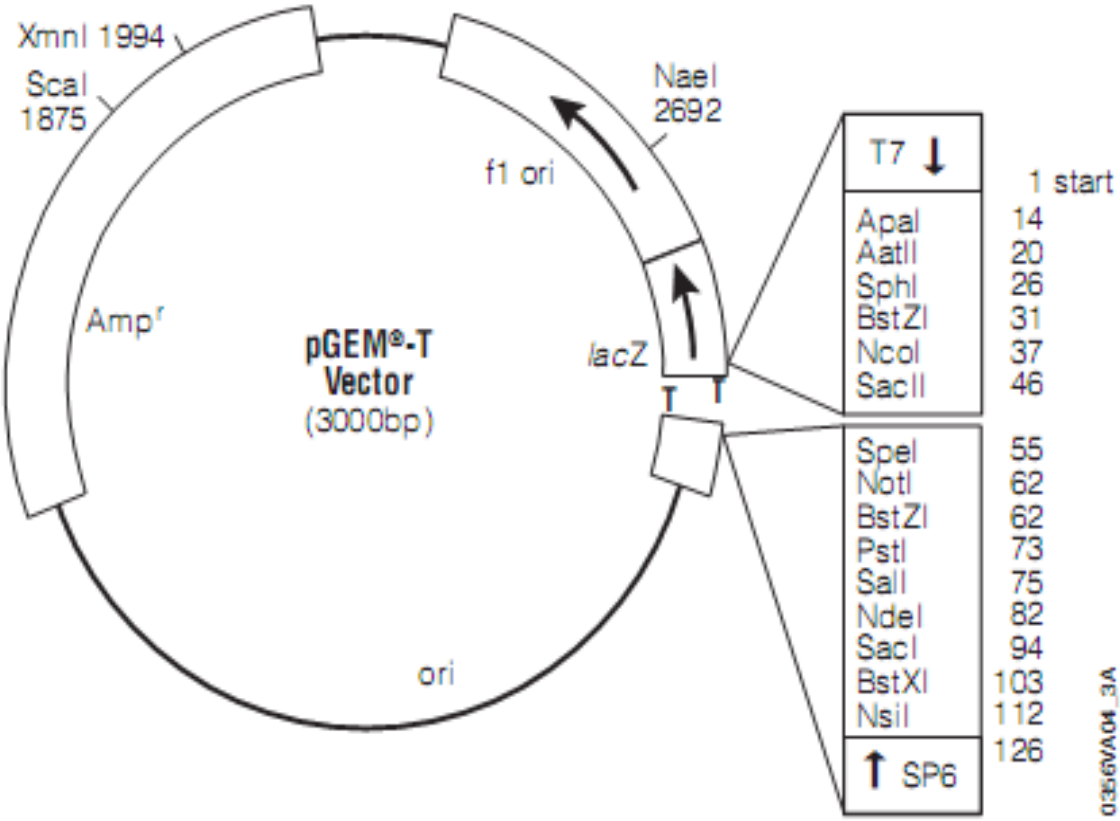


Fig. II-2. pGEM®-T vector map and sequence reference points
(http://www.promega.com/tbs/tm042/tm042.pdf)

of the reaction mix containing 1 µl of the 10X tango buffer, 10 U of the enzyme and 3 µl of the PCR product was prepared for each of the strain and incubated overnight at 37°C. The restriction fragments were separated on 3 % High Resolution agarose gel (MP Q-BIOgene, America) at 80 V for 2 hours in 1X TBE buffer. After staining with ethidium bromide, the gel was revealed under UV exposure. The size of the restriction fragments was estimated by comparison with DNA Molecular Weight Marker VIII (Roche Applied Science, France).

6.2.2.4. Establishment of 16S rDNA clone library

6.2.2.4.1. Ligation and transformation of 16S PCR products

Purified 16S rDNA amplicons were ligated to the pGEM-T easy vector (Promega, Madison, USA) (Fig. II-2). Briefly, 1 µl of the pGEM-T vector (50 ng/µl) was mixed with 5 µl of the incubation buffer (2X), 1 µl of the T4 ligase (3U/µl) and 3 µl of the 16S rDNA PCR product, and incubated overnight at 4°C. Competent cells *Echerichia coli* JM 109 (Promega, Madison, USA) were transformed with ligated plasmids through thermal shock according to the manufacturer's recommendations. The transformed cells were cultivated on the SOC medium for 1.5 hours at 150 rpm and then 100 µL aliquot was plated on LB medium plates containing ampicilline (20 µg ml^{-1}), X-Gal (40 µg ml-1) and IPTG (32 µg mL^{-1}). Recombinant clones characterized by a white phenotype were subcultured on LB ampicillin plates.

6.2.2.4.2. Screening and sequencing

The recombinant clones were screened by PCR using SP6 and T7 primers. The amplification reaction was carried out in a final 25 µl volume containing 2.5 µl of 10X Taq polymerase buffer, 0.2 mM of each dNTP, 1.5mM of $MgCl_2$, 0.5 µM of each primer and 0.625 U of Taq polymerase. 2.5 µL of the bacterial suspension of the recombinant cells (one clone suspended in 100 µL of MQ water) was used as template for PCR reaction. The PCR reaction was realized under the following conditions: 94 °C for 45 s, 35 cycles of 55 °C for 45 s, 72 °C for 90 s and a final extension step at 72 °C for 7 min. 16S rDNA amplicon was sequenced by Cogenics (Meylan, France).

6.2.2.4.3. Analysis of the sequences

The nucleotide sequences were compared to known sequences already available in the database GenBank (http://www.ncbi.nlm.nih.gov/BLAST) using the programme BlastN. Multiple alignments of the sequences were carried out using the software ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/). The alignment file (.aln and .phb) obtained from

ClustalX was used to construct a phylogenetic tree with the neighbour joining method using the software NJ Plot (http://pbil.univ-lyon1.fr/software/njplot.html).

6.2.3. Cloning and sequencing of *catA* gene

6.2.3.1. Amplification and purification of catA gene

catA gene coding for catechol degrading 1,2-dioxygenase was amplified by using catAf (5'-CCATTGAAGGGCCGCTCTATGT-3') and catAr (5'-ACCGAARTTGATCTGCGT(G,C)GTCA-3') primers (Sun et al., 2009). The amplification reaction was carried out in a final 25 µl volume containing 2.5 µl of 10X Taq polymerase buffer, 0.2 mM of each dNTP, 1.5mM of $MgCl_2$, 0.5 µM of each primer, 0.625 U of Taq polymerase and about 2.5 µl of the genomic DNA (10 ng μL^{-1}) in sterile water. PCR was carried out as follows: 30 cycles of 1 min of denaturation at 95°C, 1 min for annealing at 55°C and 0.5 min of extension at 72°C, with a final cycle of 5 min at 72°C after 30 previous cycles were complete. The quality of the *catA* amplicon was verified by electrophoresis on 1 % agarose gel run at 100 volts for 30 minutes. The gel was stained with ethidium bromide (200 µg L^{-1}) and then revealed under UV. *cat*A amplicons were purified using the MinElute PCR Purification Kit according to manufacturer's recommendations (QIAGEN, France). Briefly, 5 volumes of buffer PBI were added to 1 volume of the PCR product and mixed gently. A MinElute column was placed in a provided 2 ml collection tube and the sample was applied and then centrifuged for 1 min at 10,000 × g. The flow was discarded and MinElute column was again placed in the same tube. The DNA bound in the MinElute column was washed with 750 µl of the PE buffer and then purified *cat*A amplicons were eluted with 20 µL of EB buffer (10mM Tris-Cl, pH 8.5). Purified *cat*A amplicons were stored at -20°C until used.

6.2.3.2. Cloning and sequencing of catA amplicons

The *catA* amplicon was ligated into pGEM-T vector (Promega, Madison, USA) and transformed into *Echerichia coli* JM 109 (Promega, Madison, USA) using the procedure described above for 16S rDNA amplicon (for more details, please see 6.2.2.4.1.). *catA* recombinant clones were screened by PCR using SP6-T7 primers, sequenced and sequence analyzed phylogenetically using the procedure described for 16S rDNA.

6.2.4. Plasmid profile

The plasmid profile of the isolated strains was obtained by using the method of Eckhardt (Eckhardt, 1978) modified by Wheatcroft et al. (1990). Strain was grown in MSIPU

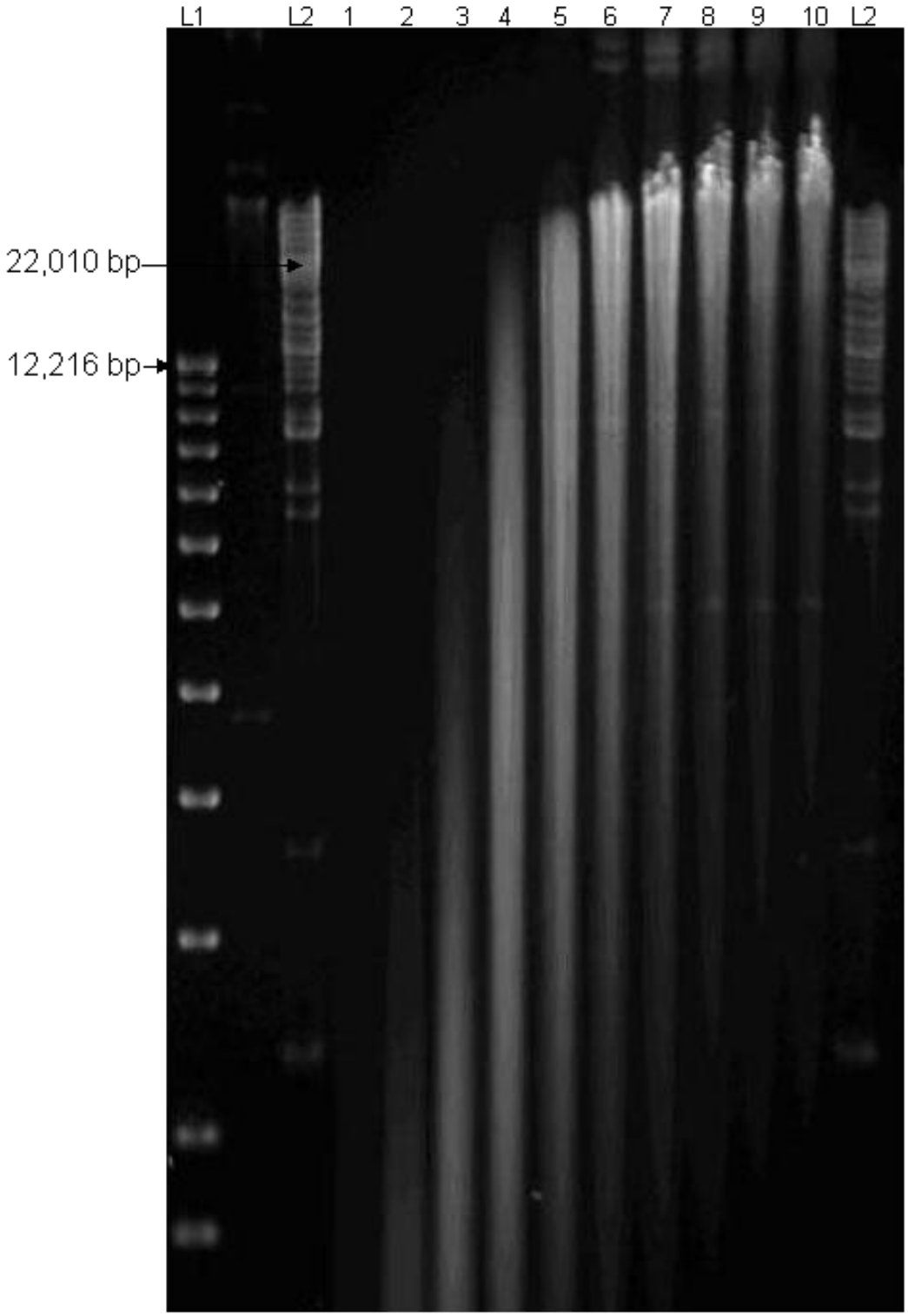


Fig. II-3. Electrophoretic separation of partially digested genomic DNA using different concentrations of *Sau3*A1 (lanes 1-10). L1 and L2 are the ladders. 1-10 represent the different concentrations of *Sau3*A1. (1=2U, 2=1U, 3=0.5U, 4=0.25U, 5=0.125U, 6=0.0625U, 7=0.03125U, 8=0.016U, 9=0.0078U, 10=0.0039U).

liquid medium and two strains (*A. tumefaciens* C58 and *R. meliloti* 41) harbouring plasmids of known size were grown in TY medium. 0.75% agarose gel was prepared in TBE buffer. For each bacterial culture, an equivalent of 1 mL at 0.2 / OD600 was harvested of all the strains by centrifugation for 5 min at 15000 rpm. The pellets were suspended in 0.5 mL of the ice cold milli-Q water. The suspensions were layered onto 1 mL of 0.3% of cold N-lauroylsarcosinate and centrifuged for 3 min at 15000 rpm. The bacterial pellets were then resuspended in 40 µl of Ficoll (20% in TE). 25 µl of 10% SDS (coloured wih xylene cyanol) was loaded into each well of the gel and was run backwards for 15 min at 100V. Then 10 µl of the lytic agent (1 ml containing 100 µl of 20 mg mL^{-1} lysozyme) was added into each suspension and 25 µl was immediately loaded into the wells. The electrophoresis was run forward at 40V for 30 min and then at 100V for 3 hours. After 3 hours, the gel was stained with ethidium bromide and revealed under UV illumination.

6.2.5. Establishment of BAC genomic library

6.2.5.1. Partial digestion of the genomic DNA

Preliminary experiment was performed to determine the amount of restriction enzyme to be used to get digestion compatible with BAC cloning. To do so, high quality genomic DNA (1 µg) was partially digested with different concentrations (like 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.016, 0.008 and 0.004) of *Sau3A1* restriction enzyme for 1.5 hours at 37°C. Digestion reaction was then stopped by adding 1 µL of 0.5 M EDTA and 10 µL of gel loading buffer. 20µL of digestion reaction was run on 0.6% agarose gel with 40 volts for 16 hours. The gel was stained with ethidium bromide and revealed under UV (Fig. II-3). The concentration of 0.125 U μL^{-1} of *Sau3A1* was chosen to get DNA fragments ranging from 20-30 kb. This concentration was applied to digest 10 µg of genomic DNA for BAC cloning.

6.2.5.2. Size selection of the partially digested genomic DNA

Partially digested genomic DNA was run on 0.6% agarose gel and DNA of selected size was extracted from the gel and placed in a 0.8% low melting point agarose gel and run for 16 h at 40 volts. DNA fragments were cut from the gel without exposing to UV (Fig. II-4) and recovered by using GELase™ Agarose Gel-Digesting Preparation protocol (Epicentre, France). Briefly, three volumes of 1X GELase buffer were added to the gel (weight/volume) and incubated at room temperature for 1 hour. GElase buffer was removed, agarose gel was melted at 70°C for about 15 minutes. Molten gel was immediately transferred to 45°C, added with GELase enzyme and incubated at 45°C for 1 hour. DNA concentration was evaluated by

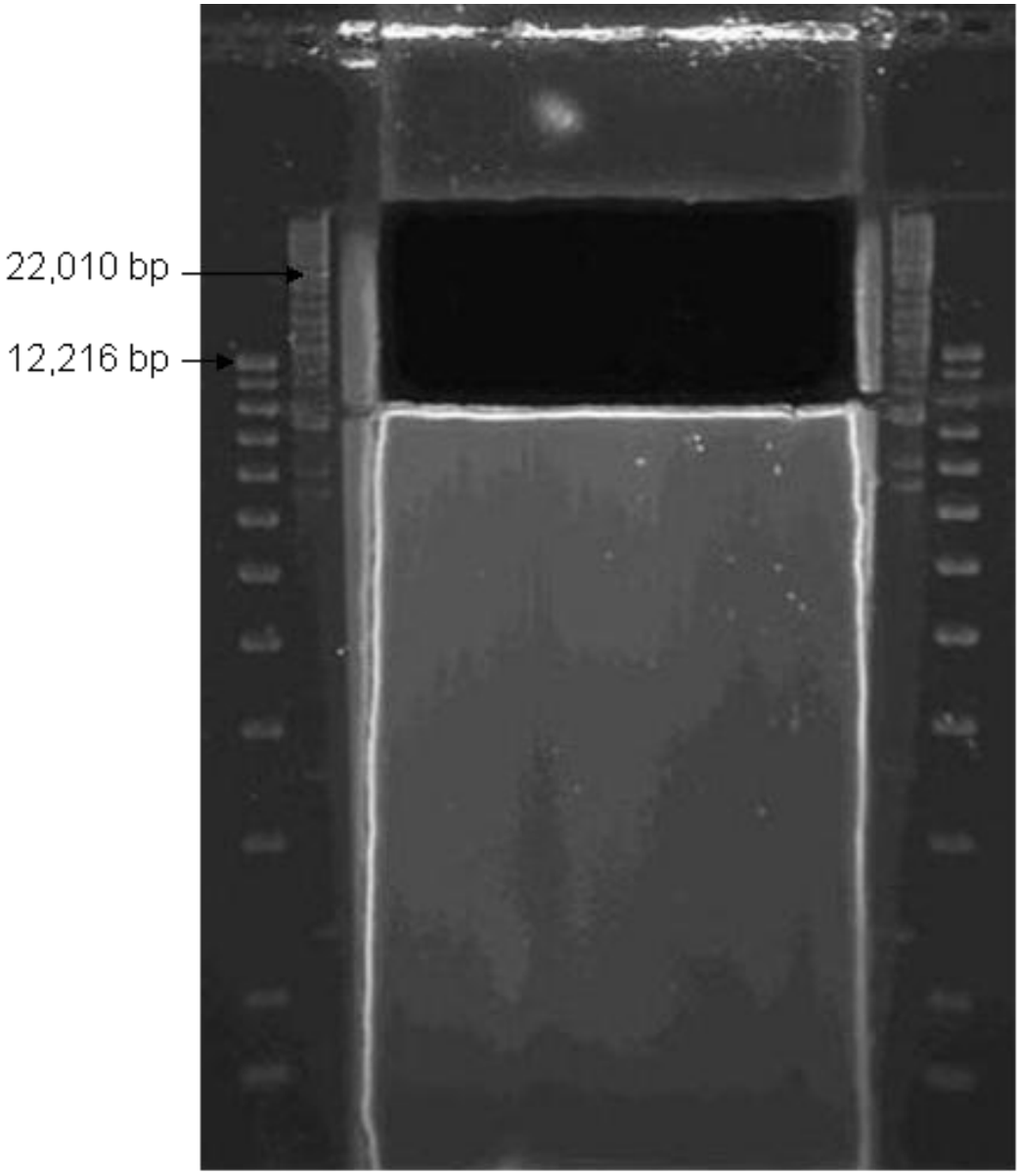


Fig. II-4. Cutting of the gel for size selection of the digested genomic DNA.

spectrophotometry at 260 nm. The size of recovered DNA fragments was determined by electrophoresis performed on 0.6% agarose gel run for 8 hours and revealing under UV after ethidium bromide deigestion. The purified partially digested genomic DNA was preserved at -20°C until used.

6.2.5.3. Ligation and transformation of size selected genomic DNA

Purified DNA was ligated into CopyControl pCC1BAC™ Vector (EPICENTRE, France) (Fig. II-5). For ligation, 100 ng of size selected DNA, 1 µL of CopyControl pCC1BAC Cloning-Ready Vector, 10 µL of 10X Fast-link ligation buffer, 1 µL of 100 mM ATP and 2 µL of Fast-Link DNA ligase were mixed in a total 100 µL volume and incubated at 16°C for 4 hours. The ligase was then heat inactivated by incubating the reaction mixture at 65°C for 15 minutes. The ligation reaction was desalted by dialysis on a 0.025 µm membrane (Millipore, Ireland).

The desalted ligated plasmid DNA was transformed into TransforMax EP1300 Electrocompetent *E. coli* (EPICENTRE, France) using the CopyControl™ BAC Cloning kit (EPICENTRE, France) according to the manufacturer's recommendations and grown overnight on LB containing chloramphenicol (12.5 mg L^{-1}), X-Gal (40 mg L^{-1}) and IPTG (0.4 mM). 3000 recombinant clones were picked and preserved at 4°C.

6.2.5.4. Screening of the BAC clone library

6.2.5.4.1. Functional screening

Functional screening of the recombinant clones was carried out based on their ability to degrade IPU and its known metabolites i.e. MDIPU, DDIPU and 4-IA. Briefly, the recombinant clones were grown overnight in LB medium containing chloramphenicol (12.5 mg L^{-1}), washed twice with KB buffer and inoculated in KB buffer containing 50 mg L^{-1} of either IPU, MDIPU, DDIPU or 4-IA. The degradation of the IPU and its metabolites was tested by HPLC analysis as described earlier.

6.2.5.4.2. Genomic screening and sequencing

PCR based screening of the BAC clone library was performed by amplifying the *cat*A gene from the recombinant BAC clones using catAf and catAr primers (Sun et al., 2009). To screen the BAC clone library, hyper pools were screened by PCR. Positive hyper pools were screened again by PCR to identify the positive pool and another step of PCR screening was performed to identify the BAC among the pool harboring *catA* amplicon. The DNA of the recombinant BAC clones harboring the *cat*A gene was extracted using the Plasmid midi and

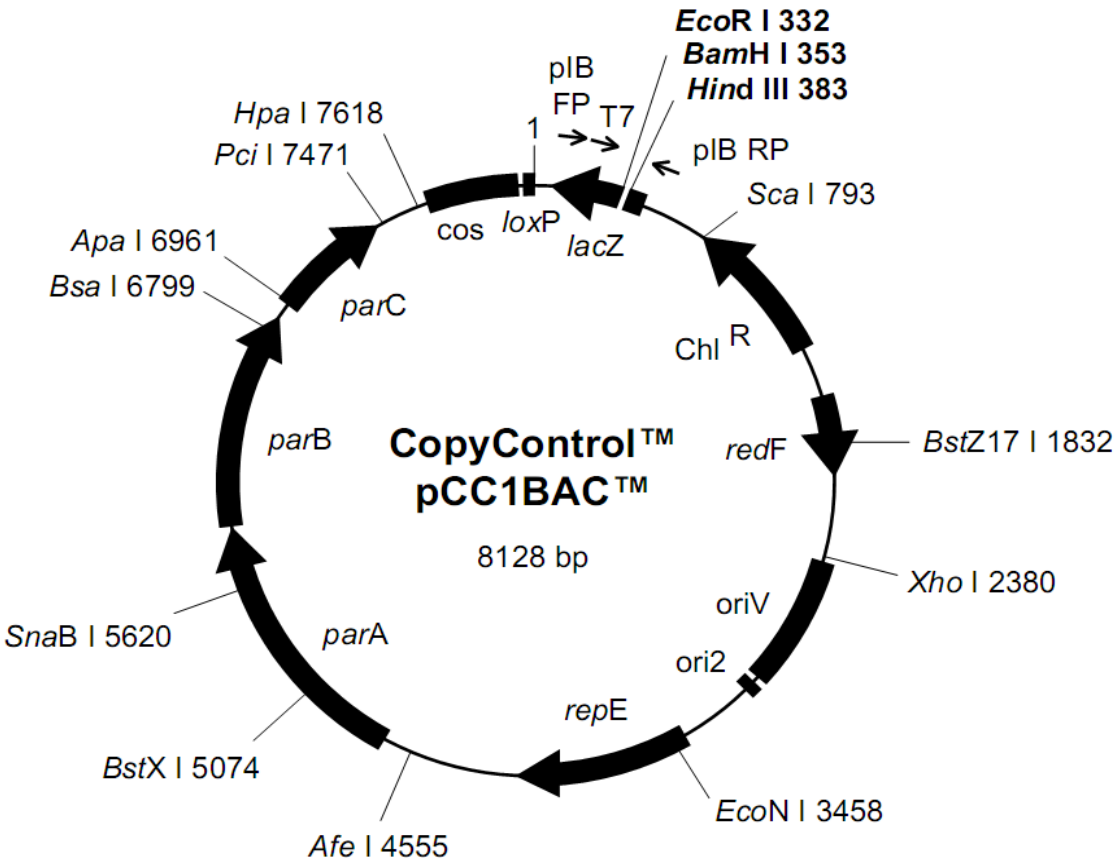


Fig. II-5. CopyControl™ pCC1BAC™ Vector (http://genomeprojectsolutions.com/pdfs/pcc1BAC.pdf).

maxi kits (QIAGEN, France) according to the providers' recommendations. The BAC clones harboring the *catA* sequence were fully sequenced by pyrosequencing using the Genome Sequencer FLX Titanium System™ (Beckman Coulter Genomic, USA). The annotation of the BAC sequence (33 kb in length) was performed using ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). For each ORF identified, blastn, blastx and blastp analysis were performed in order to identify the closest relatives. In addition, gene mapping was performed with SeqMan (Laser gene program). *cat*A sequence was compared to the known nucleotide sequences using Blastx (http://www.ncbi.nlm.nih.gov/BLAST). To construct a phylogenetic trees based on *cat*A sequences, multiple alignments were carried out using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) and the data obtained was processed using NJ Plot (http://pbil.univ-lyon1.fr/software/njplot.html) using the neighbor joining method. GC content of the DNA fragment was estimated by applet (http://www.sciencebuddies.org/mentoring/project_ideas/Genom_GC_Calculator.shtml).

7. Modelization and statistical analysis

7.1. Gompertz model

To determine the kinetics of IPU degradation, the degradation curves were fitted to a modified Gompertz model (Zwietering et al., 1990) using Sigma Plot© 4.0. The model equation was:

$y = A.\exp\{-\exp[1+\mu m.\exp(1).(\lambda-t)/A]\}$

where

y is the percentage of mineralization or degradation (%),

t is the time (h), μm is the maximum mineralization or degradation rate (% h^{-1}),

A is the maximum percentage of mineralization or degradation (%),

λ is the lag time (h).

The parameters were validated using a Student t test ($p<0.005$).

7.2. Exploratory statistical analysis

The data obtained were also analyzed by classical descriptive analysis by determining the maximum (max), minimum (min), mean, median, standard deviation and coefficient of variation.

7.3. Geostatistical analysis

Physico-chemical soil parameters and the kinetics of IPU mineralization were analyzed using geostatistical analysis. A semivariogram was calculated using the following equation (Isaaks and Srivastava, 1989)

$$\gamma(h) = 1/2N(h) \sum [z(xi+h) - z(xi)]^2$$

where g (h) is the experimental semivariogram at distance h, N (h) is the number of value pairs of samples in the distance h; z (xi) and z (xi + h) are the values of the sample at two points separated by distance h.

All pairs of points separated by distance h (lag h) were used to calculate the experimental semivariogram. A spherical model was fitted to semivariograms experiments. Model parameters were determined: the **nugget** semivariance, **range** and total semivariance (**sill**). The **nugget** semivariance is the spatial variability determined for a distance equal to zero between sampling points; **sill** is the distance (lag h) between the measures for which one value of a variable does not influence the neighboring values and the **range** is the distance at which the value of a variable becomes independent of others.

Two quality indices of spatial dependence were used to properly quantify the spatial structure. The first index is the **Q value** [(sill - nugget) / sill] which shows the spatial structure at the sampling scale (Görres et al., 1998). If it is equal to 0, no spatial structure is detected at points and the sampling scale. The higher the Q value approaches the value 1, more spatial variation over the sampling scale can be explained by the semivariogram model. The second index is the **range** which shows the limit of spatial dependence. Using these parameters, maps were calculated using the ordinary "kriging" to assess regional patterns of variation rather than local details. The geostatistical software R version 2.11.1 with the GeoR package version 1.6-27 for Windows (Gamma Design Software) was used for analysis.

SPATIAL VARIABILITY OF ISOPROTURON MINERALIZING ACTIVITY WITHIN A FRENCH AGRICULTURAL FIELD

Introduction

Isoproturon (IPU) is used for controlling the development of weeds in cereal cultures. Because of its intensive and repeated use, it has been reported to be detected in a number of surface and subsurface water samples in France (IFEN, 2003). It has been found to be harmful to living organisms including human beings. Keeping the potential harmful effects and environmental dispersion of isoproturon in view, the use of IPU in agriculture in France was restricted to 1200 g ha^{-1} in 2003. In order to investigate the fate of isoproturon after its application in agricultural fields, a number of studies have been carried out and biodegradation has been reported as a principal process for its dissipation in the soil environment. Further studies revealed that the biodegradation of isoproturon in agricultural fields was predominantly due to the adaptation of a fraction of the soil microflora which is able to use it as an energy source. IPU mineralization of has already been reported in agricultural fields located in France, Denmark and United Kingdom (Walker et al., 2001; Sorensen and Aamand, 2003; El-Sebai et al., 2005). However, it was shown that the microbial communities and the different biochemical process, including biodegradation, are not uniformly distributed in the soil. Recently, the analysis of spatial heterogeneity of the microbial communities and the biodegradation processes has appeared as a supplementary source of investigation of the natural ecosystems.

While studying the fate of isoproturon, a significant spatial heterogeneity in IPU degradation rates has been reported in different agricultural fields (Beck et al., 1996; Bending et al., 2003; El-Sebai et al., 2007). The heterogeneity in degradation rate of pesticides including isoproturon has been demonstrated to be controlled by a number of biological and physico-chemical components of the ecosystem including microbial biomass, organic matter and pH (Bending et al., 2003; El-Sebai et al., 2007), indicating that the determination of the spatial variations of the biotic and abiotic factors in the field might be helpful in further explanation of the variable microbial biodegradation process.

As biodegradation is a key determinant of the fate of pesticides including isoproturon in the soil environment, a comprehensive and precise evaluation of pesticides dissipation from

the agricultural soils is required to establish the practical methods to minimize their transfer to other components of the environment including water resources. This requires to study the microbial biodegradation not only in relation to the spatial physico-chemical characteristics of the soil but also in relation to the crop rotations and the pesticide application history.

The first part of my PhD thesis reports the investigation of IPU mineralization over a three year survey conducted on an agricultural field under winter wheat / barley / rape seed crop rotation. Isoproturon degradation was studied not only in relation to spatial variations but also temporal variations over a three year crop rotation periodically treated with isoproturon. 36 surface soil samples (0-15 cm) were taken from different points of the agricultural field in year 2008, 2009 and 2010. The activity of isoproturon degradation was determined by using the ^{14}C ring-labeled isoproturon and then characterizing the degradation parameters by modeling with modified Gompertz model. The size of the microbial communities over three years crop rotation was determined by the microbial C biomass and the genetic structure was estimated by automated ribosomal intergenic spacer analysis (A-RISA) carried out on soil DNA extracts. The physico-chemical parameters for each soil sample were determined by the laboratory of soil analysis (INRA, Arras). All the data obtained over three years was statistically analyzed by using the classical statistical determinants including minimum, maximum, mean, median, correlation and analysis of variance (ANOVA). The spatial relationship between the parameters of isoproturon mineralization and the physico-chemical and microbiological parameters of each soil sample over three year was determined with the help of geostatistics (GeoR).

Article under process:

A three year survey of in-field spatial variability of isoproturon mineralizing activity within a French agricultural soil under rape seed/winter wheat/barley rotation.

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Abstract

Mineralization activity of phenylurea herbicide isoproturon (IPU) was investigated through radiorespirometery using ^{14}C ring-labeled IPU over three year survey (2008, 2009 and 2010) in 36 samples collected from an agricultural field cropped with a winter wheat / rape seed / barley rotation periodically exposed to this herbicide. Higher IPU mineralization was observed in 2008 and 2010 as compared to that of in 2009. The assessment of spatial variability of isoproturon mineralization in relation to that of physico-chemical and biological parameters over the three year survey indicated the presence of higher variability in 2009 and 2010 as compared to that observed in 2008. Pearson's coefficient of correlation indicated for a weak relationship between the isoproturon mineralization and some physico-chemical properties like the soil pH, soil organic matter content, soil organic carbon content, cation exchange capacity (CEC) and C/N ratio. The map of isoproturon mineralization rates distribution, constructed on the basis of geostatistical data over the field, was showing important similarity to that of soil organic matter content, soil organic nitrogen content, cation exchange capacity (CEC) and C/N ratio. Geostatistics revealed that the important variability in isoproturon mineralization activity was lowered following the soil treatment with this herbicide.

Key words: IPU, mineralization, spatial variability, geostatistical analysis, crop rotation

1. Introduction

The general use of pesticides in conventional agriculture allowed safe crop production with good quality and economic benefit. However, despite their obvious interest for agricultural production, the use of pesticides has attracted much attention due to their potential harmful effects not only on the environment but also on human beings. The environmental fate of pesticides is a great determiner of the ecotoxicological impact of the pesticides and their residues, and the better knowledge of the processes governing their fate may provide an innovative solution to improve their natural attenuation for promoting the quality of the natural soil and water resources. Microbial degradation is one of the main mechanisms leading to the dissipation of pesticides in the soil environment. This key process is governed by a number of soil and environmental factors which interact together in a complex manner causing important variations in its expression at geographical level. One of the important environmental questions remaining regarding the pesticide microbial biodegradation is the extent to which this phenomenon is taking place at field scale. This is an important issue to predict pesticide fate in the soil environment. In order to address this question, we carried out a three years survey aiming at determining variation in pesticide degradation activity taking in account the spatial variability.

To conduct this study, we have chosen the herbicide isoproturon (IPU) as a pesticide model since it is one of the most extensively used pesticides in conventional agriculture in Europe (Stangroom et al., 1998; El-Sebai et al., 2007) for pre- and post-emergence control of many annual grasses and broad leaved weeds in spring and winter wheat, barley and winter rye etc (Ertli et al., 2004; El-Sebai et al., 2007). Although, IPU is moderately recalcitrant in the soil (Hussain et al., 2009b) showing DT50 ranging from 6 to 90 days (El-Sebai et al., 2007), it is frequently detected in water resources exceeding 0.1 μgL^{-1}, the European Union drinking water limit (Spliid and Koppen, 1998; Muller et al., 2002). Ecotoxicological data suggest that IPU and some of its metabolites are not only harmful for microbial communities (Widenfalk et al., 2008), aquatic invertebrates (Mansour et al., 1999) and fresh water algeae (Vallotton et al., 2009) but also carcinogenic for human being and animals (Behera and Bhunya, 1990; Hoshiya et al., 1993).

Microbial biodegradation has been reported as a primary mechanism for the dissipation of IPU in the aquatic and telluric environments (Fournier et al., 1975; Gaillardon and Sabar, 1994; Cox et al., 1996; Pieuchot et al., 1996). The activity of the pesticide degrading microbial communities greatly influences the fate of pesticides in the environment

(Aislabie and Lloyd-Jones, 1995). In response to repeated exposure to pesticides, the activity of the pesticide degrading microbial community is increased as a result of the adaptation process allowing the development of the pesticide degrading populations responsible for enhanced biodegradation of IPU (Cox et al., 1996; Karpouzas et al., 1999; Sorensen and Aamnad 2001; Bending et al., 2003; El-Sebai et al., 2005; Bending et al., 2006). Enhanced biodegradation of isoproturon has already been reported in British (Cullington and Walker, 1999), Danish (Sorensen and Aamand, 2003) and, more recently, in French (El-Sebai et al., 2005) agricultural fields yearly treated with this herbicide.

Although several soils are adapted to IPU mineralization, the activity of IPU degrading community is not evenly distributed across agricultural fields (Beck et al., 1996; Bending et al., 2001; Walker et al., 2001; Walker et al., 2002; El-Sebai et al., 2007). Indeed, Beck et al. (1996) showed that IPU half life varied from 31 to 483 days in 25 soil samples collected in the same agricultural field. This initial report was confirmed by the studies reporting in-field spatial heterogeneity of IPU degradation in the different regions of the world including Denmark, United Kingdom and France (Bending et al., 2001; Walker et al., 2001; Bending et al., 2003; Bending et al., 2006; Rodriguez-Cruz et al., 2006; El-Sebai et al., 2007). Although this phenomenon has been described, to our knowledge, there is only one publication reporting in-field spatial variability of IPU degradation activity (El-Sebai et al., 2007) and no survey has yet been conducted to estimate the variability of this parameter during a three years crop rotation.

The spatial heterogeneity in the degradation of pesticides, including IPU, has been associated with the variations in the pedoclimatic factors including intrinsic parameters such as soil pH, texture and organic matter content, and extrinsic parameters such as soil temperature, moisture and agricultural practices etc (Bending et al., 2003; El-Sebai et al., 2005; El-Sebai et al., 2007; Vieublé-Gonod et al., 2009). These parameters are known to have pronounced effects on the microbial activity and processes (Christensen et al., 1990; Drury et al., 1991; Smith et al., 1994; Bending et al., 2003). Indeed, the physico-chemical properties of the soil have been reported not only to affect the availability and biodegradability of the pesticides (Walker et al., 1992; Welp and Brummer, 1999) but also the size, diversity and activity of the pesticide degrading microbial populations (Smith et al., 1997; Hundt et al., 1998). Interestingly, spatial variability in IPU degradation within a British agricultural field at Deep Slade was correlated with the soil pH and the microbial biomass (Walker et al., 2001). Similarly, pH was shown to be responsible for the spatial variability of IPU mineralization

activity determined by using a geostatistical approach on 50 soil samples collected within a French agricultural field adapted to IPU mineralization (El-Sebai et al., 2005, El-Sebai et al., 2007). pH was shown to affect IPU degradation not only in soil (Walker et al., 2002; El-Sebai et al., 2005, El-Sebai et al., 2007) but also in pure cultures (Bending et al., 2003, Hussain et al., 2009; Sun et al., 2009). Similarly, the spatial heterogeneity of IPU mineralizing activity within an agricultural field was found to be stimulated by the addition of fresh organic matter such as compost (Vieublé-Gonod et al., 2009). Therefore, IPU mineralization activity is depending on a complex network of interacting biotic and abiotic parameters thereby influencing the pesticide fate in the soil. Although several studies have been conducted to describe the variability of IPU degrading activity, not much is known about in-field spatial level and nothing is known about the in-time variation of IPU mineralizing activity. A detailed knowledge of the evolution of the spatial variability of IPU mineralization activity is an important parameter to be addressed in order to better understand the processes responsible for IPU natural attenuation in soil.

In this context, the present study was carried out to characterize the spatial variability of IPU mineralization within an agricultural field, periodically exposed to IPU over three years period under rape seed / winter wheat / barley crop rotation, in relation to the variability of the physico-chemical and microbiological properties.

2. Materials and methods

2.1. Soil

Soil was collected from an agricultural field located at Epoisses experimental farm of the National Institute of Agronomical Research (INRA) (Breteniere, France). In April 2008, 2009 and 2010, 36 surface soil samples (approximately 500 g) were taken from 0-20 cm top layer across the field plot (9 rows and 4 columns) as described in the fig. II-1. This field has been cropped under wheat / rape seed / barley rotation for ten years and was periodically treated with IPU over this period. During the three years period investigated (2008-2010), this field was treated once with herbicide IPU before the sampling of 2008 and once with mesosulfuron methyl before 2010. The moisture content of each soil sample was estimated before beginning the experiment. The soil samples were sieved at 5 mm and subdivided into two sub-samples. One set of the sub-samples was air dried for physicochemical analysis, whereas, the other was stored at 4°C for microbiological analysis. Soil aliquots were also stored at -20°C for the soil DNA extraction.

Table III-1. Descriptive and geostatistical analysis of the physico-chemical parameters [equivalent humidity (EH), organic matter content (OM), organic carbon content (OC), nitrogen (N), C/N ratio, cation exchange capacity (CEC), pH] and biological parameters [microbial C biomass (MCB), number of cultivable bacteria (cfu), maximum percentage of IPU mineralization (A), maximum rate of mineralization (μm), lag phase (λ), abscissa of inflexion point (ti)] determined from 36 soil samples collected from the field of Epoisses.

Parameter	Year	Min	Max	Mean	Med	SD	CV (%)	P	Sill	Nugget	Range	Q	SC
	2008	22.9	28.5	25.2 c	25.0	1.6	6.1	0.7358	2.4	0	24.9	1	S
EH	**2009**	26.8	39.6	30.3 a	30.3	2.2	7.1	0.5128	3.1	1.2	68.7	0.6	M
	2010	25.3	30.7	28.6 b	28.8	1.4	4.9	0.9999	1.5	0	89.5	1	S
	2008	26.4	38.8	32.4 b	32.8	3.3	10.0	0.9999	10.3	0	31.9	1	S
OM	**2009**	30.3	53.7	37.5 a	36.5	4.8	12.8	0.9987	27.0	0	117.7	1	S
	2010	29.9	55.1	37.2 a	36.0	5.7	15.2	0.7358	26.9	0	156	1	S
	2008	15.3	22.4	18.8 b	18.9	1.8	9.8	0.9999	3.2	0	34.0	1	S
OC	**2009**	17.5	31.0	21.7 a	21.1	2.8	12.8	0.9987	8.9	0	117.6	1	S
	2010	17.3	31.9	21.5 a	20.8	3.3	15.3	0.7358	9.1	0	155.3	1	S
	2008	1.5	1.9	1.7 c	1.7	0.10	6.0	0.9999	0.003	0.0075	106.4	nd	N
N	**2009**	1.7	2.2	2.0 a	2.0	0.13	6.5	0.9999	0.013	0.002	48.9	0.8	M
	2010	1.6	2.0	1.8 b	1.8	0.11	6.0	0.9999	0.013	0	159.1	1	S
	2008	8.9	13.2	10.7 b	10.7	0.8	7.8	0.9999	0.64	0	36.8	1	S
C/N	**2009**	9.2	16.9	11.1 b	10.7	1.5	13.7	0.3380	1.95	0	50.8	1	S
	2010	10.3	18.0	12.0 a	11.5	1.9	15.5	0.5128	2.99	0	166	1	S
	2008	21.0	22.0	21.3 a	21.0	0.5	2.4	0.3380	1.5	0.74	40	0.5	M
CEC	**2009**	16.8	27.5	22.7 a	22.6	2.8	12.5	0.9999	6.9	0	279.2	1	S
	2010	15.9	27.4	22.5 a	22.5	2.9	12.8	0.9999	8.7	0	321.8	1	S
	2008	7.7	7.8	7.7 a	7.7	0.04	0.5	0.9987	0.015	0.011	113	nd	N
pH	**2009**	7.3	8.0	7.7 a	7.7	0.19	2.5	0.9999	0.029	0	108.5	1	S
	2010	7.1	8.1	7.8 a	7.9	0.27	3.4	0.9987	0.061	0	86.7	1	S
	2008	209.7	402.7	314.8 a	318.2	41.9	13.3	0.9999	1338	498.3	88	0.6	M
MCB	**2009**	91.2	467.1	311.3 a	321.5	80.2	25.8	0.9987	6249	0	36.2	1	S
	2010	199.8	413.2	295.7 a	293.3	51.2	17.3	0.9999	2465	0	59.8	1	S
	2008	7.7	8.2	8.1 a	8.1	0.10	1.3	0.9987	0.0006	0.0097	16.2	nd	N
cfu	**2009**	7.6	8.2	7.9 c	7.9	0.12	1.5	0.9999	0.0127	0	30.2	1	S
	2010	7.7	8.1	8.0 b	8.0	0.08	1.1	0.9999	0.0069	0	20.6	1	S
	2008	37.2	47.9	42.6 a	42.1	2.5	5.8	0.9987	2.4	3.0	38.9	nd	N
A	**2009**	11.0	37.5	27.1 b	29.5	7.1	26.2	0.9987	47.3	0	36.3	1	S
	2010	4.5	53.1	45.3 a	48.5	10.1	22.2	0.0694	99.7	0	38.8	1	S
	2008	2.6	5.0	4.0 a	4.0	0.6	15.6	0.9987	0.085	0.309	115.2	nd	N
μm	**2009**	0.2	1.8	0.7 b	0.7	0.4	53.7	0.9999	0.119	0.013	60.6	0.9	S
	2010	0.1	7.3	3.9 a	4.2	1.8	45.4	0.9999	2.363	1.302	186.2	0.5	M
	2008	-0.3	2.7	1.1 c	1.1	0.6	52.4	0.9900	0.15	0.13	97.4	nd	N
λ	**2009**	0.3	7.5	4.6 a	4.7	1.4	29.9	0.9999	1.70	0	30.9	1	S
	2010	-2.1	6.3	2.7 b	2.2	1.6	60.2	0.5128	1.10	1.54	79.3	nd	N
	2008	3.9	8.4	5.1 c	4.9	1.1	20.5	0.9987	0.40	0.65	78.8	nd	N
ti	**2009**	9.7	29.3	20.7 a	20.7	5.8	27.8	0.9999	29.99	2.07	54.8	0.9	S
	2010	3.9	19.2	8.2 b	6.5	4.0	48.2	0.5128	14.32	0	38.3	1	S

Min, Minimum; Max, maximum; Med, median; SD, standard deviation; CV, coefficient of variation; Q, Q-value; SC, spatial class; nd, not determined; a,b,c, Mean value differing in letter significantly differ (ANOVA, Fisher test, $p < 0.05$); p, P value of Kolmogorov-Smirnov (K-S) test

2.2. Soil physico-chemical properties

The soil physico-chemical properties including granulometric properties, equivalent humidity, organic matter content, carbon, nitrogen, C/N ratio, pH and cation exchange capacity were determined by the Laboratory of Soil Analysis (INRA, Arras, France) using the ISO procedures. The soil physico-chemical properties are shown in table III-1.

2.3. Enumeration of culturable bacteria

Culturable soil bacteria were enumerated on ten-fold diluted nutrient agar medium (DIFCO, France) containing 100 mg L^{-1} of cycloheximide (Alexander, 1965). Briefly, 10 g of each soil sample (equivalent dry weight) was suspended in 90 ml of sterilized water using a waring blender (New Hartford, USA). Resulting soil suspensions were serially ten-fold diluted in sterile water and 100 µl of 10^{-4} to 10^{-6} dilutions were plated on NA media plates in triplicate. The total heterotrophic bacteria were counted after 3 weeks of incubation at 20°C.

2.4. Microbial C biomass of the soil samples

Microbial C biomass was estimated using the fumigation-extraction method as previously described (Vance et al., 1987). Extractable C was measured by an automated UV-persulphate oxidation method in Dohrman DC80 analyser (Wu et al., 1996) and microbial C biomass was estimated using the formula:

Microbial C biomass = $(C_{\text{fumigated extract}} - C_{\text{unfumigated extract}})/K_c$

The calculated K_c value of 0.38 was used to convert extractable C into microbial biomass (Nicolhardot et al., 1984).

2.5. Soil DNA extraction

DNA was directly extracted from a 250 mg aliquot of each soil sample according to the method as previously described (Martin-Laurent et al., 2001). Briefly, samples were homogenized in 1 mL of extraction buffer [100 mM EDTA, 100 mM NaCl, 100 mM Tris (pH 8.0), 2% (w/v) sodium dodecyl sulphate, 1% (w/v) polyvinylpyrrolidone] for 30 s at 1600 rpm in a mini-bead beater cell disrupter (Mikro-Dismembrator S; Sartorius AG, Germany). Soil was removed by centrifugation at 14000 g. After sodium acetate precipitation, proteins were eliminated by centrifugation. DNA was precipitated with cold isopropanol, washed with 70% ethanol and purified by using Sepharose 4B and Polyvinylpyrrolidone (PVPP) spin columns. The quality and integrity of the soil DNA was checked by electrophoresis on 1% agarose gel. The DNA was quantified by spectrophotometry (Biophotometer, Eppendorf, germany).

2.6. Automated ribosomal intergenic spacer analysis (A-RISA) of the soil samples

The structure of the total bacterial community was determined by A-RISA. The 16S-23S intergenic spacer (IGS) of the bacterial rDNA was amplified with the 1µM 1522IRD800 (5'-TCG GGC TGG ATC ACC TCC TT-3') and 132R (5'-CCG GGT TTC CCC ATT CGG-3') primers using 3.75U of Taq polymerase, 500 ng of T4 gen 32 and 5 ng of the extracted DNA in a final reaction volume of 25 µL. The reactions were carried out in a thermocycler (PTC 200 gradient Cycler, MJ Research, Waltham, Mass) with the following programme: 1 cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 30 sec at 55°C, 1 min at 72°C and an additional 5 min cycle at 72°C. The PCR was verified by migrating 3 µL of the PCR product on 2% agarose gel. PCR products were then diluted to approximate concentration and loaded on a 3.7% solidified gel matrix (KB Plus, Li-COR, Bioscience). Electrophoretic separation was performed under denaturing conditions for 15 h at 1500V/80W on a LiCor DNA sequencer (IR2, Science Tec). The data were analyzed using the 1D-Scan software (Scan analytic programme) which permets the production of matrices (presence, absence, size and relative intensity of each band). Principal component analysis (PCA) of the ARISA fingerprints was performed on the covariance matrice derived from ADE-4 (http://pbil.univ.lyon1.fr/ADE4).

2.7. IPU mineralization potential of the soil samples

The ability of the indigenous soil microorganisms to mineralize IPU was estimated by radiorespirometry using ^{14}C ring-labeled IPU as previously described (El-Sebai et al., 2007). Analytical grade IPU (analytical grade purity, 99.0%) and ^{14}C ring-labelled IPU (specific activity 18 mCi $mmol^{-1}$; 99% radiochemical purity) were purchased from Riedel-de Haen (Germany) and International Isotopes (Munich, Germany), respectively. Briefly, 40 g (equivalent dry weight) of each of the 36 soil samples collected each year, was treated with 1.5 mg of IPU per kg of soil and 2 kBq of ^{14}C ring-labelled IPU. Soil samples were moistened to 80% of water holding capacity and incubated at 20°C in dark for about 70 days in closed respirometer jars (Soulas, 1993). $^{14}CO2$ resulting from mineralization of ^{14}C ring-labeled IPU was trapped in 5 mL of 0.2 M NaOH solution. The traps were regularly changed over the incubation period and analyzed for radioactivity content by liquid scintillation counting using ACSII scintillation fluid (Amersham). IPU mineralization parameters were deduced from mineralization kinetics by fitting the modified Gompertz model ($y = A.\exp\{-\exp[1+\mu m.\exp(1).(\lambda-t)/A]\}$) (Zwietering et al., 1990) using Sigma Plot© 4.0 software. Four

parameters were estimated, t, the time (days), μm, the maximum mineralization rate (% day^{-1}), A, the maximum percentage of mineralization (%) and λ, the lag time (days).

2.8. Determination of ^{14}C IPU residues

At the end of the incubation period, the residual ^{14}C ring-labeled IPU was extracted and analyzed as previously described (El-Sebai et al., 2005). Briefly, 20 g of soil (equivalent dry weight) was added with 40 mL of methanol and agitated overnight at 180 rpm by incubating at 20 °C. The samples were then centrifuged and 2 mL aliquot of the supernatant was analyzed for radioactivity content by liquid scintillation counting using ACSII (Amersham) scintillation fluid. Soil pellets were also recovered and air dried at room temperature.

Quantitative determination of the non-extractable IPU residues, mainly corresponding to the bound residues, was carried out by combustion of 0.5 mg of dried methanol extracted soil samples under O_2 flow at 900 °C for 4 min with the help of Biological Oxydiser OX-500 (EG & G Instruments, France) as previously described (El-Sebai et al., 2005).

2.9. Statistical Analysis

2.9.1. Exploratory statistical analysis

Classical statistical descriptors like mean, median, maximum, minimum, standard deviation and coefficient of variation were calculated for all the physico-chemical and microbiological parameters measured. The descriptive statistical analysis of the data realized using Statview 4.55 software (Abacus Concept, Inc.) indicated that all the factors were log normally distributed. Therefore, no transformation was required for the geostatistical analysis. Box plot were drawn under Excel Stat. The effect of the crop practices on the IPU mineralization parameters and other biological parameters measured was addressed using a single factor ANOVA followed by Fisher test ($n = 3$, $p < 0.05$) (Statview 4.55 software, Abacus Concept, Inc.).

2.9.2. Geostatistical analysis

All the parameters considered in this study over three years were also analyzed using geostatistics by calculating a semivariogram using the following formula;

$$\gamma(h)=1/2N(h)\Sigma[z(xi+h) - z(xi)]^2$$ (Isaaks and Srivastava, 1989)

where γ(h) is the experimental semivariogram value at distance interval h; N(h) is the number of sample pairs within the distance interval h; z(xi), z(xi + h) is the sample value at two points separated by the distance interval h. Spherical model was fitted to the semivariogram and the

parameters of the model i.e. sill, nugget and range were determined. Two indices of spatial dependence were calculated; one was the Q value [(sill-nugget)/sill] which indicates the spatial structure at sampling scale (Gorres et al., 1998). The other index is the range which indicates the limit of spatial dependence. Maps were then computed using ordinary kriging to evaluate the regional patterns of variation within the field by using the geostatistical software, GeoR version 1.6-27 under R version 2.11.1 for windows.

3. Results

3.1. Characterization of the soil physico-chemical parameters of the field

The soil texture was determined on batch samples made of 9 samples for each of the four columns and analysis was performed only once. Descriptive statistics i.e. minimum, maximum, mean, median, standard deviation (SD) and coefficient of variation (CV) for the physico-chemical parameters (equivalent humidity, organic matter content, organic carbon content, nitrogen content, C/N ratio, CEC and pH) of the 36 sub-site soil samples over three years (2008 – 2010, in total = 108) are shown in Table III-1. These values over three years survey moderately varied within the grid pattern of the field. Three of the soil physico-chemical parameters i.e. equivalent humidity (EH), nitrogen content (N) and pH were found to be weakly variable within the grid pattern with CV values below 10% over three years survey. Organic matter content (OM), organic carbon content (OC), C/N ratio and cation exchange capacity (CEC) were found to be moderately variable within the experimental field with CV values about 10 to 15% except for CEC and C/N ratio which showed CV below 10% in 2008. Although soil pH varied from 7.1 to 8.1 over the grid samples, CV was low and no significant differences could be observed over the three years. However mean values of equivalent humidity and nitrogen content of the soil samples were significantly different between years with maximal values in 2009 and minimal in 2008. Similarly, the mean values of organic matter content, organic carbon content, C/N ratio were significantly different in 2008 as compared to that of measured in 2009 and 2010.

Furthermore, each of the physico-chemical parameters estimated from the 36 sub-site soil samples of the field over three years were found to be log normally distributed with a P-value greater than 0.05 as determined by Kolmogorov–Smirnov test (K–S test) (Table III-1).

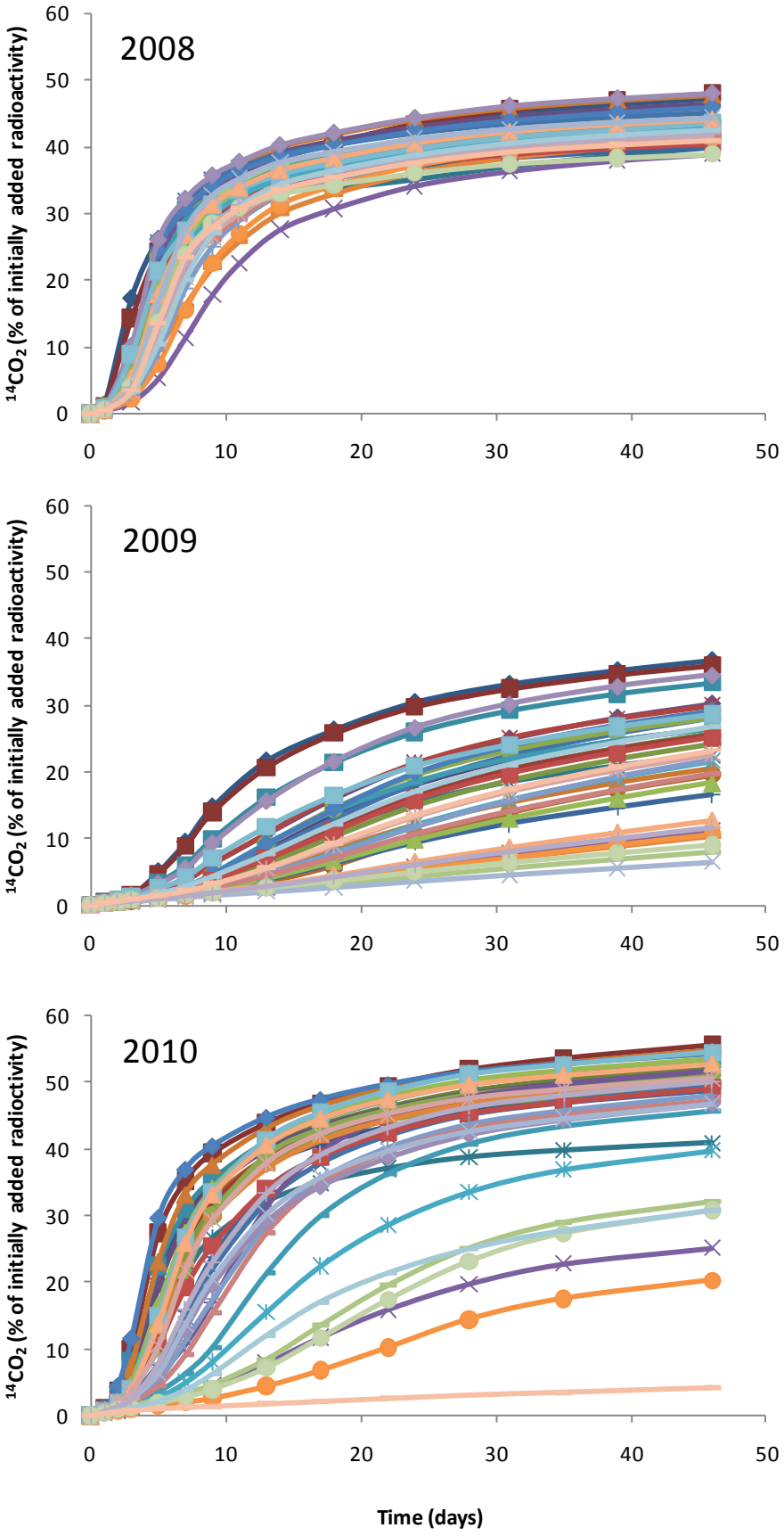

Fig. III-1. Kinetics of mineralization of ^{14}C ring-labeled IPU in 36 sub-site soil samples collected from the Epoisses field in 2008, 2009 and 2010.

3.2. Characterization of the soil microbiological parameters of the field

Descriptive statistics for the microbiological properties i.e. microbial C biomass (MCB), total culturable heterotrophic bacteria (cfu) and mineralization parameters (A, µm, λ and ti) of the 36 sub-site soil samples over three years are presented in the Table III-1. As previously observed for the physico-chemical parameters, the microbiological parameters were also log normally distributed with significant P values as determined by K-S test (Table III-1.). The microbiological properties showed higher variability within the experimental field than the physico-chemical properties except the number of culturable heterotrophic bacteria which were having a low CV (< 5%). Although microbial C biomass was showing a moderate variability within the field grid pattern with CV values ranging from 13 to 26%, the mean microbial C biomass was not significantly varying over three years survey. The majority of the 36 sub-site soil samples showed a high ability to mineralize IPU in 2008 and 2010 as compared to 2009 (Fig. III-1). Interestingly, the IPU mineralizing ability of the sub-site samples was higher in 2008 and 2010 as compared to that of in 2009. Indeed, most of the soil samples in 2008 and 2010 were able to mineralize up to 40% of the ^{14}C ring-labelled IPU within only 24 days of incubation, while in 2009, less than 20% of the ^{14}C ring-labelled IPU was mineralized after 24 days of incubation. Cumulative percentage of IPU mineralization (A) and the maximum mineralization rate (µm) were were moderately variable with CV values of 5.8 and 15.6% respectively in 2008 (Table III-1.). However, these two parameters showed higher variability in 2009 and 2010 with 25% and 50% CV values, respectively. Their mean values were significantly higher in 2008 (A = 42.6, µm = 4.0) and 2010 (A = 45.3, µm = 3.9) as compared to those measured in 2009 (A = 27.1, µm = 0.7) (Fig. III-2). The lag phase (λ) and abscissa of inflexion point (ti) were the most variable parameters within the experimental field over three years. In addition, the mean values of λ and ti were significantly higher in 2009 (λ = 4.6, ti = 20.7) as compared to those measured in 2008 (λ = 1.1, ti = 5.1) and 2010 (λ = 2.7, ti = 8.2) (Fig. III-2). The methanol extractable ^{14}C-residues were relatively low with values below 10% of the initially added ^{14}C ring-labelled IPU. Significantly higher amounts of extractable residues were quantified in 2009 than in 2008 (Fig. III-3). On the contrary, non extractable ^{14}C-residues, corresponding to bound residues, were relatively high averaging 45% of the initially added ^{14}C ring-labelled IPU. As already observed for extractable residues, significantly higher amounts of bound residues were detected in 2009 than in 2008 and 2010 (Fig. III-3). These observations suggest that besides the microbiological transformation, IPU preferentially formed bound residues.

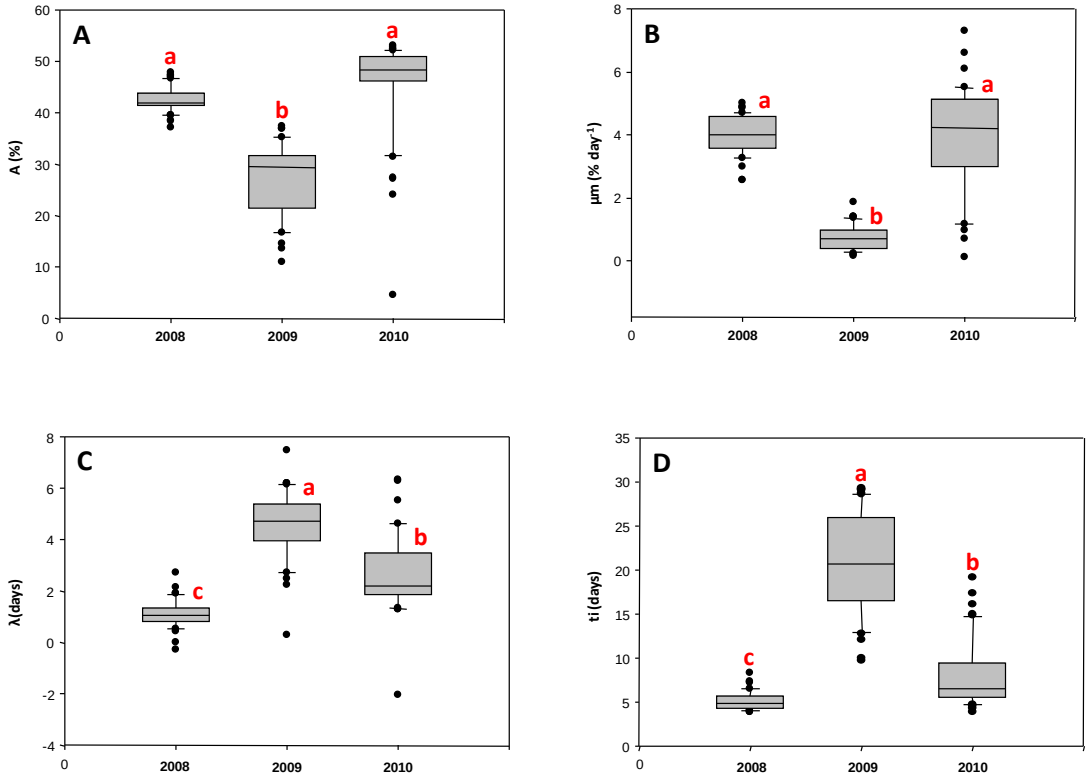

Fig. III-2. Box and whisker plot showing the distribution of parameters calculated by fitting the modified Gompertz model to isoproturon mineralization kinetics determined from each of the 36 soil samples collected in 2008, 2009 and 2010 from the experimental field of Epoisses. A, B, C and D represent the maximum percentage of mineralization (A), maximum mineralization rate (µm), lag phase (λ) and abscissa of inflexion point (ti), respectively.

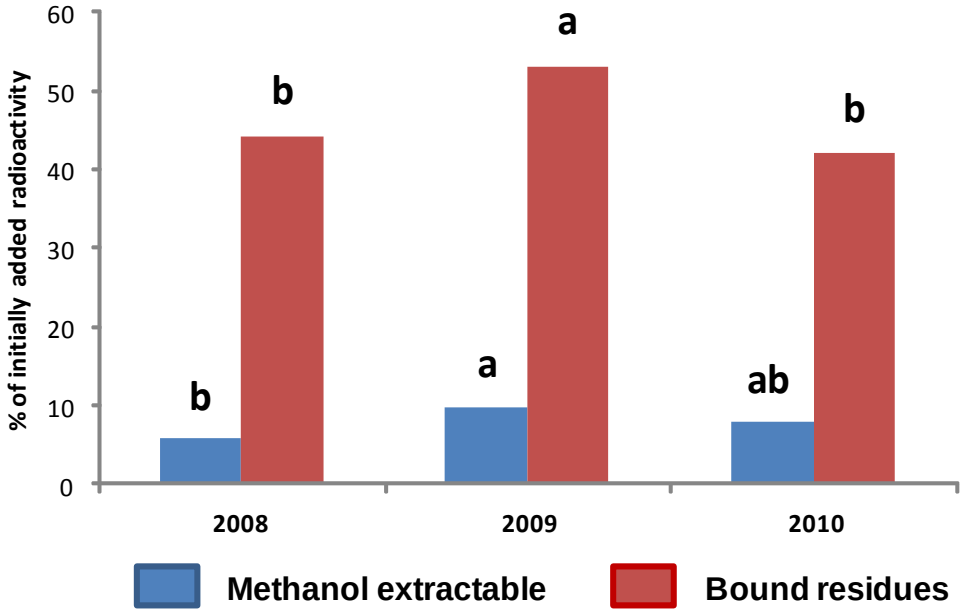

Fig. III-3. Mean values of the methanol extractable ^{14}C-residues and of the ^{14}C bound residues quantified in soil samples in 2008, 2009 and 2010 (n = 36, $p<0.05$).

The global structure of the soil microbial communities was evaluated by automated ribosomal intergenic spacer analysis (A-RISA) performed on DNA extracted directly from the soil. Relatively similar fingerprints were obtained from different sub-site soil samples (data not shown). Principal component analysis (PCA) was applied for the pair wise comparison of the number and relative intensity of the bands observed in A-RISA profile. The first principal component explained 29%, 32% and 38% of the data variance in 2008, 2009 and 2010 respectively (Fig. III-4), while, the second principal component accounted 21%, 15% and 28% of the variance in 2008, 2009 and 2010 respectively. Most of the sub-site soil samples were centered on the factorial map. In 2010, several sub-site samples (D6, D4, C7) and (A7, A5, A9 and A6 to some extent) were distinguished along axis 1.

3.3. Geostatistical analysis of the soil physico-chemical parameters

Spherical variogram model was fitted to the full data set of all physico-chemical parameters to investigate their spatial variabilities over three years survey. Based on the data obtained from the spherical variogram model, a single model of the spatial variance structure was provided for each physico-chemical parameter measured in the experimental field of Epoisses. The parameters deduced from the variogram i.e. sill, nugget, range and Q value for all the physico-chemical properties are presented in Table III-1. The physico-chemical properties showed spatial dependence with a range often superior to 50 m except equivalent humidity (EH), organic matter content (OM), organic carbon content (OC) and C/N ratio showing moderate range values of 24.9, 31.9, 34.0 and 36.8 m respectively in 2008 (Table III-1). Q values for almost all the physico-chemical parameters were more than 0.5 except for nitrogen content and pH which were having a Q value lower than 0.5 in 2008. High Q values indicate that the variance of the physico-chemical parameters could be explained by the semivariogram model. It further suggests that the physico-chemical parameters measured over three year survey presented a rather highly developed spatial structure.

Maps for the physico-chemical properties measured on the experimental field over three years were drawn by ordinary kriging based on the data within the observed ranges. Considerable spatial distribution within the field grid was clearly observed for most of the physico-chemical parameters over three years. The kriged maps of OM, CEC and soil pH are presented in fig. III-5. The kriged maps for organic matter content, organic carbon content, nitrogen and C/N ratio presented similar patterns of spatial distribution within the field grid over three years (Fig. III-5a.). However, the intensity of their spatial distribution variability within the field grid was higher in 2009 and 2010 as compared to that of in 2008. The spatial

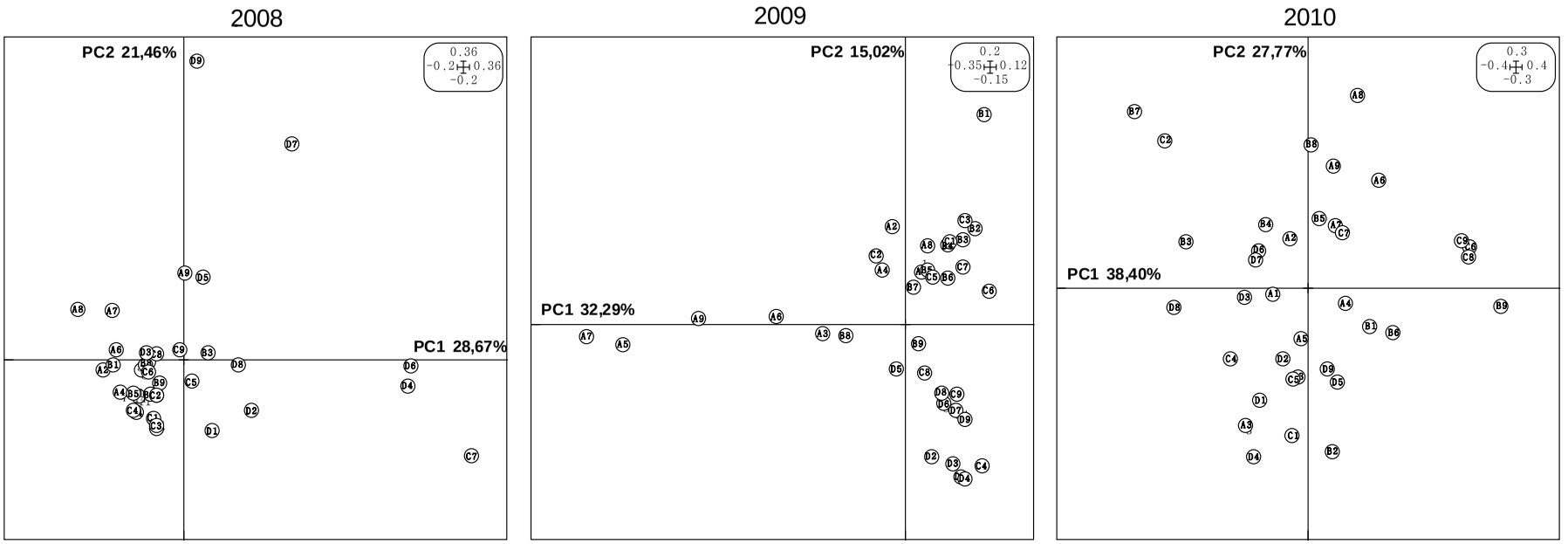

Fig. III-4. PCA ordination of the 36 soil samples based on the analysis of the soil bacterial communities

distribution of the equivalent humidity (EH) and CEC were found to be following almost the similar patterns of variability within the field grid (Fig. III-5b.). However, the patterns for the spatial distribution of these two parameters in 2009 and 2010 differed to some extent from that of in 2008. Similarly, the patterns of spatial distribution of pH in 2009 and 2010 also differed slightly from that of in 2008 with a high intensity of variability in 2009 and 2010 as compared to that of in 2008 (Fig. III-5c.).

3.4. Geostatistical analysis of the microbiological parameters

Microbiological parameters measured were also analyzed by spherical variogram model to address their spatial variability within the experimental field of Epoisses over three years survey. According to the parameters deduced from the semivariogram (i.e. sill, nugget, range and Q), the soil microbiological parameters were found to show spatial dependence with range exceeding 30 m except the number of culturable heterotrophic bacteria showing range below 30 m (Table III-1). Q values greater than 0.5 were observed in 2009 and 2010 for most of the microbiological parameters, while, weaker Q values were observed in 2008.

Kriged maps were drawn for microbiological parameters showing high Q values. As a result, we showed that the kriged maps for the mineralization parameters i.e. maximum percentage of IPU mineralization (A), maximum rate of mineralization (µm), lag phase (λ) and abscissa of inflexion point (ti) were presenting considerable spatial distribution within the experimental field of Epoisses. Kriged maps of µm calculated over three year survey are presented in fig. III-6A. Over the three year survey, µm showed almost similar patterns of spatial distribution within the experimental field with highest mineralization rate observed in the upper site of the field. It has to be noticed that the lowest mineralization rate were observed in 2009 and that the lowest variability of µm was observed in year 2008. A and µm showed similar patterns of distribution over three years. As previously showed for µm, the lag phase (λ) and abscissa of inflexion point (ti) also showed almost similar spatial pattern over three years with lowest values recorded on the upper site of the field (Fig. III-6B). Interestingly, one could observe that the spatial distribution of these two parameters was almost negative to that of A and µm parameters.

3.5. Correlation between the maximum mineralization rate (µm) and other soil parameters

In order to determine which parameter may influence the IPU mineralization ability of soil samples, the soil physico-chemical as well as microbiological parameters were related to

Table III-2. Correlation coefficient matrix between the maximum mineralization rate (µm) and other parameters.

	2009		2010	
	Pearson's r	**p-value**	**Pearson's r**	**p-value**
µm vs **Microbial C biomass**	0.346	0.042	-0.280	0.103
µm vs **Cultivable bacteria (cfu)**	-0.185	0.287	-0.115	0.510
µm vs **Organic matter**	0.553	0.001	0.439	0.008
µm vs **Equivalent humidity**	-0.433	0.009	-0.610	<0.0001
µm vs **Organic carbon**	0.555	0.001	0.439	0.008
µm vs **Nitrogen**	-0.063	0.721	-0.376	0.026
µm vs **C/N ratio**	0.560	0.0001	0.598	0.0001
µm vs **CEC**	-0.608	0.0001	-0.722	<0.0001
µm vs **pH**	0.477	0.004	0.441	0.008
µm vs **Extractable IPU residues**	-0.829	<0.0001	-0.744	<0.0001
µm vs **Bound IPU residues**	-0.371	0.028	0.065	0.710

the maximum mineralization rate (μm) using regression procedure by estimating the Pearson's coefficient of variation (r) (Table III-2). In 2009 and 2010, μm was found to have positive correlation with organic matter content (r = 0.553 and 0.439), organic carbon (r = 0.555 and 0.439), C/N ratio (r = 0.560 and 0.598) and pH (r = 0.447 and 0.441). It was also found to be negatively correlated with CEC (r = - 0.608 and -0.722) and equivalent humidity (r = - 0.433 and -0.610). However, there were no considerable correlations between the μm and other soil parameters considered in 2008.

4. Discussion

The present study was carried out not only to assess the spatial variability of IPU mineralization in relation to biotic and abiotic parameters within an agricultural field but also to estimate the IPU mineralizing variability over a three year crop rotation in relation to herbicide application. Our results indicate that the cumulative percentage of IPU mineralization (A) was ranging from 37 to 48% (CV = 5.8%), 11 to 37% (CV = 26.2%) and 5 to 53% (CV = 22.2%) in 2008, 2009 and 2010 respectively. These results confirmed that the soil microflora of the experimental field of Epoisses was adapted to IPU mineralization due to the repeated exposure of the field to this herbicide over the last decade cropped with wheat/rape seed/barley crop rotation (1 treatment/3 years, 3 treatments/10 years). Although the extent of variability in cumulative percentage of IPU mineralization (A) was low in 2008 (CV = 5.8%), it was shown to be relatively high in 2009 (CV = 26.2%) and 2010 (CV = 22.2%). In addition, similar observations could be done for the maximum mineralization rate (μm), determined by fitting the modified Gompertz model (Zwietering et al., 1990) to the mineralization kinetics, showing an important variability with 15.6, 53.7 and 45.4% CV values in year 2008, 2009 and 2010, respectively. This suggests that the in-field spatial variability in IPU mineralization activity could be important in 2009 and 2010, whereas it could be relatively low in 2008. The observed variability in IPU mineralization within the experimental field of Epoisses is in agreement with a number of previous studies reporting spatial variability in the degradation rates of several pesticides including carbofuran (Parkin and Sheloton, 1992), azoxystrobin (Bending et al., 2006), chlorsulfuron (Walker and Brown 1983), diflufenican (Bending et al., 2006), isoproturon (Walker et al., 2001; El-Sebai et al., 2007) and chlorotoluron (Walker et al., 2001). Furthermore, it was observed that cumulative means of maximum percentage of IPU mineralization (A) and maximum IPU mineralization rate (μm) measured for 36 sub-site soil samples were significantly higher in 2008 and 2010 as

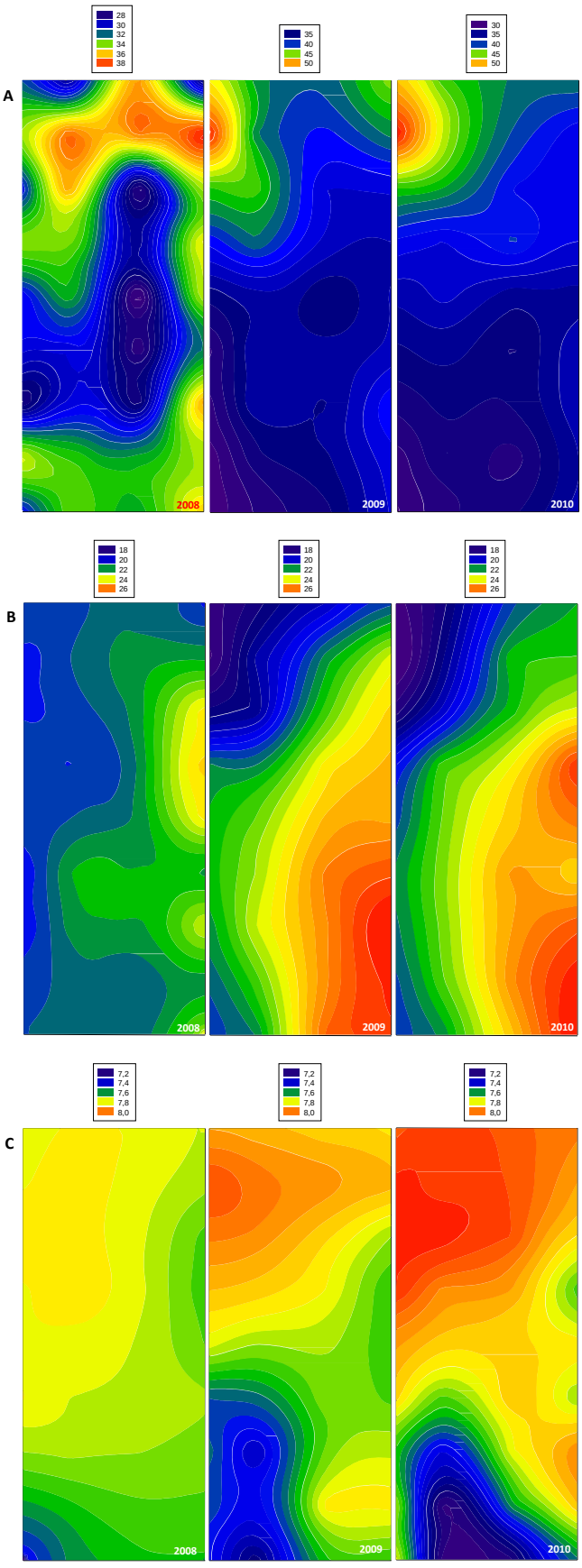

Fig. III-5. Kriged map of organic matter (panel A), cation exchange capacity (panel B) and soil pH (panel C) of the field sampled at Epoisses in 2008, 2009 and 2010.

compared to those in 2009. On the contrary, cumulative means of lag phase (λ) and abscissa of the inflexion point (ti) were significantly higher in 2009 as compared to those in 2008 and 2010. These observations revealed a higher IPU mineralization activity in year 2008 and 2010 than in year 2009. This difference in IPU mineralization activity might be related to the scheme of herbicide application to the field over three years crop rotation which implies a treatment with IPU (1.2 kg ha^{-1}) in 2008, with dinitroaniline herbicide trifluralin in 2009 and sulfonylurea herbicides (mesosulfuron-methyl and iodosulfuron-methyl) in 2010. Based on our results, one could hypothesize that IPU treatment applied in 2008 might have promoted the growth of the microbial population able to mineralize this herbicide. It has already been described that in a soil adapted to enhanced pesticide degradation, repeated treatment with the pesticide led to increase the size of the pesticide degrading community. This has also been demonstrated for atrazine (Piutti et al., 2002) as well. In addition, it has been shown that pesticide treatment may also contribute to decrease the lag phase due to an increase in the size of the pesticide degrading community. This was observed that the means of lag phase (λ) and abscissa of the inflexion point (ti) were about half in 2008 as compared to that of in 2010 and about 4 times low in 2008 as compared to that of in 2009. It is in accordance with the previous study reporting a considerable decrease in ti due to repeated application of IPU (El-Sebai et al., 2005). In response to IPU treatment, the increase in IPU mineralization activity in almost all sub-site soil samples led to the increased growth of the IPU degrading population thereby lowering the variability of IPU mineralization activity in 2008. Interestingly, although IPU was not applied in 2010, it seems that the treatment with sulfonylurea, which presents similarity in structure with phenylurea (i.e. having phenyl ring and urea side chain), could have promoted the activity of IPU degrading community. This observation suggests that IPU degrading community might also be able to degrade sulfonylurea residues.

Although the pesticide treatment seems to have a great importance on the overall IPU degrading activity, our results showed that it was also considerably varying over the field. In order to explore in more details the processes responsible for in-field spatial variability in isoproturon mineralization, the physico-chemical and microbial properties measured on 36 sub-site soil samples were analyzed by using the geostatistical tools. All the parameters considered in this study were log-normally distributed and could be used for analysis by univariate geostatistical tools. Log-normal distribution of the biotic and abiotic parameters estimated here is in agreement with a number of previous studies reporting the normal distribution of the soil parameters within a field (Lopez-Granados et al., 2002; El-Sebai et al.,

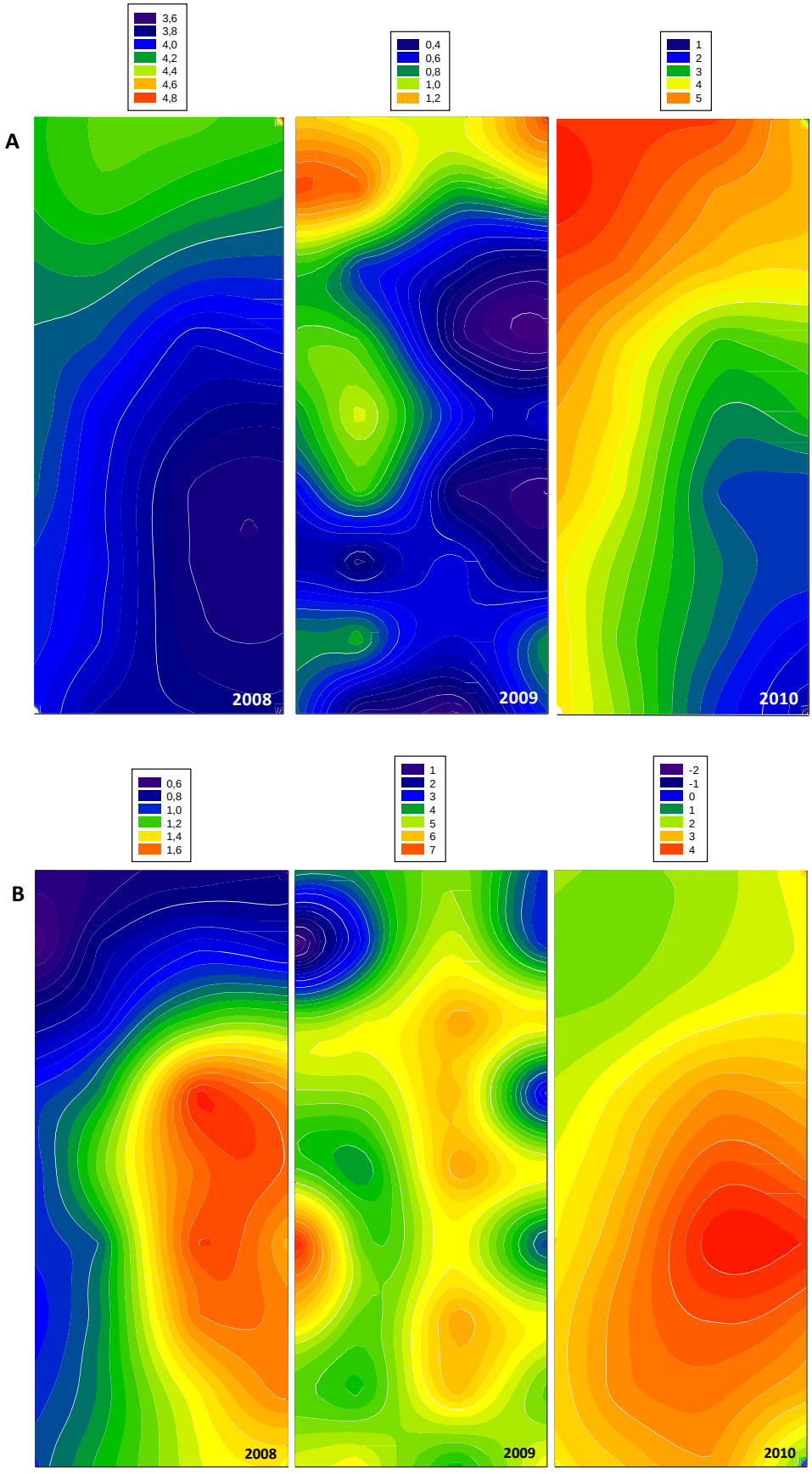

Fig. III-6. Kriged map of maximum mineralization rate (μm) (Panel A) and lag phase (λ) (Panel B) of the experimental field of Epoisses in 2008, 2009 and 2010.

2007). Most of the parameters showed high spatial variability but some parameters including equivalent humidity, nitrogen content, pH and number of culturable heterotrophic bacteria were characterized by relatively low coefficient of variation (CV < 10%) over three years survey. This low coefficient of variation for these four parameters is in agreement with the previous studies (Walker et al., 2001; El-Sebai et al., 2007). One could observe that lower CV values in 2008 as compared to that of in 2009 and 2010 might also be helpful in explaining the low variability in IPU mineralization activity in this year.

The biotic and abiotic parameters measured in this study were analyzed with geostatistical tools which have recently been used to investigate the spatial variation of IPU mineralization within a French agricultural field (El-Sebai et al., 2007). The interest of using geostatistics is to explore the spatial variation of the regionalized variables, to perceive the spatial structure of soil parameters and to predict the values of soil attributes at unsampled locations by kriging. These tools can be used to estimate the scale of spatial variation in the rate of IPU mineralization and several physico-chemical and microbiological properties measured in this study. The variability of the parameters measured within the field over three years survey was estimated by measuring the spatially-dependent variability based on Q values. Most of the parameters measured in 2009 and 2010 showed significant Q values (Q > 0.8), while, several parameters (N, pH, CEC, MCB, cfu, A, μm, λ and ti) showed low Q values in 2008 which were either non-significant (Q < 0.5) or moderately significant (0.5 < Q < 0.8). Based on Q values, one could observe that highly developed spatial structure (Q > 0.8) was observed for all the parameters in 2009 and 2010 except equivalent humidity in 2009 and μm in 2010 which had moderately developed spatial structure (Q ≈ 0.5). This existence of varying spatial classes in soil parameters is in accordance with some of the previous studies reporting such spatial structures (Yanai et al., 2001; Lopez-Granados et al., 2002; El-Sebai et al., 2007). The spatial variability results from a combination of intrinsic and extrinsic parameters. The first resulting from the natural variations in soil and the last from human activities, such as crop production, interact with each other to define the spatial variability of the biotic paramaters.

Geostatistical maps were drawn to compare the variability of different physico-chemical and microbiological parameters measured in the experimental field over three years survey. Comparison of the geostatistical maps revealed that some of the physico-chemical parameters (organic matter content, organic carbon content, C/N ratio and CEC) showed in-field variability comparable to the variability in mineralization activity within the field.

Indeed, the kriged maps obtained with maximum percentage of mineralization (A) and maximum mineralization rate (μm) were found to have almost similar patterns of variability over the field. The kriged maps obtained with μm in 2009 and 2010 indicated the presence of a patch with a high mineralization rate within the 0 to 30 meters strips (Fig. III-6A). Almost similar patches were also observed in kriged map of μm in 2008 but the extent of variability over the field was low. When the units of axes of the μm kriged maps were homogenized, the maps clearly indicated that the μm over the field was higher in 2008 and 2010 as compared to that of in 2009 with a higher intensity of spatial variability in 2010 as compared to that of in 2008 (Appendix-III). These results reinforce the above results indicating a low mineralization activity in 2009 as compared to that of in 2008 and 2010 and strengthen that although soil microflora showed an enhanced mineralization in 2008 and 2010, the spatial variability in mineralization activity was considerable higher in 2010 as compared to that of in 2008. The kriged maps obtained with lag phase (λ) and abscissa of inflexion point (ti) were observed to have the patches of lowest values within the strips where μm and A were maximum. Some of the physico-chemical parameters like organic matter content, organic carbon content and C/N ratio were observed to have almost similar kriged maps as shown for organic matter content (Fig. III-5A). The kriged maps obtained with these parameters in 2009 and 2010 were comparable with that of μm in that the patches with higher values existed at the same strip (0 to 30m) as already observed for μm. Simply, we can say that the patches exhibiting high mineralization activity in 2009 and 2010 were also characterized by higher values of organic matter content, organic carbon content and C/N ratio. In addition, determination of Pearson's coefficient of correlation (r) indicted that μm in 2009 and 2010 was considerably correlated with organic matter content ($r = 0.553$ and 0.439), organic carbon content ($r = 0.555$ and 0.439) and C/N ratio ($r = 0.560$ and 0.598). Thus, the present study points out the importance of soil organic matter content in determining the spatial variability of pesticide mineralization activity. It is in accordance with a number of previous studies indicating the influences of organic matter on the pesticide biodegradation in soil environment by proliferating the growth and activity of the microbial communities (Spark and Swift, 2002; Saison et al., 2006; Briceno et al., 2007). Similarly, soil pH was also observed to be having a correlation ($r = 0.477$ and 0.441) with μm in 2009 and 2010. Kriged map obtained with pH was also indicating the patches of high pH in almost the same strip where high μm was observed. Soil pH is considered as a key parameter in controlling the pesticide biodegradation and the importance of soil pH in having pronounced effects on microbial pesticide biodegradation has been described in a number of previous studies not only in soil environment (Bending et al.,

2001; El-Sebai et al., 2005, El-Sebai et al., 2007) but also in pure cultures (Hussain et al., 2009; Sun et al., 2009). In contrast to these parameters, the kriged maps obtained with cation exchange capacity (CEC) and equivalent humidity in 2009 and 2010 were observed to have patches with lowest values at the locations which were characterized by the patches with higher values of μm. This was further confirmed by the Pearson's coefficient of correlation (r) negatively relating μm with CEC (r = -0.608 and -0.722) and equivalent humidity (r = -0.433 and -0.610). This suggests that in this study CEC and equivalent humidity might also be playing role in modifying the isoproturon mineralization activity. Our results indicate that although the physico-chemical parameters are having moderate correlations with the mineralization, they might be contributing to the microbial isoproturon degradation potential and ultimately leading to heterogeneity in rate of biodegradation (Vinther et al., 2001). The μm and A maps did not share any similarities with the maps representing the genetic structure of the bacterial communities and the maps representing the number of culturable heterotrophic bacteria and microbial C biomass. This suggests that overall genetic structure of the bacterial communities is not related to mineralization activity within this field which is in accordance with the previous studies indicating the same type of results (El-Sebai et al., 2007).

Based on the results presented in this study, it can be concluded that spatial and temporal variability in IPU mineralization is observed in the experimental field of Epoisses. The mineralization activity and heterogeneity are modified by the contribution of not only the varying soil physico-chemical parameters like organic matter content, C/N ratio and CEC but also by the pesticide treatments plans. Our results mainly suggest that variations in soil physico-chemical parameters and pesticides treatment schedules should be considered while modeling the pesticide kinetics in the soil environment.

Conclusions and Perspectives

The results obtained in this study provide another example confirming the adaptation of the soil microflora to enhanced IPU biodegradation. The process of microbial adaptation is considered as a dominant phenomenon affecting the pesticide biodegradation in the soil environment and has already been reported for a number of pesticides including isoproturon (Sorensen and Aamand, 2003; El-Sebai et al., 2005), 2,4-D (Soulas, 1993), carbofuran (Charnay and Fournier, 1994), atrazine (Yassir et al., 1999) and isoxaben (Arrault, 2001). Although the soil microflora adapted to biodegradation of pesticides in soil environment, this phenomenon is not equally distributed at the field scale. The statistical analysis of the results obtained with soil samples from the Epoisses field over three year survey showed considerable variations in IPU mineraization in soil samples collected in 2009 and 2010. However, the extent of variation of IPU mineralization was considerably lowered in 2008. Kriged maps resulting from geostatistics also indicated that the maximum rate of IPU mineralization presented a spatially dependent variation over the field. These maps were sharing similarities with those obtained for organic matter content, organic carbon content, C/N ratio and cation exchange capacity (CEC). This finding led us to hypothesize that in-field spatial variability of IPU mineralization rate might be at least partially attributed to the spatial variation of physico-chemical parameters. It was further confirmed when relatively important correlations (Pearson's coefficient of variation) were found between the IPU mineralization rate and above physico-chemical parameters. This further confirms the previous studies reporting a correlation between the mineralization activity and the physico-chemical properties in the soil environment (Bending et al., 2003; El-Sebai et al., 2005; El-Sebai et al., 2007). Furthermore, this study also highlights the importance of knowing the pesticide application history while studying the spatial variability of degradation and fate of pesticide. The results obtained here indicated that the spatial variability in isoproturon mineralization activity over the field was considerably lower in 2008 as compared to that of in 2010. This lower variability in 2008 might be attributed to the treatment of the field with isoproturon. Due to isoproturon application in 2008, the soil microflora in the samples presenting low mineralization activity might also have adapted through different ways which remain to be elucidated, thus, decreasing the extent of variability among them. However, higher mean mineralization activity in 2010 might be due to application of sulfonylurea herbicides (mesosulfuron-methyl and iodosulfuron-methyl) which shared similar structural components (phenyl ring with a urea side chain) with that of IPU in their molecule. Overall, the results

obtained in this study led us to conclude that while studying the pesticide biodegradation in the soil, the physico-chemical properties and pesticide treatment history of the soil should be considered to better understand these processes. Keeping in view that microbial activity is responsible for IPU mineralization activity observed at field level in view, there was need to carry out further research to isolate, identify and characterize the bacterial communities and the bacterial populations responsible for IPU mineralization in order to better describe the metabolic pathway and the genes involved in IPU mineralization.

CHARACTERIZATION OF DEGRADING ABILITY OF AN ISOPROTURON MINERALIZING ENRICHED BACTERIAL CULTURE

Introduction

My PhD work aimed at investigating the IPU degrading microbial community from the field to the genes in order to understand and highlight the processes involved in this degradation. The first part of my PhD work showed the spatial variability of IPU degradation potential at the field scale in relation to the variability of both the physico-chemical and microbiological properties. These results revealed the existence of the soil microbial communities able to mineralize IPU in response to repeated exposure of the soil microflora. In order to better understand the microbial processes involved in biodegradation, we developed a research aiming at characterizing the IPU mineralizing natural microbial populations. In this aspect, several attempts were made to isolate the soil microbial communities through enrichment cultures and a number of fungal and bacterial strains able to metabolize different phenylurea herbicides have been isolated and characterized (Cullington and Walker, 1999; Khadrani et al., 1999; Sorensen et al., 2001; Turnbull et al., 2001; El-Sebai et al., 2004; Sorensen et al., 2008; Sun et al., 2009). However, from the previously exposed agricultural soils, some recent studies reported the isolation of the mixed bacterial cultures degrading different herbicides including linuron (Dejonghe et al., 2003), metobromuron (El-Fantroussi, 2000) and atrazine (Udikovic-Kolic et al., 2008). The advantages of employing the mixed cultures in biodegradation and bioremediation are attributed to the synergistic interaction occurring among its members. Sorensen et al. (2002) described that the metabolic activity of an isoproturon mineralizing bacterial strain, *Sphingomonas* sp. SRS2, was significantly enhanced when it was co-cultured with another bacterial strain SRS1. Similar observations were also done for atrazine degrading populations which were found to work in a cooperative way. Although naturally occurring mixed bacterial cultures have been isolated and characterized for different phenylurea herbicides (diuron and linuron), there is no report for the involvement of such cultures in the degradation of isoproturon.

Therefore, in this context, enrichment cultures were established from IPU adapted soil (El-Sebai et al., 2005) to isolate and characterize a mixed bacterial culture carrying out the mineralization of isoproturon. Culturable members of this enriched bacterial culture were isolated by using different types of media. Culturable isolates were characterized for the degradation of isoproturon and its metabolites. The composition of the bacterial culture was

also studied by applying molecular approaches relying on construction and screening of a 16S rDNA clone library. Physiological characterization of the enriched bacterial culture was carried out by testing its ability to mineralize IPU and degrade different related phenylurea herbicides like diuron, chlorotoluron, linuron and monolinuron. Further characterization was also carried out by studying the effect of pH on IPU degradation kinetics by the enriched bacterial culture. The initial steps and metabolites involved in isoproturon metabolism by this bacterial culture were identified by analyzing the samples collected during IPU mineralization kinetics by ultra performance liquid chromatography (UPLC).

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Characterization of an isoproturon mineralizing bacterial culture enriched from a French agricultural soil

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ABSTRACT

The phenylurea herbicide isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), was found to be rapidly mineralized by a bacterial culture isolated from an agricultural soil regularly exposed to IPU. Molecular analysis of the bacterial culture by DNA fingerprinting, cloning and sequencing of the 16S rRNA genes revealed that it consisted of six different members among whom the dominant was related to *Sphingomonas* sp. Six bacterial strains belonging to genera *Ancylobacter*, *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium*, *Variovorax* and *Agrobacterium* were isolated from the IPU-degrading culture. None of these were able to degrade IPU in pure culture and only the intact culture sustained the ability to mineralize IPU. The composition of the culture appeared stable suggesting that yet unknown interactions are involved in IPU mineralization. IPU degradation involved the transitory accumulation of three known IPU metabolites 3-(4-isopropylphenyl)-1-methylurea, 3-(4-isopropylphenyl)-urea, and 4-isopropylaniline and their further degradation. Thus, it indicates a metabolic pathway initiated by two successive N-demethylations, followed by cleavage of the urea side chain. This culture did not degrade other structurally related phenylurea herbicides. The degrading activity of the bacterial culture was deeply influenced by the pH, being completely inhibited at pH 5.5 and optimal at pH 7.5.

Key words: Isoproturon, bacterial culture, mineralization, metabolites, pH regulation

1. Introduction

The phenylurea herbicide isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), is among the most extensively used herbicides in conventional agriculture in Europe. It is applied for the prevention of the pre- and post-emergence weed developments in spring and winter wheat, barley and winter rye. It is relatively recalcitrant in the environment with 60% of the initial added amount remaining in the soil three months after its application (Nicholls et al., 1993). Due to its intensive and repeated usage as well as its persistence in the environment, IPU is frequently detected in the surface- and groundwater bodies at concentrations exceeding 0.1 µg L^{-1}, the European Union drinking water limit (Muller et al., 2002). Ecotoxicological data suggest that IPU and several of its main metabolites are carcinogenic for animals and humans (Behera and Bhunya, 1990; Hoshiya et al., 1993) and harmful to aquatic invertebrates (Mansour et al., 1999), fresh water algae (Vallotton et al., 2009) and microbial communities (Widenfalk et al., 2008). So, there is a crucial interest in studying the fate of IPU in the soil environment which acts as a recharge zone for underlying groundwater aquifers as well as nearby rivers, streams and lakes.

Microbial degradation is the primary mechanism for dissipation of IPU and related phenylurea herbicides in agricultural soils (Fournier et al., 1975; Cox et al., 1996). The degradation rate of pesticides is influenced by their bioavailability (Heitzer et al., 1992) and physico-chemical parameters such as pH and soil type (Aislabie and Lloydjones, 1995; Andrea et al., 2000). It is noteworthy that soil pH is among the most important factors controlling pesticide biodegradation and a positive correlation between soil pH and degradation rates has been described in several recent studies (Bending et al., 2003; El-Sebai et al., 2005; Rasmussen et al., 2005; Sun et al., 2009). Several studies have reported the adaptation of soil microflora to the enhanced IPU degradation in agricultural soils regularly exposed to this herbicide (Walker et al., 2001; Bending et al., 2003; El-Sebai et al., 2005). These observations have stimulated the research aimed at isolating and characterising microbial strains able to entirely mineralize IPU. *Sphingomonas* sp. SRS2 (Sørensen et al., 2001), *Arthrobacter* sp. N2 (Tixier et al., 2002), *Sphingomonas* sp. F35 (Bending et al., 2003) *Methylopila* sp. TES (El-Sebai et al., 2004), and *Sphingobium* strains YBL1, YBL2 and YBL3 (Sun et al., 2009) are the bacterial strains isolated and characterized for degradation of IPU and other phenylurea herbicides. These bacterial isolates have different capabilities for degradation of IPU and other structurally related phenylurea herbicides.

Although described in details for *Sphingomonas* sp. SRS2, the IPU metabolic pathway still remains to be elucidated for other isolates. Transient accumulation of MDIPU (3-(4-isopropylphenyl)-1-methylurea) following demethylation of the dimethylurea side chain of IPU has already been described as the initial step of degradation pathway (Sørensen et al., 2001; Sun et al., 2009). Some of the metabolites [MDIPU, DDIPU ((3-(4-isopropylphenyl)-urea) and 4-IA (4-isopropylaniline)] formed during the IPU degradation by *Sphingomonas* sp. SRS2 were identified and it was proposed that IPU metabolism is initiated by two successive N-demethylations, followed by the cleavage of the urea side chain and finally by the mineralization of the phenyl structure (Sørensen et al., 2001). Sørensen et al. (2003) also proposed different parallel degradation pathways for IPU by defined microorganisms in agricultural soils.

It should be noticed that most of the studies related to IPU degradation have been carried out with pure bacterial isolates from agricultural soils. Several studies have indicated that multi-member bacterial consortia are involved in the degradation of different pesticides (Udikovic-Kolic et al., 2008). One previous study reports the enhancement of IPU-degrading capability of *Sphingomonas* sp. SRS2 when grown in co-culture with strain SRS1, not able to degrade IPU (Sørensen et al., 2002). This observation suggests that bacterial consortia may also be involved in IPU degradation and that such synergistic interactions may have been overlooked previously. The current study was aimed at examining the complexity of an IPU degrading bacterial community isolated from an agricultural soil treated regularly for over a decade with IPU. In this study, we have reported for the first time the characterization of an IPU-mineralizing bacterial culture enriched from a French agricultural soil. The initial steps of the IPU metabolic pathway used by this bacterial culture were proposed based on the transient accumulation of three IPU metabolites during the mineralization process. The capability of this bacterial culture to degrade different phenylurea herbicides and the effect of pH on the IPU degradation kinetics were studied in liquid culture. Some of the bacterial members of this culture were isolated and tested for IPU degradation as well as for determining the diversity of the culture.

2. Materials and Methods

2.1. Soil

Soil was sampled from an agricultural field located at "le Souich, France". This field has annually been treated with IPU (1.8 kg ha^{-1}) for ten years. Soil physico-chemical characteristics were: clay 17.2%, fine silt 29.7%, coarse silt 44.2%, fine sand 8.8%, coarse sand 1.2%, moisture content 25%, organic carbon 14.6 g kg^{-1}, organic nitrogen 1.9 g kg^{-1}, C/N ratio 7.8, Cation Exchange Capacity (CEC) 11.12 mMol 100 g^{-1}, and pH (in water) 7.4 (El-Sebai et al., 2004). Surface soil samples (0-15 cm) were collected, sieved to 5 mm and stored at 4 °C until used.

2.2. Herbicides

IPU (99.0% purity), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, 99.4% purity), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) and monolinuron (3-(4-dichlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) were purchased from Riedel de Haen (Germany). Chlorotoluron (3-(3-chloro-p-tolyl)-1,1-dimethylurea, 99.7% purity) was obtained from Chem Service (West Chester, USA). Analytical grade standards of the IPU metabolites MDIPU (99.4% purity), DDIPU (purity) and 4-IA (99.5% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsberg, Germany). ^{14}C ring labelled IPU (specific activity 18 mCi.$mmol^{-1}$; 99% radiochemical purity) was purchased from International Isotopes (Munich, Germany).

2.3. Culture Media

A mineral salt (MS) medium (Rousseaux et al., 2001) was used to estimate the capability of the bacterial culture to degrade IPU (provided as sole source of carbon and nitrogen) during enrichment cultures. For liquid culture studies, 50 mg L^{-1} of IPU was added, whereas, a solid IPU-MS medium was obtained by adding 15 mg L^{-1} agar (Biokar Diagnostics, France) and 2 mL L^{-1} of methanol dilution of IPU giving a final concentration of 500 mg L^{-1}. Isolation of the bacterial strains was done on Luria-Bertani medium (LB) [tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (5 g L^{-1})] and MS medium supplemented either with 100 mg L^{-1} of casamino acids (Difco, Becton Dickinson, France) or with a defined mixture of different amino acids (Sørensen et al., 2001). A mineral salt (Knapp) buffer (Devers et al., 2004) was used to determine the capabilities of the bacterial culture to degrade different phenylurea herbicides. To assess the pH effect on degradation kinetics, pH was adjusted to targeted values by using 37% HCl and 3 M NaOH.

2.4. Enrichment cultures and isolation of bacterial strains

Selective enrichment cultures were established from soil slurries in order to isolate the IPU degrading bacteria. Briefly, 10 g of the soil of "le Souich" (dry weight equivalent) was added to 90 mL of IPU-MS medium and incubated on an orbital shaker (150 rpm) at 20 °C. When about 50% of the initially added IPU was degraded, 10 mL of the soil slurry was transferred to 90 mL of fresh IPU-MS medium and incubated again under the same conditions. After the first 4 enrichments, 12 successive enrichment cycles were performed by transferring 100 µL of the IPU degrading culture to 10 mL of the fresh IPU-MS medium. A bacterial culture having a stable community structure revealed by ARDRA (Amplified Ribosomal DNA Restriction Analysis) was obtained after performing about 16 enrichments. The bacterial culture was serially diluted and 100 µL aliquot of the fresh enrichment culture dilutions (10^{-3} to 10^{-6}) was plated on IPU-MS, MS supplemented with casamino acids, MS supplemented with amino acids and LB agar media. Different types of bacterial strains appearing on the media were purified by successive isolations on Petri plates and then preserved as stock in 25% sterile glycerol and kept frozen at -80 °C for further experiments.

2.5. Determination of purity and of the ARDRA fingerprint of the bacterial culture

The composition of the bacterial culture was determined by amplifying the 16S and 18S rRNA genes by polymerase chain reactions (PCR), whereas, the fingerprint of the bacterial culture, revealing its dominant members, was assessed by ARDRA profiles at different times during the enrichment procedure.

2.5.1. 16S and 18S rRNA gene amplification

The cells of the bacterial culture were treated with proteinase K (Cheneby et al., 2004) before the PCR. Fungal 18S rRNA gene was amplified from the proteinase K extract of the bacterial culture by PCR using the primers nu-SSU-0817-5' (TTAGCATGGAATAATRR AATAGGA) and nu-SSU-1536-3' (ATTGCAATGCYCTATCCCCA) with the same PCR conditions as previously described by Edel-Hermann et al. (2004). Bacterial 16S rRNA gene was amplified using the universal primers 27f (5'-AGA GTT TGA TCH TGG CTC AG-3') and 1492r (5'-TAC GGH TAC CTT GTT ACG ACT T-3') (Gurtler and Stanisich, 1996). The amplification reaction was carried out in a final 25 µL volume containing 2.5 µL of 10X Taq polymerase buffer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 µM of each primer and 0.625 U of Taq polymerase. 2.5 µL of proteinase K extract was used as template for the PCR

reactions. PCR was performed in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Mass) according to the following program: 1 cycle of 4 min at 94°C; 39 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C and one final cycle of 5 min at 72 °C. The amplified 16S rRNA products were purified by MinElute PCR Purification Kit according to manufacturer's recommendations (QIAGEN, France).

2.5.2. ARDRA bar coding

For the determination of ARDRA profiles, 16S rRNA PCR products were digested with restriction enzyme *Alu*I (Fermentas, France). The restriction fragments obtained were separated on 3% High Resolution (MP Q-BIOgene, America) agarose gel. ARDRA profiles and the size of the fragments were estimated by comparing with DNA Molecular Weight Marker VIII (Roche Applied Science, France) run on the same gel.

2.6. Extraction of genomic DNA from the bacterial culture

For phylogenetic characterization, the genomic DNA of the IPU-degrading bacterial culture was extracted using QIAGEN Genomic DNA Isolation Kit (QIAGEN, France) according to the provider's recommendation.

2.7. ARDRA, cloning and sequencing of 16S rRNA gene of the bacterial culture

16S rRNA genes of the genomic DNA of the bacterial culture were amplified by PCR and purified as described above. The purified PCR products were cloned in pGEMT-EasyII vector according to manufacturer's recommendations (Promega, Madison, WI, USA). ARDRA profiles were determined after amplifying the 16S rRNA genes of the recombinant clones in the same way as described above. Based on the ARDRA profiles, the recombinant clones from the genomic DNA of the bacterial culture were categorized into different families and one member from each family was amplified by PCR using the SP6 and T7 universal primers under the following conditions: 94 °C for 45 s, 35 cycles of 55 °C for 45 s, 72 °C for 90 s and a final extension step at 72 °C for 7 min. Partial sequencing was performed using the 341f (5'-CCT ACG GGA GGC AGC AG-3') and 926r (5'-CCG TCA ATT CMT TTR AGT TT-3') primers with the CEQ2000 Dye Terminator Cycle Sequencing following the provider's recommendations (Beckman Coulter, France). Partial 16S rRNA gene sequences were deposited in the GenBank database under GQ365182-GQ365191 accession numbers. These sequences were compared to the known nucleotide sequences using the program BlastN (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignments of the sequences were

carried out using the software ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) and the data obtained were treated with the software NJ Plot to construct a phylogenetic tree according to neighbour joining method.

2.8. Rarefaction and microbial diversity analysis of the bacterial culture

To estimate the microbial diversity based on the presence of different ARDRA families obtained from the bacterial culture, rarefaction calculations were done by using the software Analytic Rarefaction (version 2.1; Stratigraphy laboratory, University of Georgia). The diversity was also estimated by the determination of the Simpson's Index of diversity (1-D) and reciprocal (1/D) of the Simpson's index (Simpson, 1949).

2.9. Phylogenetic analysis of the bacterial isolates

The phylogenetic analysis of the isolates was carried out by performing genetic fingerprinting using ARDRA and further sequencing of 16S rRNA genes as described above.

2.10. IPU mineralization kinetics of the bacterial culture

IPU mineralization kinetics of the bacterial culture was determined by radiorespirometery using ^{14}C ring-labelled IPU in Knapp buffer. Briefly, the culture was suspended in 10 mL of 50 mg L^{-1} of IPU and 1 kBq ^{14}C ring-labelled IPU to obtain an optical density (600 nm) of 0.5. Microbial cultures (n = 3) were incubated at 28 °C under agitation (125 rpm) in closed respirometer jars. $^{14}CO_2$ resulting from the mineralization of ^{14}C-IPU was trapped in 5 mL of 0.2 M NaOH. Traps were regularly replaced with fresh ones and $^{14}CO_2$ in the NaOH traps was estimated by liquid scintillation counter (Packard 1900TR-Tricarb) using 10 mL of scintillation fluid (ACSII, Amersham). The parameters of the mineralization kinetics were determined by fitting modified Gompertz model (Zwietering et al., 1990) to the mineralization data using the Sigma Plot 4.0 program.

2.11. Capability of the bacterial culture to degrade phenylurea herbicides

The capability of the bacterial culture to degrade IPU and other phenylurea herbicides i.e. diuron (λ_{max} = 248 nm), linuron (λ_{max} = 246 nm), monolinuron (λ_{max} = 243 nm) and chlorotoluron (λ_{max} = 240 nm) was estimated by HPLC analysis of liquid media samples. The culture was suspended in 50 mL of 50 mg L^{-1} of the respective phenylurea herbicides to obtain an optical density (600 nm) of 0.5. The samples were incubated at 28°C under agitation (125 rpm). An aliquot (800 µL) from each of the bacterial suspensions was taken regularly and then centrifuged (3220 *g*, 10 min) to have clear supernatant (650 µL). The supernatant of

each aliquot sample was then analysed by HPLC to quantify the remaining phenylurea herbicides in the media during the course of biodegradation kinetics. The HPLC consisted of an automatic injector (Star-410), a Microsorb-MV C18 column (25 cm long with 4.6 mm id, Varian) and a UV detector (Wavelength 243 nm). The HPLC analysis was performed under isocratic conditions and the mobile phase was composed of a mixture of acetonitrile and water (75/25, v/v). The flow was maintained at 1.0 mL min^{-1} and the column was placed at 20 °C.

2.12. Effect of pH on degradation kinetics by the bacterial culture

The effect of pH on IPU degradation kinetics by the culture was determined in Knapp buffers containing 50 mg L^{-1} of IPU. The pH values of the buffers were adjusted to 5.5, 6.5 or 7.5. The bacterial cells grown in the liquid MS medium were centrifuged, washed twice with respective buffers and then inoculated in triplicate to an optical density (600 nm) of 0.2 in the Knapp buffers having pH 5.5, 6.5 and 7.5. They were incubated at 28 °C with 125 rpm and samples were regularly taken for HPLC analysis. The impact of pH on IPU degrading parameters (lag phase, maximum rate of IPU degradation) estimated by fitting modified Gompertz model was assessed by subjecting them to a single factor analysis of variance followed by a Fisher procedure ($n = 3$, $p < 0.001$) (Statview 4.55 software, Abacus Concept, Inc.).

2.13. Study of mineralization pathway and identification of intermediate IPU metabolites

Degradation of IPU (λ_{max} = 241 nm) and transitory occurrence of known IPU metabolites i.e. MDIPU (λ_{max} = 241 nm) , DDIPU (λ_{max} = 239 nm) and 4-IA (λ_{max} = 242 nm) at different intervals during the IPU degradation kinetics were measured in the liquid medium by an isocratic Ultra Performance Liquid Chromatography (UPLC)-method employing a UPLC-system (Waters, ACQUITY UPLC) with a photodiode array detector scanning the interval of 204–260 nm. Chromatography was performed on a Waters ACQUITY UPLC BEH C18 (1.7 µm, 2.1 x 50 mm) column with a mobile phase consisting of methanol and 0.01% formic acid (32:68, v/v), a column temperature of 35 ^{o}C and a flow rate of 0.65 mL min^{-1}. The UPLC retention times of the metabolites (MDIPU, DDIPU and 4-IA) were used to identify metabolites occurring during IPU degradation. MassLynx software, version 4.1 (Waters) was used for data acquisition and processing. Samples for UPLC were prepared by filtration through sterilized 0.2 µm filters into UPLC glass vials and kept frozen at -20 °C until analysis.

2.14. Capability of the bacterial isolates to degrade IPU and its known metabolites

All the isolated bacterial strains were grown overnight individually in the liquid LB medium at 28 °C. For each isolate, the grown cells were harvested by centrifugation, washed twice with Knapp buffer and then resuspended in MS media. For pure culture studies, each bacterial suspension was used to inoculate the different liquid MS media containing 50 mg L^{-1} of either IPU or its known metabolites. All the six bacterial isolates were also tested in co-culture with each other. All the samples were incubated at 28 °C under agitation (125 rpm). The degradation of IPU and its metabolites was studied by HPLC as described above.

3. Results and Discussion

3.1. Isolation of IPU mineralizing bacterial culture

Enrichment culture experiments were carried out for the isolation of IPU mineralizing bacterial culture as previously described in several studies regarding the isolation of phenylurea herbicides degrading bacterial strains (Cullington and Walker, 1999; Sørensen et al., 2001; El-Sebai et al., 2004; Sun et al., 2009). Enrichment cultures were performed from the soil samples of "le Souich" (France) characterized by enhanced IPU mineralization (El-Sebai et al., 2004). Enrichment cultures were initiated in a mineral salt medium containing IPU as a sole source of carbon and nitrogen. Successive transfers of the enrichment cultures to fresh IPU-containing liquid medium were performed to obtain a stable bacterial culture able to degrade IPU as observed by HPLC analysis of the culture media (data not shown). In a first approach, the composition of the IPU degrading bacterial culture was examined by PCR amplification of either the bacterial 16S rRNA or the fungal 18S rRNA genes. A signal was obtained only for 16S rRNA gene showing the presence of the bacterial species (data not shown). For the assessment of stability of the composition of the bacterial culture, ARDRA profile analysis was conducted at different enrichments. The same type of ARDRA profiles (data not shown) were observed in different steps of the enrichments, thus indicating that the community structure of the bacterial culture remains stable after 16 rounds of subculturing.

3.2. Characterization of IPU mineralization capabilities of the bacterial culture

The ability of the bacterial culture to rapidly mineralize IPU was determined by radiorespirometric experiments using the ^{14}C-ring labeled IPU. Over an incubation period of

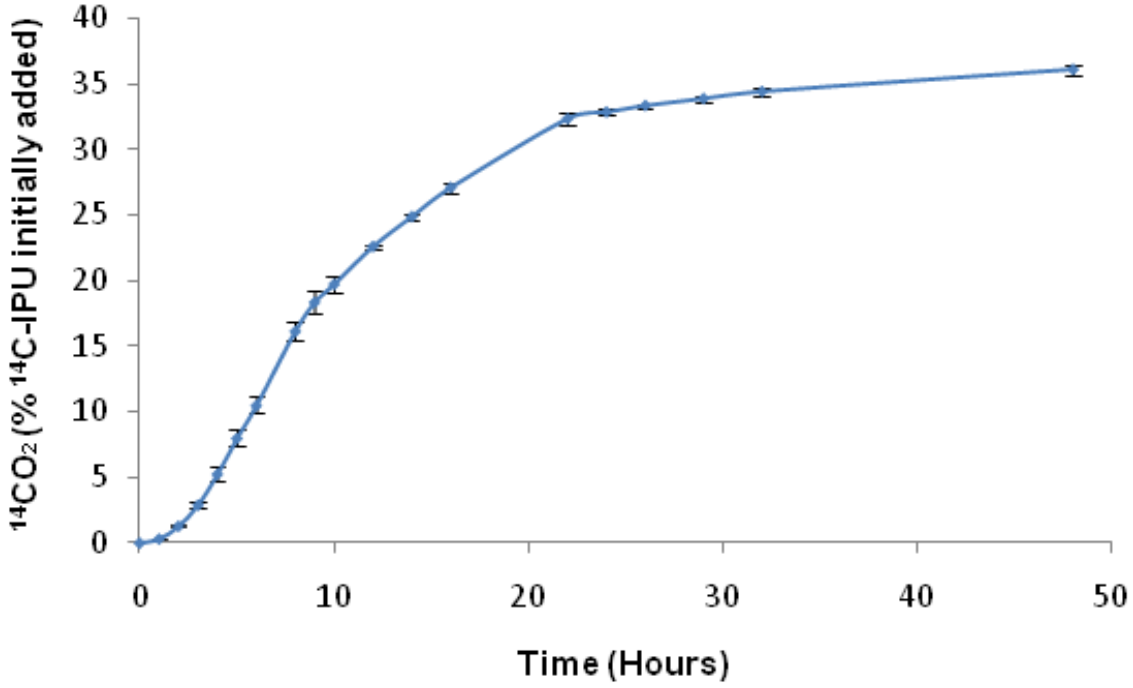

Fig. IV-1. Kinetics of mineralization of ^{14}C-ring labelled isoproturon by the bacterial culture (initial optical density of 0.5) incubated at 28°C in Knapp buffer under agitation (150 rpm). Error bars represent the standard error of the means.

48 h, about 95% of the initially added IPU disappeared from the liquid culture medium and the culture mineralized about 36% (±0.4%) of the initially applied ^{14}C-ring labeled IPU to $^{14}CO_2$ (Fig. IV-1). Determination of the remaining radioactivity revealed that up to 35% (±1.5%) of the initially added radioactivity was detected in the bacterial pellet suggesting that it was incorporated in the bacterial biomass and only about 5% remained in the culture media. The ^{14}C mass balance was not equilibrated. This could either be attributed to the limitations due to $^{14}CO_2$ trapping particularly during the exponential phase or due to some possible interactions of the bacterial components with the liquid scintillation. The parameters of IPU mineralization kinetics were described by using the modified Gompertz model (Zwietering et al., 1990) as already used for many other degradation studies (El-Sebai et al., 2005; Li et al., 2005). The parameters of mineralization calculated by modeling revealed that a short lag phase (λ) of 1 h was observed when mineralization was not initiated and the maximum rate of mineralization (μm) was 1.2 mg L^{-1} h^{-1}. Up to now different bacterial strains having varied abilities to degrade IPU have been isolated from the soils under diverse climatic conditions (Sørensen et al., 2001; Turnbull et al., 2001; Tixier et al., 2002; El-Sebai et al., 2004; Sun et al., 2009). *Sphingomonas* sp. SRS2 (Sørensen et al., 2001), *Methylopila* sp. TES (El-Sebai et al., 2004), *Sphingomonas* sp. F35 (Bending et al., 2003) and *Sphingobium* sp. Strains YBL1, YBL2, YBL3 (Sun et al., 2009) are the bacterial strains which have been characterized for the IPU mineralization, whereas, the strains *Arthrobacter globiformis* D47 (Cullington and Walker, 1999; Turnbull et al., 2001) and *Arthrobacter* sp. N2 (Tixier et al., 2002) could not mineralize IPU and only had the ability to transform IPU to its corresponding aniline derivative (4-IA) by the hydrolysis of the urea side chain. Isolation of this bacterial culture is considered as a second report showing a bacterial culture involved in IPU mineralization (Sørensen et al., 2002) and as a second study related to the IPU mineralization in French agricultural soils (El-Sebai et al., 2005). The amount of $^{14}CO_2$ evolved from the mineralization of ^{14}C-ring labeled IPU was almost similar to that of *Methylopila sp.* TES, already isolated from the soil of "Le Souich" (El-Sebai et al., 2004).

IPU degradation kinetics by the bacterial culture was also studied in detail by using the HPLC analysis. It was found that IPU (50 mg L^{-1}), added to the medium as sole source of carbon and nitrogen, was almost entirely degraded within 20 h of incubation (Fig. IV-2a). Analysis of the samples collected during the IPU degradation kinetics by UPLC to search for known IPU metabolites revealed the transitory accumulation of three known IPU metabolites (MDIPU, DDIPU and 4-IA) which were observed at different times during the degradation

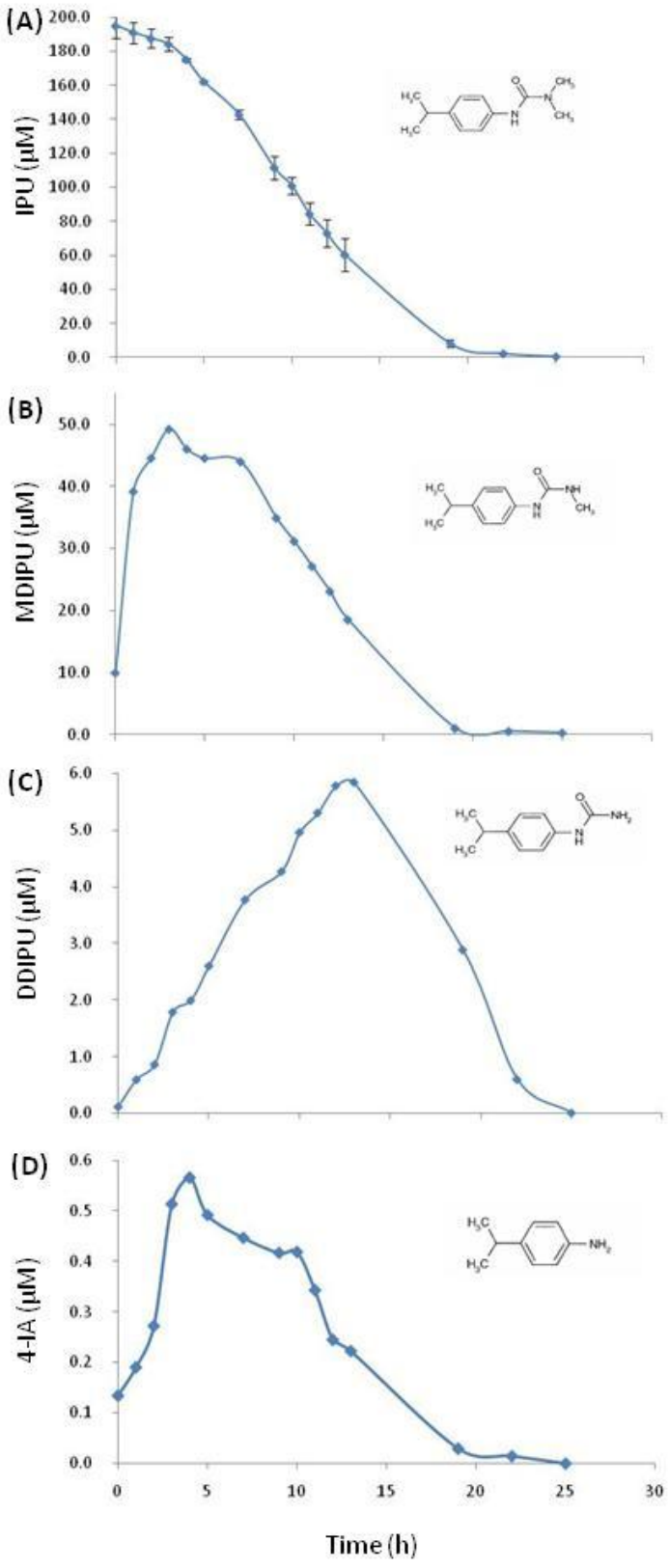

Fig. IV-2. Transitory accumulation and degradation of IPU metabolites during the IPU degradation kinetics. **A-** IPU degradation kinetics by the bacterial culture inoculated in Knapp buffer at 28°C under agitation (150 rpm) with **B-**, **C-** and **D-** showing the transitory accumulation and degradation of IPU metabolites MDIPU, DDIPU and 4-IA, respectively, during the IPU degradation kinetics.

kinetics (Fig. IV-2.). MDIPU has been reported to be the main metabolite produced during IPU degradation in several agricultural soils (Cox et al., 1996; Berger, 1999; Juhler et al., 2001). The intermediate accumulation of MDIPU during the IPU degradation by this culture shows that the metabolic pathway is initiated by N-demethylation of the side chain which has already been described as the first and apparently limiting step in IPU degradation by *Sphingomonas* sp. SRS2 (Sørensen et al., 2001) and *Sphingobium* sp. YBL2 (Sun et al., 2009). The transient accumulation of DDIPU and 4-IA observed successively during the degradation suggested that the metabolic pathway proceeds by N-demethylation of MDIPU to form DDIPU followed by cleavage of the urea side chain to form 4-IA. It is noteworthy that this sequential degradation has already been reported for *Sphingomonas* sp. SRS2 (Sørensen et al., 2001). So, we could conclude that this bacterial culture follows an IPU metabolic pathway similar to that proposed for *Sphingomonas* sp. SRS2 (Sørensen et al., 2001). The culture however appeared to mineralize IPU faster than both strain SRS2 (Sørensen et al., 2001) and the previously described coculture (Sørensen et al., 2002) suggesting that synergistic interactions within this multimember bacterial culture ensures a fast and efficient IPU degradation.

The capability of the bacterial culture to degrade other phenylurea herbicides i.e. diuron, linuron, monolinuron and chlorotoluron was assessed. Surprisingly, we showed that this bacterial culture, at least in resting cell experiments, did not degrade any of these structurally related phenylurea herbicides during incubation for up to 80 h (data not shown). Keeping the previous studies in view, it can be noticed that different bacterial isolates have been characterized for the degradation of IPU and other phenylurea herbicides (Sørensen et al., 2001; Turnbull et al., 2001; El-Sebai et al., 2004; Sun et al., 2009) but all of them had different extents and degrees of degradation. *Sphingomonas* sp. SRS2 (Sørensen et al., 2001) was reported to degrade the closely related dimethylurea-substituted herbicides, such as diuron and chlorotoluron. The *Sphingobium* strains YBL2 and YBL3 (Sun et al., 2009) harboured similar metabolic capabilities as that of *Sphingomonas* sp. SRS2. In contrast to *Sphingomonas* sp. SRS2, the strain *A. globiformis* D47, isolated from Deep Slade agricultural field (UK), had the ability to transform IPU and many other phenylurea herbicides to their corresponding aniline derivative by the hydrolysis of the urea side chain (Cullington and Walker, 1999; Turnbull et al., 2001). *Sphingobium* sp. YBL1 had the ability to degrade most of the phenylurea herbicides as well as related anilines (Sun et al., 2009). Up to now only one bacterial isolate, *Methylopila* sp. TES, was shown to exclusively mineralize IPU without any

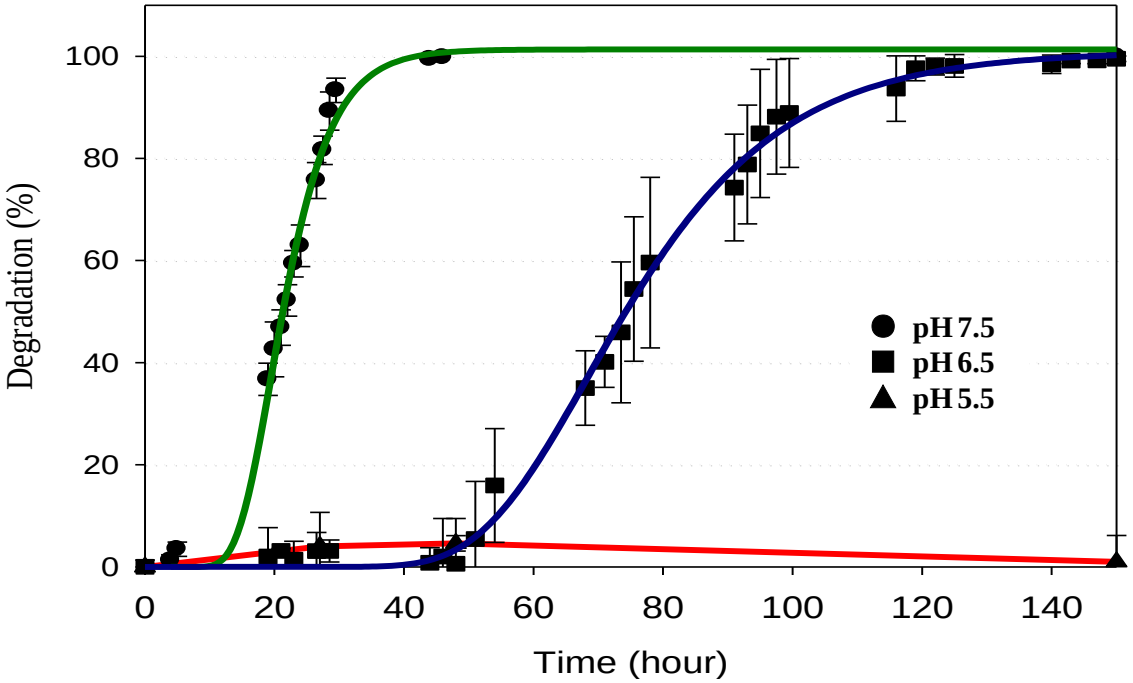

Fig. IV-3. IPU degrading kinetics by the bacterial culture observed at pH values of 5.5, 6.5 and 7.5 in the Knapp buffer. Error bars represent the standard errors of the mean.

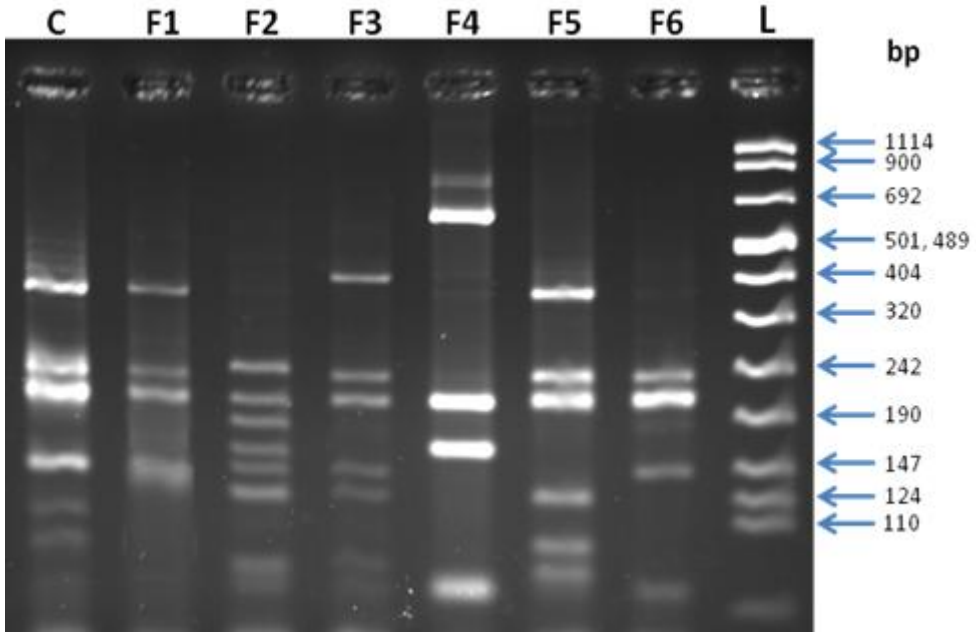

Fig. IV-4. ARDRA patterns of the six clone families (F1-F6) generated by digestion with *AluI*. The lane L is the BVIII molecular weight marker (sizes indicated in base pairs). The lane C is the ARDRA of the bacterial culture. The lanes F1, F3, F5 and F6 represent the *Sphingomonas* families with F1 as a dominant one. The lanes F2 and F4 represent the ARDRA profiles of *Agrobacterium* and *Pseudomonas* respectively.

activity towards other structurally related phenylurea herbicides (El-Sebai et al., 2004). Based on these findings, it was hypothesized that this strain might be using an IPU-metabolic pathway different from that of *Sphingomonas* sp. SRS2. Here, we showed that although this bacterial culture was able to mineralize IPU only, it still seems to follow the same metabolic pathway as that of *Sphingomonas* sp. SRS2. So, keeping the above observations in view, one could hypothesize that *Methylopila* sp. TES (El-Sebai et al., 2004) and the current IPU-mineralizing culture harbor the enzymatic capacities highly specific for IPU degradation which is different from that of the *Sphingomonas* (Sørensen et al., 2001; Bending et al., 2003) and *Sphingobium* (Sun et al., 2009) strains apparently showing a broader substrate range.

3.3. pH range for IPU degradation

It is well known that pesticide biodegrading activity of the soil micro biota is deeply influenced by pedoclimatic conditions (Smith et al., 1997; Andrea et al., 2000). Among the physico-chemical properties, pH is considered to play a key role being responsible for the regulation of pesticide degrading capabilities of microorganisms (Bending et al., 2001; Walker et al., 2001; Bending et al., 2003; Rasmussen et al., 2005). An experiment was performed to assess the effect of pH on IPU mineralization capabilities of the bacterial culture. We showed that IPU degradation was almost entirely inhibited at pH 5.5, whereas, efficient IPU degradation was observed at pH values of 6.5 and 7.5 (Fig. IV-3). Modeling of IPU degradation kinetics further confirmed that IPU degradation rate was strongly affected by pH. Analysis of the variance revealed that the µm was significantly higher at pH 7.5 (7.1±0.4 h^{-1}) as compared to that at pH 6.5 (2.2±0.2 h^{-1}). At the same time, it was found that the λ was reduced at pH 7.5 (14.3±0.6 h^{-1}) as compared to that at pH 6.5 (51.4±5.1 h^{-1}). Complete degradation of IPU was observed after about 120 and 30 h of incubation at pH values of 6.5 and 7.5 respectively. These results revealing a clear effect of pH on IPU degradation are in accordance with that of *Sphingomonas* sp. SRS2 (Bending et al., 2003) and *Sphingobium* sp. YBL2 (Sun et al., 2009). These results are also a further confirmation of many of the previous studies which had demonstrated a significant positive correlation between the soil pH and the degradation of different pesticides (Smith et al., 1997; Houot et al., 2000; Bending et al., 2001; Walker et al., 2001). Recently, geostatistical analysis further highlights the importance of soil pH to regulate IPU mineralization activity (El-Sebai et al., 2007). Thus, the effect of pH on biodegradation seems to be a general feature of the pesticide-degrading communities. Although pH effect is clearly shown, yet the underlying mechanisms responsible for the regulation of the IPU degradation remain to be elucidated.

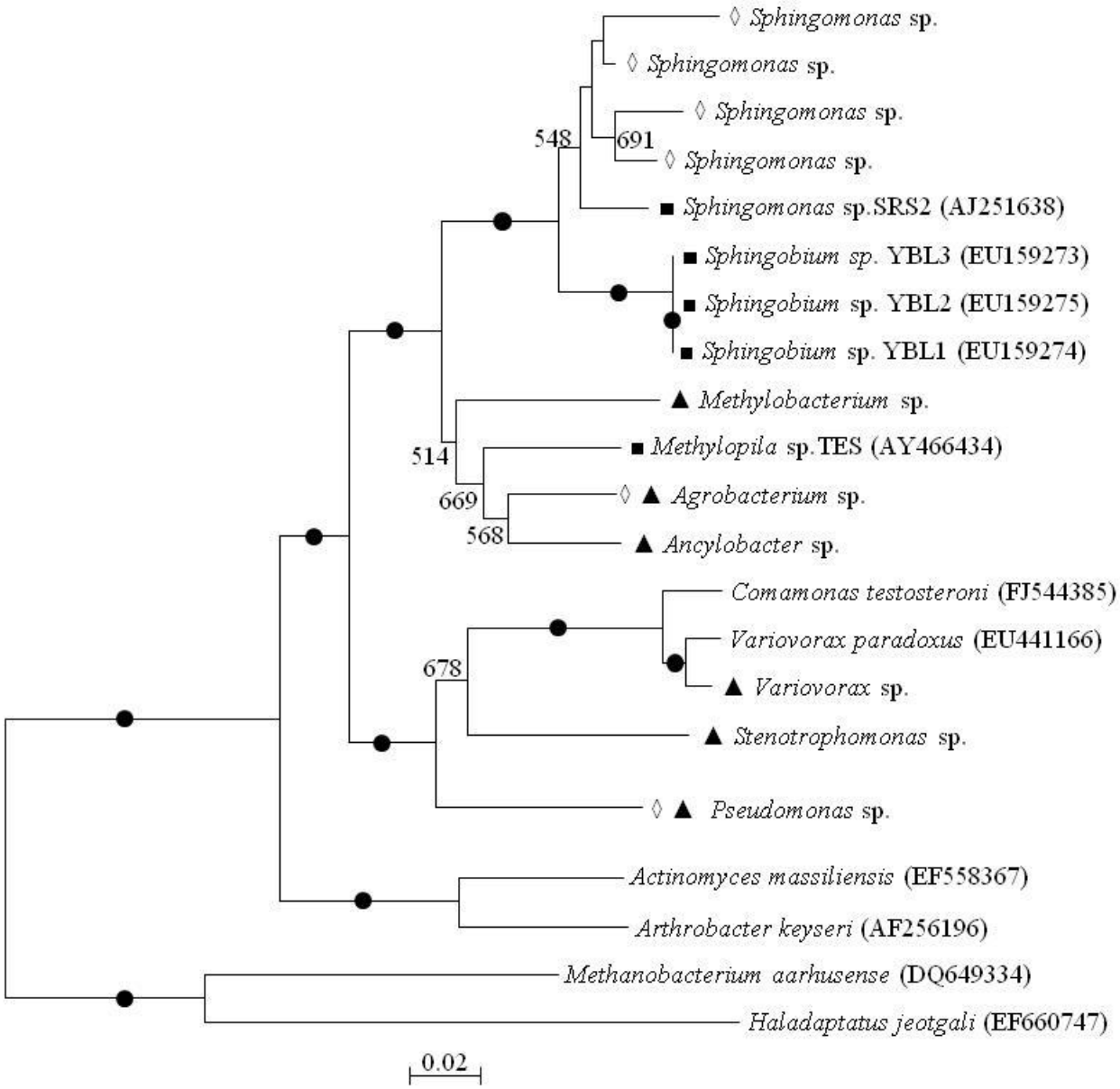

Fig. IV-5. Neighbour-joining phylogenetic analysis based on 16S rRNA gene fragments showing the relationship between the major ARDRA families of the genomic DNA of the bacterial culture, the isolated bacterial strains and IPU degrading bacterial strains already found in the GenBank database. The bacterial strains isolated from the bacterial culture are indicated with a black triangle (▲); the bacterial strains belonging to different ARDRA families revealed during the cloning of 16S rRNA gene of the genomic DNA of the culture are indicated with a rectangle (◊), whereas, the black square (■) indicates some of the IPU degrading bacterial strains already found in the GenBank database. The accession numbers of the strains from the GenBank database, used for phylogenetic analysis, are given in brackets.

3.4. Isolation and characterization of the culture members

A library based on 16S rRNA gene cloning and sequencing was prepared to estimate the diversity within the IPU-mineralizing bacterial culture. In a first approach, the analysis of the ARDRA profile of the bacterial culture consisted of numerous bands (data not shown) indicating a relatively high diversity within the IPU-mineralizing culture. By the cloning of 16S rRNA gene amplified from the genomic DNA of the bacterial culture, only six different clone families were identified after screening 35 clones (Fig. IV-4). When the sequences of the six clone families were compared to the known sequences available in the GenBank database, four were found to be closely related to the genus *Sphingomonas* (> 98% similarity for each one) and two other families belonged to the genera *Pseudomonas* and *Agrobacterium* (Fig. IV-5) with one of the *Sphingomonas* strains presented as a dominant member (> 80% abundance) of the bacterial culture. It was noted that the ARDRA profile of this *Sphingomonas* strain resembled the dominant ARDRA profile of the culture (Fig. IV-4). In total, *Sphingomonas* strains represented about 90% abundance of the members in this bacterial culture. It is noteworthy that several studies have already reported the involvement of the bacterial strains belonging to the Sphingomonadaceae family including *Sphingobium* (Sun et al., 2009) and *Sphingomonas* (Sørensen et al., 2001, 2002; Bending et al., 2003) isolates in IPU mineralization isolated from geographically distant soils including Denmark, United Kingdom and China. A similar pattern with involvement of closely related *Sphingomonas* spp. strains has also been indicated in a study involving DNA extractions from soil without any cultivation or enrichment involving media (Bending et al., 2003). This indicates that this observation is not merely a bias caused by enrichment and cultivation under laboratory conditions. The presence of highly similar genotypes in our culture builds on this and suggests that this group of bacteria has a wide geographical distribution and rapidly adapts to IPU exposure seemingly being able to use this herbicide as a source of carbon and nitrogen.

In order to estimate the diversity of the bacterial culture, rarefaction curve (Fig. IV-6) analysis was performed on the families of the clones of its genomic DNA. This curve indicated a relatively lower degree of diversity based on the number of clones selected to determine it. The diversity of the culture was also quantitatively measured by using 1-D and 1/D indices. The values calculated on the basis of the clone families of the genomic DNA of the bacterial culture were 0.31 and 1.46 for 1-D and 1/D, respectively, indicating a relatively lower degree of diversity of the members of the bacterial culture which is in accordance to the

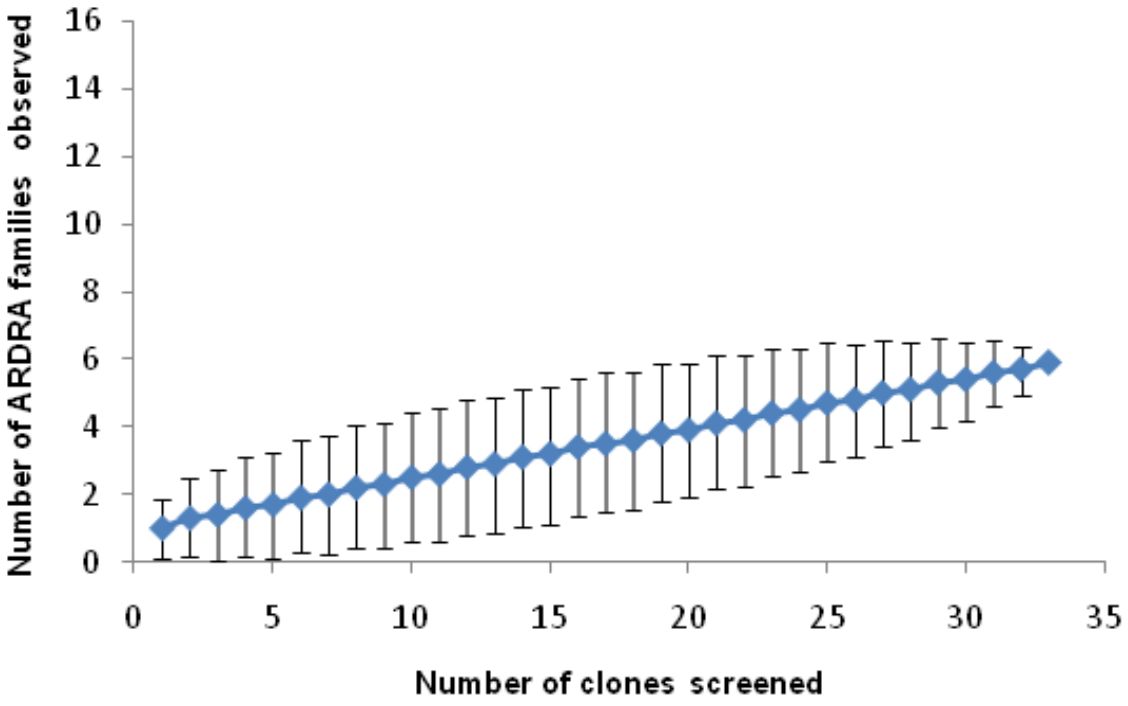

Fig. IV-6. Rarefaction curve for the observed diversity of ARDRA families of the clones from 16S rRNA cloning of genomic DNA of the bacterial culture

rarefaction curve. It can be assumed that the enrichment procedure involving mineral salt medium provided with IPU as sole source of carbon and nitrogen, exerted a high selection pressure thereby decreasing the diversity and selecting for a stable culture where carbon and nitrogen are shared. Similar networks of substrate sharing have been proposed for other bacterial consortia (De Souza et al., 1998; Pelz et al., 1999).

Pasteurian approaches were applied to isolate culturable members of the culture by plating as previously described (Sørensen et al., 2001; El-Sebai et al., 2004; Sun et al., 2009). As a result, several members were isolated and identified as belonging to six different phylotype families close to (> 98% similarity for each one) *Ancylobacter, Pseudomonas, Stenotrophomonas, Methylobacterium, Variovorax* and *Agrobacterium* genera (Fig. IV-5). All these isolates were characterized for the degradation of IPU and of its known metabolites. Unfortunately, none of them were found to be able to degrade these compounds although isolated on specific media. Co-culturing of all the six isolates further confirmed the absence of IPU degrading capability of these isolates.

It is noteworthy that among the six isolates, only two (*Pseudomonas* sp. and *Agrobacterium* sp.) were related to the 16S rRNA clone families of the bacterial culture. In addition, we were not successful in isolating *Sphingomonas* strains, representing the dominant family of the clones, on the different types of agar media used. It is in accordance with the previous study indicating that some of the putative species were difficult to culture although they were detectable by DGGE but were not cultivable on agar plates (El-Fantroussi, 2000). It was also hypothesized that different bacterial partners, instead of a single bacterial strain, act as a culture contributing to IPU mineralization, however, non-cultivable dominant *Sphingomonas* strains might play a key role in IPU mineralization. It is in accordance with previous degradation studies indicating that the degradation of some herbicides may require mixed bacterial cultures (Jimenez et al., 1991; Roberts et al., 1993; Assaf and Turco, 1994). Further work will aim at better describing the IPU degrading pathway through the identification of the genes coding the enzymes responsible for IPU mineralization by applying a genomic approach. The synergistic interactions underlying the rapid IPU mineralization performed by this culture will also be studied in further details.

4. Acknowledgments

The PhD work of Sabir Hussain was funded by Higher Education Commission, HEC (Pakistan) and the doctoral school of the University of Burgundy (France). The work of Sebastian R. Sørensen was funded by the Center for Environmental and Agricultural Microbiology, CREAM, funded by the Willum Kann Rasmussen Foundation.

Conclusion and Perspectives

We report here the characterization of a mixed bacterial culture enriched from the soil of Le Souich previously reported to demonstrate IPU mineralizing activity. The enriched bacterial culture a high ability to mineralize IPU but it was unable to degrade any of related phenylurea herbicides. Based on this finding, it was hypothesized that the genes coding IPU degrading enzymes are specific for isoproturon. Furthermore, the characterization of the metabolites formed during IPU transformation by UPLC analysis revealed the formation of three known IPU metabolites i.e. monodemethylisoproturon (MDIPU), didemthylisoproturon (DDIPU and 4-isopropylaniline (4-IA). Based on this observation, we hypothesized that the bacterial culture initiates IPU mineralization by two successive demethylations followed by cleavage of urea side chain and ultimately mineralization of the phenyl ring. This is in accordance with the metabolic pathway described from *Sphingomonas sp.* SRS2 (Sorensen et al., 2001). It was interesting and noteworthy that although this enriched bacterial culture and *Sphingomonas sp.* SRS2 had varying capabilities towards the degradation of different phenylurea herbicides, yet they seem to follow the same initial steps of IPU metabolism. The enriched bacterial culture showed an optimal IPU metabolic activity at pH 7.5. However, this bacterial culture was not able to degrade IPU at pH 5.5. None of the six bacterial strains isolated from this enriched bacterial culture was able to degrade either IPU or its metabolites. The bacterial culture was mainly composed of the bacterial populations related to genus *Sphingomonas* based on 16S rDNA cloning and sequencing. This result highlights the involvement of this genus in the degradation of a number of xenobiotics including substituted phenylureas. Keeping the results of this study in view, further research was carried out to isolate pure strain mineralizing IPU.

ISOLATION AND CHARACTERIZATION OF AN ISOPROTURON MINERALIZING BACTERIAL STRAIN

Introduction

In the previous chapter, my PhD work led to the isolation and the characterization of a bacterial culture able to mineralize isoproturon. However, this work did not lead to the isolation of a pure strain able to mineralize IPU. In order to get a pure isolate to conduct further work to characterize the genetic potential coding for IPU degrading enzymes. Although enhanced biodegradation of isoproturon has been well described in several agricultural soils (Sorensen et al., 2001; El-Sebai et al., 2005; El-Sebai et al., 2007) and several isoproturon degrading bacterial strains have been isolated (Sorensen et al., 2001; El-Sebai et al., 2004; Sun et al., 2009), yet the genes coding the degrading enzymes responsible for IPu degradation remain unknown. Indeed, the possibility of isolating a pure pesticide degrading bacterial strain still remains one of the simplest and best solutions to elucidate the genetic, physiological and metabolic processes involved in its degradation. The isolation procedure was consisting of serial enrichment cultures with IPU adapted soil in mineral salt medium using isoproturon as a sole source of carbon and nitrogen. After purification, the isolated strain was identified by 16S rDNA amplification, cloning and sequencing. The ability of the pure strain to mineralize isoproturon was determined by radiorespirometery using ^{14}C ring-labelled isoproturon. Degrading capability of the isolated bacterial strain was tested by studying the degradation of known isoproturon metabolites (MDIPU, DDIPU and 4-IA) and other structurally related phenylurea herbicides (linuron, monolinuron, chlorotoluron, diuron). pH regulation of the isoproturon degradation was also investigated by estimating IPU degradation at different pH values ranging from 5.5, 6.5, 7.5 and 8.5. In order to target the genes, possibly involved in the lower pathway, *catA* sequence coding for catechol 1,2-dioxygenase was amplified, cloned and sequenced. Catechol 1,2-dioxygenase which is involved in the *ortho*-cleavage of catechol, was recently detected during the degradation of aniline by an IPU degrading bacterial strain *Sphingobium* sp. YBL2 (Sun et al., 2009)

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Isolation and characterization of an isoproturon mineralizing *Sphingomonas* sp. strain SH from a French agricultural soil

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Abstract

The phenylurea herbicide isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), was found to be rapidly mineralized in an agricultural soil in France that had been periodically exposed to IPU. Enrichment cultures from samples of this soil isolated a bacterial strain able to mineralize IPU. 16S rDNA sequence analysis showed that this strain belonged to the phylogeny of the genus *Sphingomonas* (96% similarity with *Sphingomonas* sp. JEM-14, AB219361) and was designated *Sphingomonas* sp. strain SH. From this strain, a partial sequence of 1,2-dioxygenase (*cat*A) gene coding for an enzyme degrading catechol putatively formed during IPU mineralization was amplified. Phylogenetic analysis revealed that the *cat*A sequence was related to *Sphingomonas* sp. and showed a lack of congruence between the *cat*A and 16S rDNA based phylogenies, implying horizontal gene transfer of the *cat*A gene cluster between the soil microbiota.

The IPU degrading ability of the strain SH was strongly influenced by the pH with maximum degradation taking place at pH 7.5. SH was only able to mineralize IPU and its known metabolites including 4-isopropylaniline and it could not degrade other structurally related phenylurea herbicides such as diuron, linuron, monolinuron and chlorotoluron or their aniline derivatives. These observations suggest that the catabolic abilities of the strain SH are highly specific to the metabolism of IPU.

Keywords: biodegradation, isoproturon, phenylurea, catechol dioxygenase, pH

1. Introduction

Much attention has been paid to the use of crop protection products in conventional agriculture in recent years owing to rising public concern about environmental contamination and their impact on human health. Phenylurea herbicides are among the most widely used class of crop protection products and are of particular significance as several have been recorded as contaminants of agricultural catchments and water resources in various parts of the world including Europe (Field et al., 1997; Stangroom et al., 1998; Thurman et al., 2000; Sorensen et al., 2003). They are used as selective weed killers in cereal cultivation and are moderately mobile in soil (Sorensen et al., 2003). In view of the poor mineralization rate of the phenyl ring and potential carcinogenic effects of phenylurea herbicides and their metabolites (Scassellati-Sforzolini et al., 1997; Tixier et al., 2001), improved biodegradation of these compounds is of great importance.

Isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea], IPU, is one of the phenylurea herbicides used to prevent pre- and post-emergence weed development in winter cereals. IPU is used intensively, persists in soil, contaminates water resources where it is frequently detected in groundwater bodies at concentrations often exceeding the EU limit for drinking water (0.1 µg L^{-1}) (Spliid and Koppen, 1998; Muller et al., 2002). Although this compound has been banned in several countries or restricted to 1.2 kg $ha^{-1}year^{-1}$ since 2003 in several countries including France, it is still extensively used worldwide. Several studies have indicated the harmful effects of IPU and its metabolites on aquatic invertebrates, freshwater algae and microbial communities (Mansour et al., 1999; Widenfalk et al., 2008; Vallotton et al., 2009). IPU and its metabolites have also been suspected to be carcinogenic to human beings and animals (Behera and Bhunya, 1990; Hoshiya et al., 1993). Given these harmful effects, it is important to eliminate or minimize IPU contamination in soils and waters. Microbial biodegradation, which is the primary mechanism for dissipating IPU as well as other phenylurea herbicides from the soil (Fournier et al., 1975; Gaillardon and Sabar, 1994; Pieuchot et al., 1996), could prove a reliable, cost-effective remediation technique for IPU abatement. Several studies have reported the adaptation of soil microflora to IPU degradation in response to repeated exposure to this herbicide over a long period on fields under cultivation (Bending et al., 2003; Sorensen and Aamand, 2001; El-Sebai et al., 2005). The IPU degrading ability of soil microflora has been found to be influenced by various soil and environmental factors (Aislabie and Lloydjones, 1995; Andrea et al., 2000; Bending et al., 2003; El-Sebai et al., 2007), among which pH is considered to be a key factor regulating

biodegradation not only in pure cultures (Bending et al., 2003; Hussain et al., 2009b; Sun et al., 2009) but also in soils (Houot et al., 2000; El-Sebai et al., 2005).

The presence of an IPU degradation potential has been reported in a variety of bacterial and fungal species isolated from the soils of various regions throughout the world (Vroumsia et al., 1996; Khadrani et al., 1999; Castillo et al., 2001; Sorensen et al., 2001; Tixier et al., 2002; Bending et al., 2003; El-Sebai et al., 2004; Badawi et al., 2009; Hussain et al., 2009b; Sun et al., 2009). Of IPU degrading isolates, only *Sphingomonas* sp. SRS2 (Sorensen et al., 2001), *Methylopila* sp. TES (El-Sebai et al., 2004) and *Sphingobium* sp. strains YBL1, YBL2, YBL3 (Sun et al., 2009) were found to mineralize IPU completely to CO_2 and biomass.

Although the complete IPU metabolic pathway still remains to be determined, the initial steps and processes involved have been described in recent studies (Sorensen et al., 2001; Sorensen et al., 2003; Hussain et al., 2009b; Sun et al., 2009). Demethylation of the dimethylurea side chain of IPU resulting in transient accumulation of MDIPU (3-(4-isopropylphenyl)-1-methylurea) has been described as an initial, limiting step during IPU degradation (Sorensen et al., 2001; Hussain et al., 2009b; Sun et al., 2009). Based on the accumulation of the metabolites, it has been suggested that the IPU metabolic pathway is initiated by two successive N-demethylations, followed by the cleavage of the urea side chain resulting in transient accumulation of 4-isopropylaniline and finally by the mineralization of the phenyl structure (Sorensen et al., 2001; Hussain et al., 2009b). Various IPU metabolic pathways by defined microorganisms in agricultural soils were proposed by Sorensen et al. 2003). Recently, Sun et al. (2009) described the accumulation of catechol and the first metabolite after phenyl ring cleavage, cis,cis-muconic acid, during the mineralization of aniline by the IPU degrading *Sphingobium* sp. strain YBL2. A catechol degrading 1,2-dioxygenase gene coding for *cat*A, catalyzing the opening of phenyl ring of catechol in many Gram positive and Gram negative bacteria, was also amplified by PCR from the IPU degrading strain YBL2. It was suggested that catechol, which is considered as a key intermediate during the phenyl ring cleavage of aromatic compounds, could also be an intermediary metabolite during IPU mineralization.

This study was carried out to isolate and characterize a bacterial population able to mineralize IPU by applying enrichment cultures to an agricultural soil from France that had adapted to IPU mineralization as a result of repeated exposure to IPU. Taxonomic and genetic

Table V-1. Soil physical and chemical parameters [equivalent humidity, organic matter content, organic carbon, total nitrogen, C/N ratio, pH and cation exchange capacity (CEC)] and biological parameters [number of cultivable bacteria, microbial C biomass, maximum percentage of mineralization (A), maximum mineralization rate (μm) and lag phase (λ)] determined from 9 different top soil (0-20 cm) samples collected at the experimental station of INRA of Epoisses (Breteniere, France)

Parameter	Maximum	Minimum	Mean	SD	CV (%)
Equivalent Humidity (g $100g^{-1}$)	26.9	23.5	24.8	1.26	5.07
Organic matter (g kg^{-1})	37.2	26.4	30.8	4.1	13.4
Organic Carbon (g kg^{-1})	21.5	15.8	18.4	1.9	10.5
Total Nitrogen (g kg^{-1})	1.87	1.65	1.71	0.07	4.02
C/N	11.3	8.9	10.2	0.9	8.8
pH	7.83	7.66	7.76	0.06	0.76
CEC ($cmol^{+}kg^{-1}$)	22.3	21.0	21.59	0.38	1.77
Cultivable bacteria (log10 CFU g^{-1})	9.24	8.74	9.06	0.14	1.52
Microbial C Biomass (mg kg^{-1})	374.9	295.9	336.1	27.8	8.3
A (% $^{14}CO_2$)	45.3	39.5	42.1	1.8	4.3
μm (% $^{14}CO_2$ day^{-1})	4.57	2.48	3.68	0.71	19.23
λ (days)	0.76	0.42	0.57	0.10	17.19

characterization of the bacterial isolate was performed by cloning and sequencing 16S rDNA as well as 1,2-dioxygenase genes. The ability of the bacterial strain to degrade IPU metabolites, various phenylurea herbicides and their aniline derivatives was also characterized. As pH is considered to be one of the most important factors affecting the degrading capabilities of the soil microorganisms, its effect on IPU mineralization kinetics was also investigated.

2. Materials and methods

2.1 Soil sampling and characteristics

Nine soil samples (0-20 cm) were collected from an agricultural field periodically exposed to IPU located at the experimental farm of the French National Institute of Agronomical Research of Epoisses (Breteniere, France). The soil particle size distribution was clay 47.8%, fine silt 26%, coarse silt 18%, fine sand 3.5%, and coarse sand 4.7%. The physical and chemical characteristics of the soil samples are given in table V-1. The soil samples were sieved to 5 mm and stored at 4°C for less than one month until used.

2.2. Chemicals

Analytical grade IPU (99.0% purity), chlortoluron (3-(3-chloro-p-tolyl)-1,1-dimethylurea, 99.7% purity), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, 99.4% purity), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) and monolinuron (3-(4-chlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) were purchased from Riedel-de Haen (Germany). The IPU metabolites MDIPU (1-(4-isopropylphenyl)-3-methylurea, 99.0% purity), DDIPU (1-(4-isopropylphenyl) urea, 99.0% purity) and 4-IA (4-isopropylaniline, 99% purity) were purchased from Dr. Ehrenstorfer-Schafers (Augsburg, Germany). Aniline derivatives of various phenylurea herbicides [3-Chloroaniline, 4-Chloroaniline, 4-Bromoaniline, 3-Chloro-4-methylaniline, 3,4-Dichloroaniline (3,4-DCA)] were purchased from Sigma-Aldrich (Germany). ^{14}C-ring-labeled IPU (specific activity 18 mCi mmol^{-1}; 99% radiochemical purity) was purchased from International Isotopes (Munich, Germany).

2.3. Enrichment and growth media

Enrichment and microbial cell cultures were performed using a mineral salt medium (Rousseaux et al., 2001) containing IPU as the sole source of carbon and nitrogen at a concentration of 50 mg L^{-1} for liquid medium (MS-IPU) and 500 mg L^{-1} for solid medium (MSA-IPU). The bacterial strain was isolated on MSA-IPU and Nutrient agar (NA) medium

(Difco, France). Resting cell experiments were performed in a Knapp buffer (Devers et al., 2004). To assess the pH effect on degradation kinetics, the pH of the Knapp buffer was modified using 37% HCl or 3 M NaOH.

2.4. IPU mineralization potential of soil

The IPU mineralization potential of the soil samples was determined by radiorespirometry using ^{14}C-ring-labeled isoproturon as previously described (El-Sebai et al., 2005). 40 g equivalent dry weight of each soil was treated with 1.5 mg of IPU per kg of soil and 2 kBq of ^{14}C-ring-labeled IPU. The soil moisture was adjusted to 80% of water holding capacity and incubated at 20°C in the dark for 90 days in closed respirometer jars (Soulas, 1993). ^{14}CO2 resulting from mineralization of ^{14}C-ring-labeled isoproturon was trapped in 0.2M NaOH solution. The traps were changed regularly over the incubation period and analyzed for radioactivity content by liquid scintillation counting using ACSII scintillation fluid (Amersham).

2.5. Enrichment cultures and isolation of IPU degrading bacterial strain

In order to isolate IPU-mineralizing bacterial strains, enrichment cultures were performed using a composite soil sample made of the nine samples. 10 g of soil equivalent dry weight was suspended in 90 mL of MS medium and incubated at 20°C on an orbital shaker (150 rpm). Aliquots were taken regularly and the IPU remaining in the medium was quantified by HPLC as already described (Hussain et al., 2009b). When about 50% of the IPU added initially has been degraded, 10 mL of this suspension was transferred to 90 mL of fresh MS medium and incubated under the same conditions. Twelve successive enrichment cycles were carried out in the same way and 1 mL aliquots of each enrichment culture were preserved at -20°C. After 12 enrichments, the culture was serially 10-fold diluted and 100 µl of the 10^{-3} to 10^{-6} dilutions were inoculated onto MSA-IPU and NA plates. The plates were incubated at 20°C. Single colonies isolated from the plates were tested for IPU degradation in liquid MS-IPU media at 28°C under agitation (150 rpm). After a few days, the cells were harvested (3220 g, 10 min) and the supernatant was analyzed to estimate the remaining IPU using HPLC.

2.6. Extraction of DNA

Crude DNA was prepared from the frozen aliquots of the serial enrichment cultures using proteinase K and thermal shock treatment as previously described (Cheneby et al., 2004). Genomic DNA was extracted from the isolated IPU-degrading bacterial strain

collected at the late exponential stage of growth using QIAGEN Genomic DNA Isolation Kit (QIAGEN, France) according to the manufacturer's recommendations.

2.7. PCR Amplification of 16S rDNA and *catA* sequences

The 16S rDNA sequence was amplified using the universal primers 27f (5'-AGA GTT TGA TCH TGG CTC AG-3') and 1492r (5'-TAC GGH TAC CTT GTT ACG ACT T-3') (Gurtler and Stanisich, 1996). The amplification reaction was carried out in a final 25 µL volume containing 2.5 µL of 10X Taq polymerase buffer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 µM of each primer and 0.625 U of Taq polymerase. 2.5 µL of crude DNA or 25 ng of the genomic DNA was used as template for the PCR reactions. PCR was performed in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Mass) according to the following program: 1 cycle of 4 min at 94°C; 39 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C and a final extension step at 72 °C for 5 min. *cat*A gene was amplified by PCR using *cat*Ar and *cat*Af primers as recently described (Sun et al., 2009).

For further analyses, 16S rDNA and *cat*A PCR products were purified using the MinElute PCR Purification Kit according to manufacturer's recommendations (QIAGEN, France).

2.8. Amplified ribosomal DNA restriction analysis (ARDRA)

The purified 16S rDNA amplicons were analyzed by restriction fragment analysis. The PCR products were digested using the restriction enzyme *Alu*I (Fermentas, France) and separated on 3% High Resolution (MP Q-BIOgene, America) agarose gel along with the DNA Molecular Weight Marker BVIII (Roche Applied Science, France).

2.9. Cloning and sequencing of 16S rDNA and *cat*A of the isolated bacterial strain

The purified 16S rDNA and *cat*A amplicons were cloned in pGEMT-EasyII vector according to the manufacturer's recommendations (Promega, Madison, WI, USA). The recombinant clones were screened by PCR using the SP6 and T7 universal primers and the following program: 94°C for 45 s, 35 cycles of 55°C for 45 s, 72°C for 90 s and a final extension step at 72°C for 7 min. Cloned 16S rDNA amplicon was sequenced by Cogenics (Meylan, France). The sequence was deposited in the GenBank database under the accession number HM191725. Cloned *cat*A PCR product was sequenced using T7 primer with the CEQ8000 Dye Terminator Cycle Sequencing following the manufacturer's recommendations

(Beckman Coulter, France). The partial *cat*A sequence was deposited in the GenBank database under the accession number HM191726.

2.10. Phylogenetic analysis of 16S rDNA and *catA* sequences

Both 16S rDNA and *cat*A sequences were compared to the known nucleotide sequences using BlastN (http://www.ncbi.nlm.nih.gov/BLAST). To construct phylogenetic trees based on 16S rDNA or *cat*A sequences, multiple alignments were carried out using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) and the data obtained was processed using NJ Plot (http://pbil.univ-lyon1.fr/software/njplot.html) using the neighbor joining method.

Pairwise Comparison of phylogenetic tree based on 16S rDNA with the one based on *cat*A sequences was carried out using an applet (http://www.mas.ncl.ac.uk/~ntmwn/phylo_comparison/pairwise.html).

2.11. IPU mineralization kinetics of the isolated strain

The IPU mineralization kinetics of the isolated bacterial strain were determined by radiorespirometry using ^{14}C-ring-labeled IPU in Knapp buffer (Devers et al., 2004). The cells were harvested from MS-IPU medium at the late exponential phase, washed twice with Knapp buffer and re-suspended to an optical density of 0.5 in Knapp buffer containing 50 mg L^{-1} of IPU and 2 kBq of ^{14}C-ring-labeled IPU. Cell suspensions (n=3) were incubated for three days at 20°C on an orbital shaker (150 rpm) in closed sterile respirometer jars containing NaOH traps. The traps were changed regularly during the incubation period and analyzed for radioactivity content by liquid scintillation counting using ACSII scintillation fluid (Amersham). At the end of incubation, the amount of radioactivity remaining in the cell-free medium as well as in the bacteria pellet was also analyzed by ACSII scintillation counting.

2.12. Degradation of IPU, its metabolites, various phenylurea herbicides and their aniline derivatives

Resting cell experiments were performed in order to estimate the ability of the isolated bacterial strain to degrade IPU, its known metabolites (i.e. MDIPU, DDIPU and 4-IA), various phenylurea herbicides (i.e. isoproturon, diuron, linuron, monolinuron and chlorotoluron) and their aniline derivatives (i.e. 4-IA, 3,4-DCA, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline and 3-chloro-4-methylaniline). The isolated strain was grown in MS medium, washed twice in Knapp buffer and then suspended in Knapp buffer containing 50 mg L^{-1} of the pesticide or metabolite, to obtain an optical density (600 nm) of 0.2. Three

replicates were performed for each compound. The cells were incubated at 28°C under agitation (125 rpm) and aliquots from each suspension were taken regularly during the incubation period. The suspensions were centrifuged (3220 g, 10 min) and the supernatants were subjected to HPLC analysis in order to quantify the compound remaining in the suspension.

2.13. Impact of pH on IPU degradation kinetics

Resting cell experiments were performed as described above to determine the impact of pH on IPU degradation kinetics. The pH of the Knapp buffer was adjusted to 5.5, 6.5, 7.5 or 8.5. At the end of incubation (3 days), cells were enumerated on Petri dishes. Cell suspensions were serially 10-fold diluted and the 10^{-4} to 10^{-6} dilutions were plated on NA medium. The plates were incubated at 28°C and the CFU were enumerated after 10 days of incubation.

2.14. Determination of the kinetics parameters of the degradation of IPU

To determine the kinetics of the degradation of IPU, the degradation curves were fitted to a modified Gompertz model (Zwietering et al., 1990) using Sigma Plot© 4.0. The model equation was:

$y = A.\exp\{-\exp[1+\mu m.\exp(1).(\lambda-t)/A]\}$

where

y is the percentage of mineralization or degradation (%),

t is the time (h), μm is the maximum mineralization or degradation rate (% h^{-1}),

A is the maximum percentage of mineralization or degradation (%),

λ is the lag time (h).

The parameters were validated using a Student t test ($p<0.005$).

2.15. Statistical analysis

In order to assess the impact of pH on IPU degradation, the kinetics parameters were subjected to a single-factor analysis of variance (one-way ANOVA) followed by a Fisher procedure ($n=3$, $p<0.001$) (Statview 4.55 software, Abacus Concept, Inc.). A similar approach was applied to assess the impact of pH on the estimation of the abundance of SH colonies.

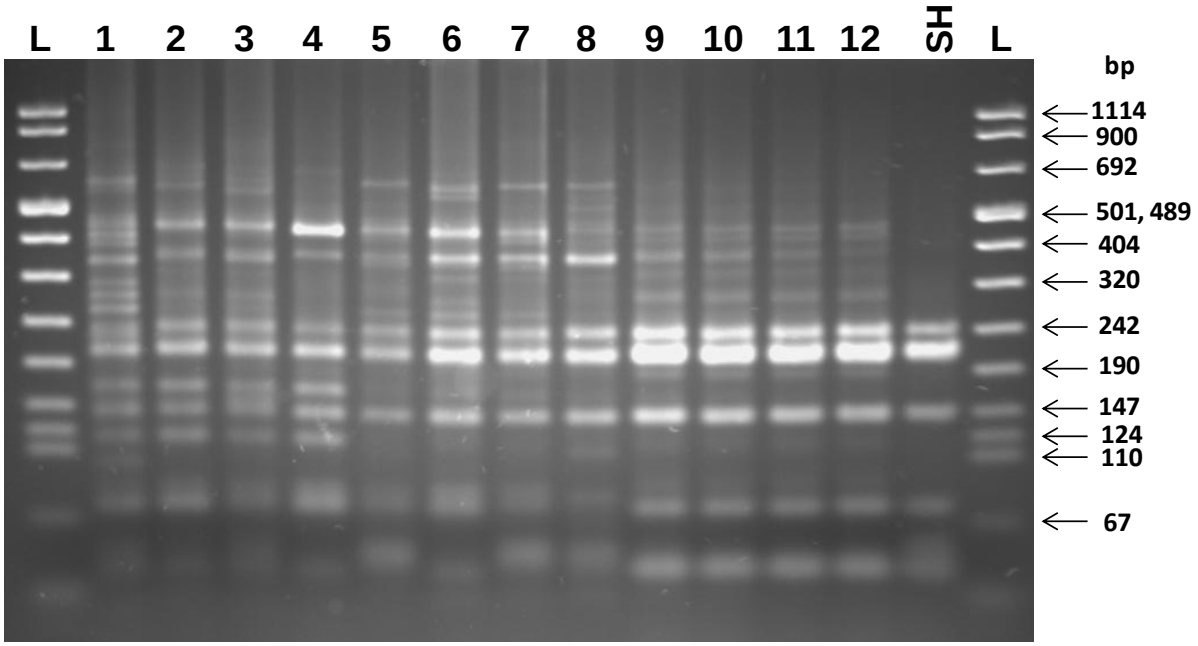

Fig. V-1. ARDRA fingerprints produced from DNA samples extracted from enrichment culture aliquots. Lanes 1-12 represent the ARDRA fingerprints of twelve consecutive enrichment cultures (from enrichment 1 to 12). Lane SH indicates the ARDRA fingerprint of the isolate *Sphingomonas* sp. SH and the lane L is the BVIII molecular weight marker (size indicated in base pairs).

3. Results

3.1. IPU mineralization kinetics in soil

This study was performed using nine soil samples collected from a field under rape seed / winter wheat / barley crop rotation. This field had been treated with IPU (2 applications per year for 2 years of every 3 year rotation) for ten years. The physical and chemical properties of the soil samples were found to be relatively homogenous with a low coefficient of variation (CV) (Table V-1). The ability of the soil microflora to mineralize this herbicide estimated by radiorespirometry showed that all these soil samples were able to mineralize IPU under laboratory conditions. Approximately 30% to 40% of the ^{14}C-ring-labeled IPU initially added was transformed to $^{14}CO_2$ over two weeks of incubation. The maximum IPU mineralization (A), estimated by fitting the modified Gompertz model to the IPU mineralization kinetics of the soil samples, averaged 42.1% $^{14}CO_2$ with a CV of 4.3% (Table V-1). However, the other two kinetic parameters, the lag phase (λ) and the maximum mineralization rate (μm), averaged about 0.6 days and 3.68 % $^{14}CO_2$ day^{-1} respectively and were found to be moderately variable with a CV of 17.1% and 19.2% respectively (Table V-1).

3.2. Enrichment and isolation of an IPU mineralizing bacterial strain

In order to further characterize the IPU degrading populations responsible for the IPU mineralization observed in the field at Epoisses, the IPU-mineralizing bacterial strain was isolated using enrichment cultures starting from a composite soil sample formed from the 9 soil samples that had a high IPU mineralization ability. This composite soil sample was transferred to fresh MS-IPU medium containing IPU as the sole source of carbon and nitrogen. When about 50% of the IPU added initially was degraded an aliquot was transferred to fresh MS-IPU. Twelve successive enrichment cultures were carried out. During the serial enrichment cultures, the structure of the microbial communities in the soil slurries was evaluated by ARDRA fingerprinting. These analyses revealed a relatively complex microbial community of ten different bands (Fig. V-1). During the early enrichments, the ARDRA profiles were simplified and very similar profiles were observed for the last four enrichments (9-12). In order to isolate a pure IPU-degrading strain, the IPU mineralizing bacterial culture obtained after twelve enrichments was serially diluted and inoculated on NA and MSA-IPU plates. After five days of incubation, different types of colonies appeared on NA media plates one of which was dominant (>80%). Similarly, the growth of different types of colonies was

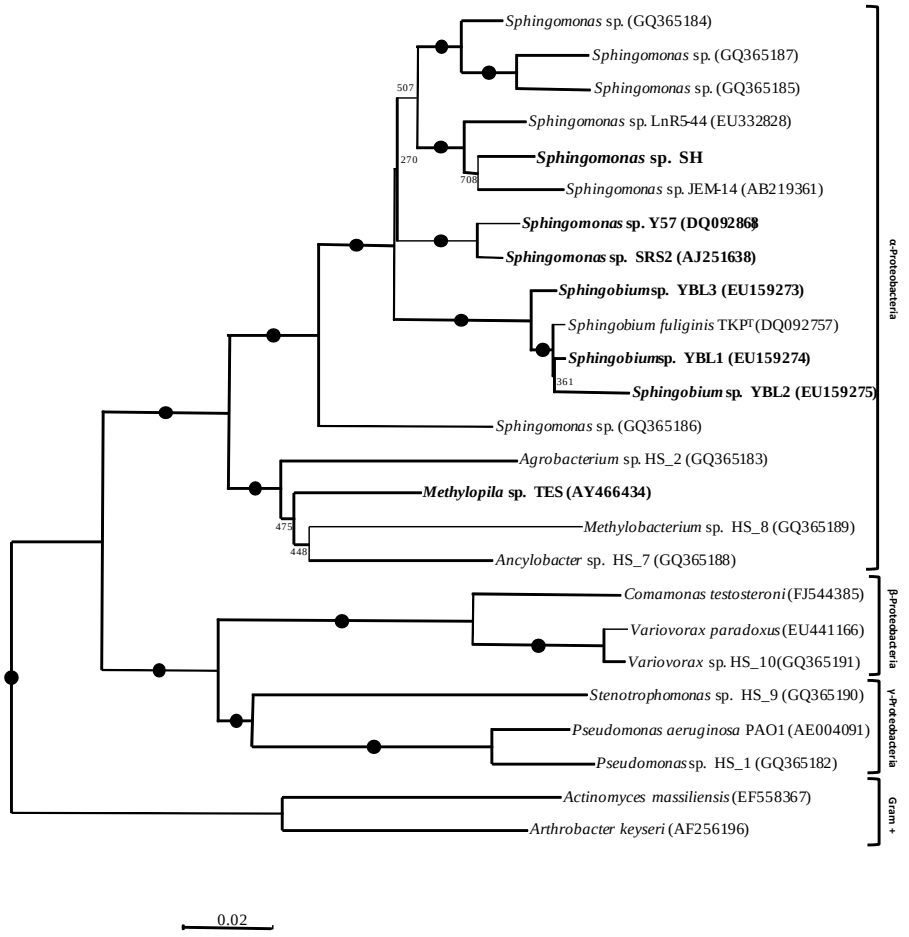

Fig. V-2. Neighbor-joining phylogenetic analysis resulting from the multiple alignment of 16S rDNA gene sequences of *Sphingomonas* sp. SH with those of other IPU degrading strains found in GenBank database. IPU degrading bacterial isolates are in bold. GenBank accession numbers are given in brackets. Bootstrap values greater than 900‰ are marked as black circles.

observed on MSA-IPU plates after about fifteen days of incubation. The IPU degrading ability of the different types of colonies appearing on NA and MSA-IPU was tested in liquid MS-IPU medium. This showed that the dominant bacterial colony present on the NA and MSA-IPU media plates was able to degrade IPU. On NA plates, the IPU degrading colonies were opaque, buff, smooth, circular and convex. On MSA-IPU media plates, the IPU degrading colonies formed a clear zone owing to the degradation of IPU crystals. The purity of the strain was ensured by passing it twice from MS-IPU liquid to MSA-IPU plates and then testing its IPU degrading activity by HPLC.

3.3. Identification and phylogenetic characterization of IPU mineralizing strain

The 16S rDNA amplicon (1447 bp) amplified from the isolated IPU degrading bacterial strain was sequenced and deposited in the GenBank database under the accession number HM191725. The highest degree of similarity (96%) of this sequence was obtained with 16S rDNA gene sequence of *Sphingomonas* sp. JEM-14 (Ac. No. AB219361), an estrogen degrading bacterium. Phylogenetic analysis revealed that the isolated bacterial strain was grouped (i.e. bootstrap >95%) in a cluster of several *Sphingomonas* spp. (Fig. V-2). Given the phylogenetic relationship of this bacterial strain with several *Sphingomonas* spp., this isolate was named *Sphingomonas* sp. SH. It was classified as bacteria, phylum proteobacteria, class Alphaproteobacteria, order *Sphingomonadales*, family *Sphingomonadaceae*, genus *Sphingomonas*.

By performing a PCR assay targeting catechol 1,2-dioxygenase gene (Sun et al., 2009), a DNA fragment of about 417 bp was obtained (data not shown). The *cat*A amplicon was sequenced and deposited in the GenBank database under the accession number HM191726. Comparison of the sequence on BLAST showed that this gene had 98% identity with the *cat*A gene of *Sphingomonas* sp. KA1 (GenBank Ac. No. AB270530).

An *in silico* study was performed to establish the level of congruence between the corresponding *cat*A and 16S rDNA sequence based phylogenies of 14 Proteobacteria including *Sphingomonas* sp. SH. The sequences were retrieved from the GenBank database by selecting known 16S rDNA and *cat*A sequences identified in the same bacterial strain. The structure of the phylogenetic tree based on the alignment of 16S rDNA was consistent with the expected taxonomy exhibiting three clusters (i.e. 100% bootstrap) consisting of α-, β- and γ-Proteobacteria respectively (Fig. V-3a). Unlike 16S rDNA phylogeny, the *cat*A based tree did not have these three significantly distinct clusters (Fig. V-3b). However, *Sphingomonas*

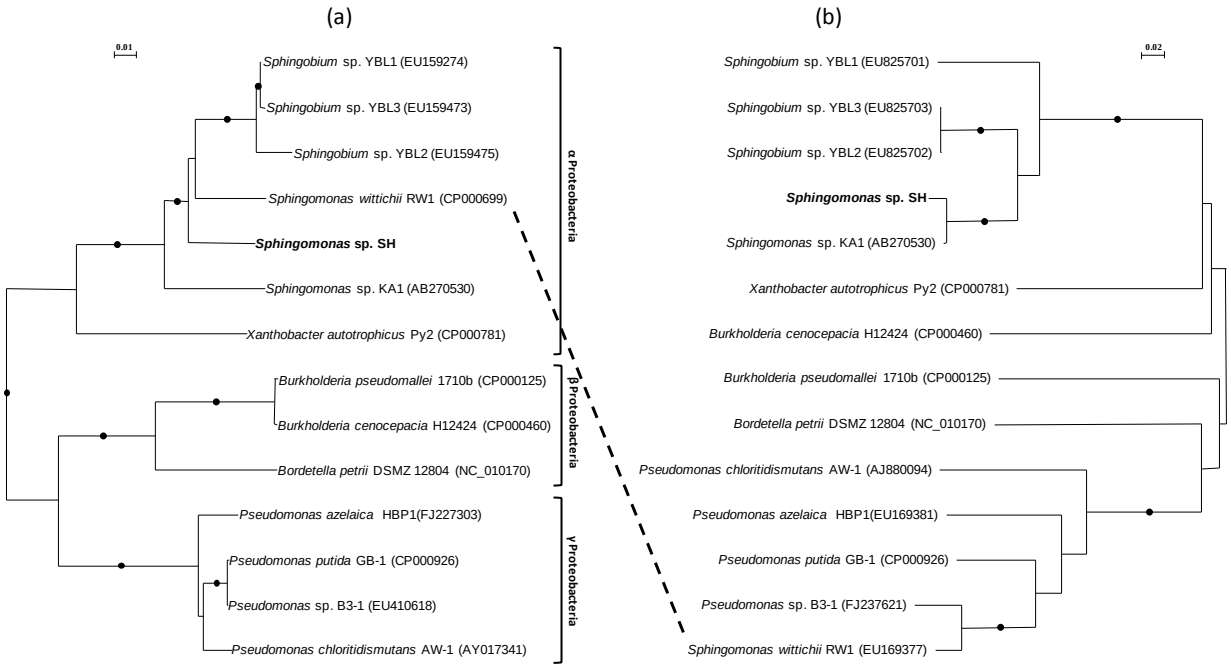

Fig. V-3. Comparison of 16S rDNA and *catA* phylogenies of 14 bacterial strains. (a) Phylogenetic tree of 16S rDNA sequences resulting from the multiple alignment of 14 bacteria. The different clusters of different classes of bacteria are indicated. (b) Phylogenetic tree based on *cat*A sequences of the 14 bacterial strains. The broken line between the two trees shows an example of non-congruence between the two phylogenies. GenBank accession numbers of the *cat*A and 16S rDNA sequences are given in brackets. Bootstrap values greater than 900‰ are marked as black circles.

and *Sphingobium* spp. belonging to α-Proteobacteria were grouped together in the same cluster with 100% bootstrap value except for *Sphingomonas wittichii* RW1 which was found in another cluster (100% bootstrap value) of γ-Proteobacteria consisting of *Pseudomonas* spp. Similarly, the bacterial strains belonging to β-Proteobacteria were significantly grouped together in the 16S rDNA based phylogenetic tree. However, they were found to be spread and non-significantly (Bootstrap <70%) grouped with α-Proteobacteria and γ-Proteobacteria in *cat*A based phylogenetic tree. It can, therefore, be concluded that the 16S rDNA and *cat*A based phylogenies were not congruent with each other because the same taxonomical structure was not found. This was also statistically confirmed by the pairwise comparison of *cat*A and 16S rDNA phylogenies which revealed a probability of similarity of only 52.3%.

3.4. Degrading capabilities of *Sphingomonas* sp. SH

The degrading ability of the bacterial strain was estimated by performing resting cell experiments coupled with HPLC. IPU degradation kinetics by the strain SH indicated that the complete degradation (>99%) of IPU occurred within 12 hours of incubation. Transient accumulation of a metabolite showing the same retention time as that of MDIPU was detected during the IPU degradation kinetics. The isolated bacterial strain was also able to degrade its known metabolites (i.e. MDIPU, DDIPU and 4-IA). Complete degradation (>99%) of MDIPU, 4-IA and DDIPU occurred after 12, 23 and 30 hours of incubation, respectively (Fig. V-4.). The maximum degradation rate [µm (% h^{-1})] calculated by modeling the degradation kinetics was found to be highest for IPU and MDIPU (µm = 14.2 and µm = 13.6, respectively) while the lowest rates were observed for DDIPU and 4-IA (µm = 5.4 and µm = 6.2, respectively).

Although *Sphingomonas* sp. SH was shown to degrade IPU and its metabolites successfully, it could not degrade other phenylurea herbicides (diuron, linuron, monolinuron and chlorotoluron) even after 5 days of incubation (data not shown). Moreover, it was unable to degrade any of the aniline derivatives of other phenylurea herbicides i.e. 3,4-DCA, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline and 3-chloro-4-methylaniline after 90 hours of incubation.

Results of the radiorespirometric experiments indicated that over 40 hours of incubation, the isolated bacterial strain had mineralized about 42% (±0.5%) of the ^{14}C IPU added initially (Fig. V-5). Determination of the remaining radioactivity revealed that up to 39% (±2.4%) of the radioactivity added initially was detected in the bacterial pellet,

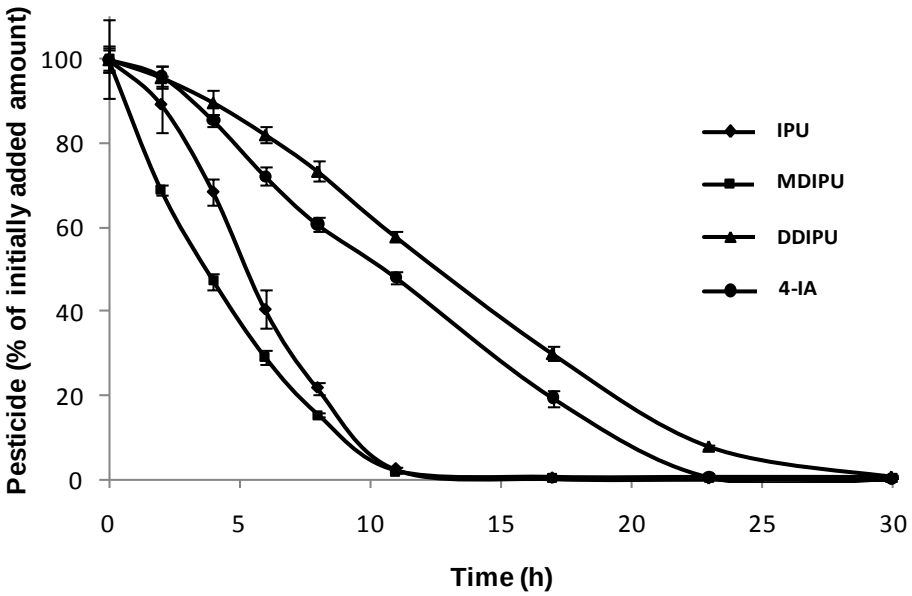

Fig. V-4. Degradation kinetics of IPU, DMIPU, DDIPU and 4-IA by *Sphingomonas* sp. SH incubated in Knapp buffer at 28°C under 150 rpm agitation. Error bars indicate standard error (n=3).

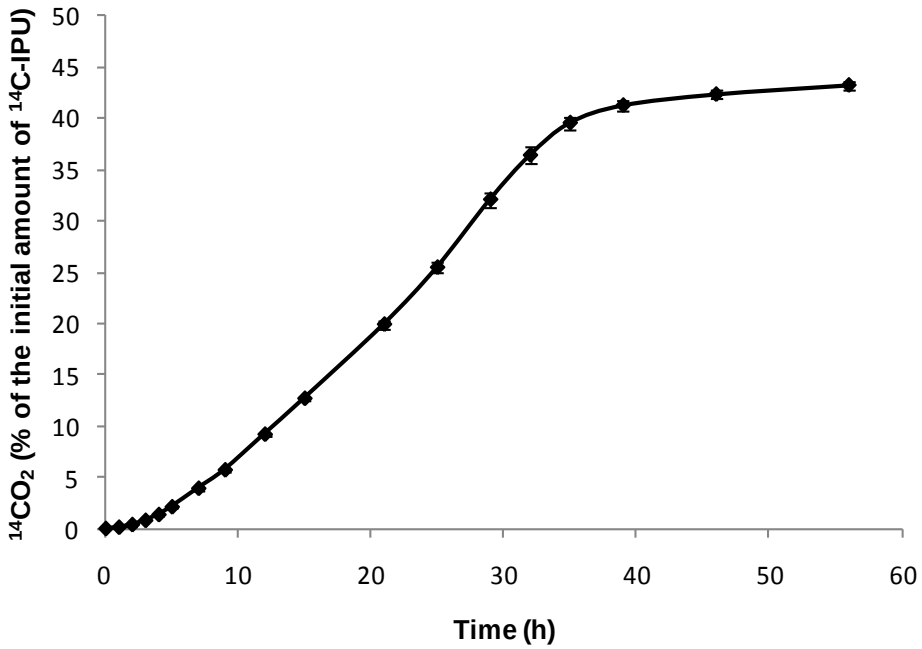

Fig. V-5. Kinetics of ^{14}C-ring-labeled IPU mineralization by *Sphingomonas* sp. SH incubated at 28°C in Knapp buffer under agitation (150 rpm). Error bars indicate standard error (n=3)

suggesting that it was incorporated in the bacterial biomass, and only about 8% (±0.7%) remained in the culture media. The parameters of IPU mineralization kinetics deduced by modeling the mineralization data revealed a lag phase (λ) of 2h and a maximum mineralization rate (µm) of 1.5 % h^{-1}.

3.5. Effect of pH on IPU degradation kinetics

The effect of pH on the IPU degrading ability of the *Sphingomonas* sp. SH was estimated. The pH was shown to have a strong effect on the IPU degradation kinetics of the isolated bacterial strain in liquid culture (Fig. V-6). *Sphingomonas* sp. SH had completely degraded IPU (>99%) after 44, 24, 18 and 36 hours of incubation at pH values of 5.5, 6.5, 7.5 and 8.5 respectively. By fitting the modified Gompertz model to the degradation data, the highest µm (19.9) was observed at pH 7.5, while the lowest µm (3.5) was observed at pH 5.5 (Fig. V-6A). Statistical analysis confirmed that µm was significantly higher at pH 7.5 than at other pH values ($p < 0.05$), while there was no significant difference between the µm at pH 5.5 and that at pH 8.5. However, µm for pH 6.5 (9.1) was significantly higher than for pH 5.5 and 8.5 but significantly lower than for pH 7.5 ($p < 0.05$). Thus, 7.5 was found to be the optimum pH value for the IPU degrading ability of *Sphingomonas* sp. SH and that the IPU degradation rate was significantly affected as the pH shifted towards more acidic or alkaline conditions.

In order to determine the effect of pH on *Sphingomonas* sp. SH, the colony forming units (CFU) of this strain were counted at different pH values at the end of the incubation period. Statistical analysis indicated that the abundance of the strain was significantly affected by the different pH values following the same pattern as that for the degradation. CFU was found to be significantly correlated (µm = 7E-08cfu mL^{-1} – 5.0183, R^2 = 0.945) with the maximum degradation rate (µm) at different pH values (Fig. V-6B).

4. Discussion

Accelerated degradation of IPU has already been reported in several soils repeatedly treated with this herbicide at various places in Europe including UK, Denmark and France (Sorensen et al., 2001; Bending et al., 2003; El-Sebai et al., 2005; El-Sebai et al., 2007). Microbial degradation has been described as a primary mechanism responsible for IPU dissipation as well as other phenylurea herbicides from the soil (Fournier et al., 1975; Gaillardon and Sabar, 1994; Pieuchot et al., 1996). This widespread IPU degradation in the

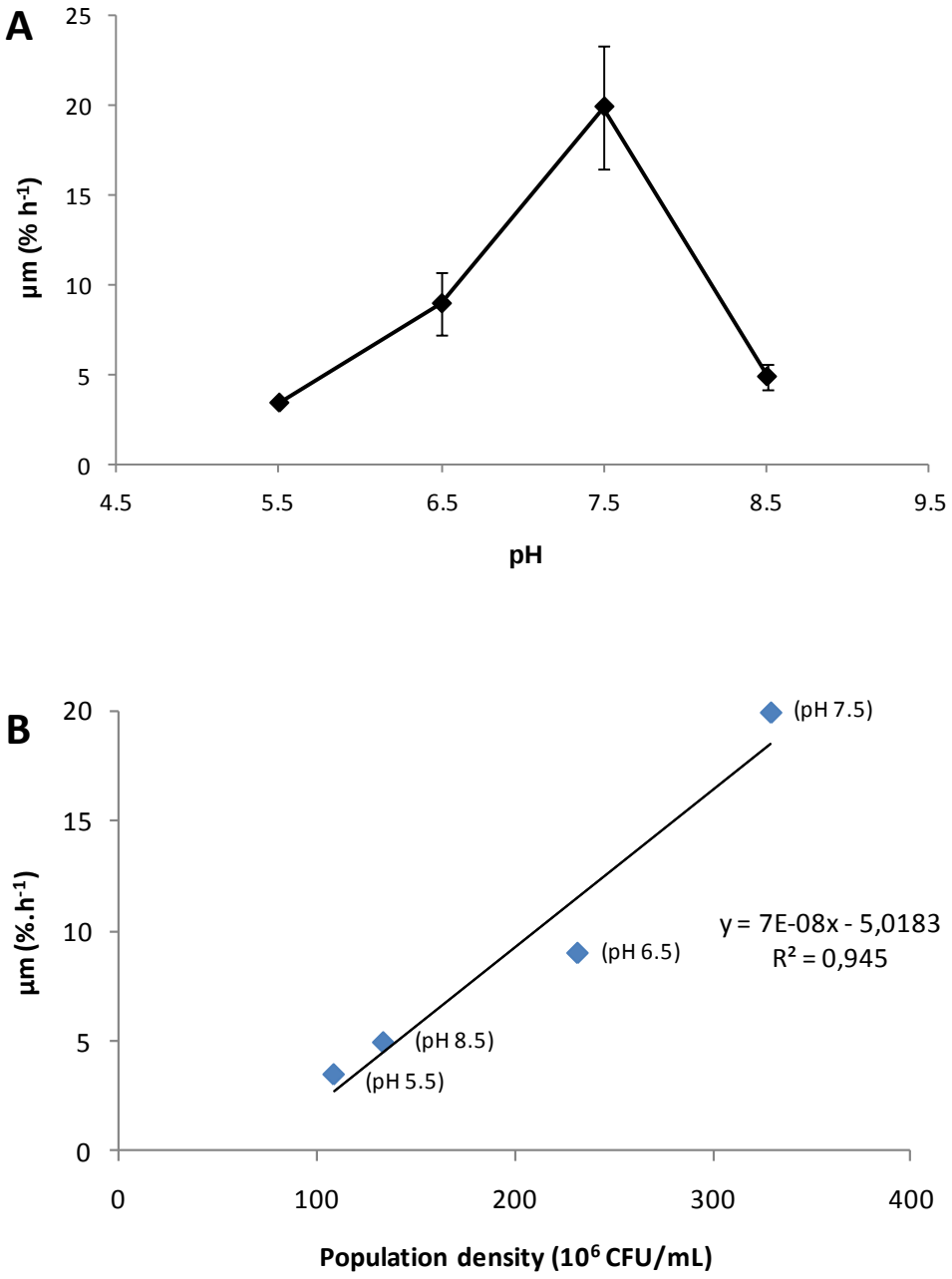

Fig.V-6. Panel A. Effect of pH on the maximum degradation rate (µm) of IPU by *Sphingomonas* sp. SH in liquid culture. Error bars indicate standard error (n=3). Panel B.Correlation between the maximum degradation rate (µm in % of IPU degraded per hour) and the number of *Sphingomonas* sp. SH colonies enumerated on nutrient agar medium (CFU mL^{-1}) after their incubation at different pH in Knapp buffer added with IPU.

soil environment is the result of the adaptation of the microbial community in which the abundance of a specific population able to degrade and grow on IPU is increased. This study indicated that all the sub-site soil samples collected from a field under rape seed / winter wheat / barley crop rotation, periodically exposed to IPU for ten years, were able to mineralize rapidly about 40% of the ^{14}C-ring-labeled IPU initially added to $^{14}CO_2$ after only 31 days of incubation. These results revealed the IPU mineralizing ability of the experimental field in Epoisses which might result from the adaptation of the indigenous soil microflora to metabolize IPU in response to repeated exposure. The parameters of IPU mineralization, i.e. maximum percentage of mineralization (A), maximum mineralization rate (μm) and lag phase (λ) determined using the modified Gompertz model on the mineralization data of the sub-site samples showed a relatively low variability which was unexpected since pesticide degrading activity, like other microbial activities, often varies considerably. This reduced variability in IPU mineralization ability of these samples might be attributed to a low variation in the physical, chemical and microbial properties known to influence the pesticide biodegrading ability of the soil microflora (Bending et al., 2003; Briceno et al., 2007; El-Sebai et al., 2007; Vieuble-Gonod et al., 2009).

Although rapid IPU degradation in agricultural fields has often been reported, few IPU degrading bacterial strains have been described and up to now, the gene coding for IPU degrading enzymes has not been determined. In order to characterize the bacterial populations responsible for IPU mineralization, enrichment cultures were performed starting from a composite sample to isolate an IPU degrading strain (Rousseaux et al., 2001; El-Sebai et al., 2004). Throughout the enrichment process, the evolution of the 16S rDNA genetic structure of the bacterial community was monitored by ARDRA fingerprinting. After successive enrichments, the complexity of the structure of the bacterial community exposed to IPU was simplified. After 9 enrichment cycles, a stable ARDRA fingerprinting profile was observed. The dominant ARDRA profile observed from enrichments 9 to 12 was very similar to that of the isolated IPU degrading strain. It is very likely that the successive enrichments led to the disappearance of certain uncultivable bacterial strains and of the bacterial populations unrelated to IPU degradation leading to the dominance of the IPU degrading bacterial population. This observation is in accordance with *in vitro* studies indicating that enrichment procedures using pesticides as a selection pressure induce significant changes in bacterial community composition (El-Fantroussi et al., 1999; Bending et al., 2003; El-Sebai et al., 2004). This study also confirms the effectiveness of molecular based approaches for exploring

the changes in microbial community structure during enrichment cultures allowing the identification of the numerically dominant catabolic species within a degrading community (Ralebitso-Senior et al., 2003; El-Sebai et al., 2004).

Phylogenetic analysis based on 16S rDNA revealed that the strain SH showed up to 96% similarity with the 16S rDNA of *Sphingomonas* sp. JEM-14 and was clustered with several *Sphingomonas* strains. This genus comprises Gram-negative, non spore forming, chemoheterotrophic, rod-shaped aerobic bacteria (White et al., 1996). Within the *Sphingomonas* cluster that includes the strain SH, several isolates have been characterized for the degradation of various xenobiotics. The bacterial isolates belonging to the Sphingomonadaceae family including *Sphingobium* (Sun et al., 2009) and *Sphingomonas* strains (Sorensen et al., 2001, 2002; Bending et al., 2003), isolated from geographically distant soils (including Denmark, United Kingdom and China), are known to mineralize IPU. Furthermore, the recent characterization of an IPU mineralizing enriched bacterial culture represented by *Sphingomonas* sp. present as a dominant partner (Hussain et al., 2009b) as well as isolation of the strain SH further reinforces the idea that members of this genus, which are ubiquitous in the environment, harbor broad catabolic capabilities (White et al., 1996; Fredrickson et al., 1999; Sorensen et al., 2001), can rapidly adapt to IPU exposure and develop the ability to break it down as a source of carbon and nitrogen (Hussain et al., 2009b).

Catechol, which is a key intermediate of the β-ketodipate pathway responsible for the degradation of many aromatic compounds (Alva and Peyton, 2003; Nayak et al., 2009) and cis,cis-muconic acid, its aliphatic metabolite, (Nayak et al., 2009), have also been also detected during the mineralization of aniline by an IPU degrading bacterial strain (Sun et al., 2009). It was recently reported that the *cat*A gene coding for catechol 1,2-dioxygenase was detected in the IPU degrading strains YBL1, YBL2 and YBL3 thereby suggesting that phenyl ring of IPU might be degraded through ortho-cleavage of the catechol. These results show that the strain SH also harbors the *cat*A gene. Phylogenetic analysis of the *cat*A amplicon revealed that it shared about 98% homology with the *cat*A of the *Sphingomonas* sp. KA1 (AB270530) and about 82% homology with that of the *Sphingobium* sp. YBL3 (Sun et al., 2009). In addition, the *cat*A sequence of the strain SH was closely related to the *cat*A genes present in various xenobiotic degrading members of the family *Sphingomonadaceae*. This may suggest that, for the strain SH, the mineralization of the IPU phenolic ring would have occurred through the formation of and degradation of catechol as an intermediate compound at some

stage after 4-IA, as previously described (Sun et al., 2009). This finding may also be helpful in describing the later steps of IPU metabolism after 4-IA.

16S rDNA and *cat*A phylogenies of 14 bacterial strains including *Sphingomonas* sp. SH were constructed and checked for congruence. The taxonomical structure of the selected 14 bacterial strains was reflected in 16S rDNA phylogeny but not in the *cat*A phylogeny. In addition, pairwise comparison revealed a low probability (52.3%) further indicating that these two phylogenies are not congruent and suggesting that *cat*A has been the subject of horizontal gene transfer, most likely as a result of the selection pressure exerted by the exposure of the soil microflora to contaminants including pesticides such as phenylurea herbicides..

The strain SH showed an important ability to mineralize IPU with up to 40% of the ^{14}C IPU evolved as $^{14}CO_2$ and almost the same amount incorporated into bacterial biomass. It is noteworthy that the amount of IPU evolved in $^{14}CO_2$ by the strain SH (42 ± 0.5%) was comparable to that of other IPU mineralizing bacterial strains like *Sphingomonas* sp. SRS2, *Methylopila* sp. TES and enriched bacterial culture (Sorensen et al., 2001; El-Sebai et al., 2004; Hussain et al., 2009). These results confirmed that the strain SH was able to completely mineralize IPU as efficiently as *Sphingomonas* sp. SRS2 (Sorensen et al., 2001), *Methylopila* sp. TES (El-Sebai et al., 2004) and *Sphingobium* sp. Strain YBL1, YBL2, YBL3 (Sun et al., 2009). This study is thought to be the second isolating an IPU mineralizing strain from agricultural soils in France after the isolation of *Methylopila* sp. TES (El-Sebai et al., 2004).

The transient accumulation of a metabolite with the same retention time as that of MDIPU during the IPU degradation by the bacterial strain SH and its further degradation suggests that IPU degradation might have been initiated by N-demethylation of the urea side chain as previously proposed (Sorensen et al., 2001; Hussain et al., 2009b; Sun et al., 2009). The production of MDIPU as the main metabolite during IPU degradation has already been reported in agricultural soils (Berger, 1999; Juhler et al., 2001) and it was suggested as a first, limiting step during IPU degradation (Sorensen et al., 2001; Hussain et al., 2009b; Sun et al., 2009). Sorensen et al. (2001) and recently Hussain et al. (2009) have reported the initial steps of the IPU metabolic pathway by two different IPU degrading cultures. Both cultures initiated IPU degradation by two successive N-demethylation producing MDIPU and DDIPU which were further transformed to 4-IA by the cleavage of the urea side chain and ultimately by mineralization of the phenyl ring. Based on the characterization of ability of the strain SH to degrade known IPU metabolites (i.e. MDIPU, DDIPU and 4-IA) and transient accumulation

of a metabolite during the degradation kinetics of IPU, one hypothesizes is that SH has a metabolic pathway similar to that of *Sphingomonas* sp. SRS2 (Sorensen et al., 2001) and that of the IPU mineralizing bacterial culture (Hussain et al., 2009b).

In addition, the strain SH had the ability to degrade IPU and its known metabolites, MDIPU, DDIPU and 4-IA (aniline derivative of IPU). However, it could not degrade any of the related phenylurea herbicides i.e. diuron, linuron, monolinuron and chlorotoluron or their aniline derivatives. This suggests that the degrading capabilities of SH are highly specific for IPU and its known metabolites. The enzymatic specificity of SH towards the IPU metabolic pathway was further highlighted by its ability to degrade only 4-IA (aniline derivative of IPU) and none of the aniline derivatives of other phenylurea herbicides (i.e. 3,4-DCA, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline and 3-chloro-4-methylaniline). Up to now different strains, able to degrade phenylurea herbicides along which IPU, have been isolated from geographically diverse regions of the world (Sorensen et al., 2001; Turnbull et al., 2001; Tixier et al., 2002; El-Sebai et al., 2004; Hussain et al., 2009b; Sun et al., 2009). Among the known IPU degrading bacterial strains, *Arthrobacter globiformis* D47 (Turnbull et al., 2001) and *Arthrobacter* sp. N2 (Tixier et al., 2002) are able to transform phenylurea herbicides to their corresponding aniline derivatives. However, *Sphingomonas* sp. SRS2 (Sorensen et al., 2001) as well as *Sphingobium* strains YBL2 and YBL3 (Sun et al., 2009) have been reported to mineralize IPU and to degrade closely related phenylurea herbicides such as diuron and chlorotoluron. In addition, more recently, *Sphingobium* sp. YBL1 (Sun et al., 2009) was shown to metabolize most of the phenylurea herbicides and their aniline derivatives. Unlike these strains, *Methylopila* sp. TES was found to mineralize only IPU without showing any degrading activity towards other structurally related phenylurea herbicides (El-Sebai et al., 2004). Recently, an enriched bacterial culture able to mineralize only IPU and none of other phenylurea herbicides has been reported (Hussain et al., 2009b). *Sphingomonas* sp. SH has catabolic abilities similar to those of *Methylopila* sp. TES (El-Sebai et al., 2004) and the enriched bacterial culture (Hussain et al., 2009b). It is possible that the strain SH as well as *Methylopila* sp. TES and the enriched bacterial culture possess a degrading ability specific for IPU.

pH is one of the most important factors which has been reported to have significant effects on microbial pesticide degradation not only in soils (El-Sebai et al., 2005; Bending et al., 2006; El-Sebai et al., 2007) but also in liquid pure culture studies (Bending et al., 2003; Hussain et al., 2009; Sun et al., 2009). This study also highlights the effect of pH on the IPU

degradation kinetics by the strain SH. The results indicate that the strain SH had the ability to completely degrade IPU between the pH range of 5.5 to 8.5 with an optimum activity at pH 7.5 (μm = 19.9) and reduced activity at pH 5.5 (μm = 3.5) and at pH 8.5 (μm = 4.95). These results are in accordance with similar patterns in pH regulation of IPU degradation kinetics by the bacterial species in liquid cultures (Bending et al., 2003; Hussain et al., 2009b; Sun et al., 2009). They also confirmed studies reporting a significant correlation between the soil pH and the degradation of various pesticides (Houot et al., 2000; El-Sebai et al., 2005; El-Sebai et al., 2007). Although the effect of pH on IPU degradation by soil microflora is well described by many previous studies (Bending et al., 2003; Hussain et al., 2009b; Sun et al., 2009), the mechanisms responsible for this regulation have not been explained. However, the pH is known to affect the growth and survival of microbial populations (Russell and Dombrowski, 1980; Sun et al., 1998; Rousk et al., 2009) which ultimately affects their activity. Enumeration of the SH population at the end of the incubation period for each pH value revealed that the maximum degradation rate (μm) was strongly correlated (R^2 = 0.945) with the number of SH bacteria counted at the end of incubation period (Fig. V-6B). This suggests that pH might have an effect on the growth or survival of the SH bacterial population which might partly explain the variation in IPU degradation rate observed at different pH values. However, a more specific effect of pH on IPU degradation (IPU speciation and IPU transformation etc.) cannot be excluded and further research should be carried out to study the transcriptional and enzymatic regulation occurring in strain SH on exposure to IPU at different pH values.

In conclusion, the isolation of *Sphingomonas* sp. SH is the second report after *Methylopila* sp. TES (El-Sebai et al., 2004) of a soil borne bacterial isolate from agricultural soils in France, which is able to mineralize IPU and has degrading capabilities that are highly specific for metabolizing IPU. This study is possibly the first report showing the correlation between physiological effects and the IPU degradation capabilities of a bacterial isolate in response to variations in pH. It also suggests the involvement of *cat*A in the lower degradation pathway of IPU. Further research is required to throw more light on the IPU metabolic pathway by identifying some of the genes coding the enzymes involved in IPU mineralization through meta-genomic approaches.

5. Acknowledgments

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Conclusions and perspecitves

This work led to the isolation of a pure bacterial strain able to mineralize IPU from a composite soil sample made of nine IPU mineralizing soil samples collected from the experimental field of Epoisses. Analysis of the 16S rDNA sequence using bioinformatic tools revealed that isolate was belonging to genus *Sphingomonas* and was designated *Sphingomonas* sp. SH. Like the enriched bacterial culture described in the previous chapter, the strain SH also had the ability to degrade only isoproturon and none of other structurally related phenylurea herbicides. This observation further suggests the specificity of the catabolic enzymes for IPU degradation. The specificity of the strain SH and of the enriched bacterial culture for IPU was also reported for *Methylopila* sp. TES (El-Sebai et al., 2004) isolated previously from the soil of Le Souich. As *Sphingomonas* sp. SH had the ability to degrade known IPU metabolites including 4-isopropylaniline, it might follow the metabolic pathway described for the *Sphingomonas* sp. SRS2 (Sorensen et al., 2001) and the enriched bacterial culture (Hussain et al., 2009b). The optimal degradation activity was observed at pH 7.5 and was shown to be considerably decreased at pH values lower and higher than 7.5. Based on the presence of the *catA* gene in the strain SH and on the work of Sun et al. (2009), we hypothesized that phenyl ring cleavage IPU may occur through *ortho*-cleavage of catechol putatively formed downstream of 4-isopropylaniline. In order to further characterize the IPU metabolic pathway, we developed a genomic approach relying on the construction of a BAC clone library screened by a functional approach and by a molecular approach targeted on *catA* gene.

GENOMIC CHARACTERIZATION OF A CATECHOL DEGRADING GENE CLUSTER

Introduction

Although a lot of work is available on characterization of microbial biodegradation of different pesticides at field level and in isolated bacterial and fungal cultures, there are relatively few reports about the characterization of the genes and related enzymes catalyzing the biodegradation process. So, there is a dire need to identify and characterize the genes and enzymes involved in biodegradation of xenobiotics including pesticides to better understand their metabolic processes. Even though, there are a number of reports about the genes and enzymes involved in biodegradation of different pesticides including organochlorine insecticides (Boltner et al., 2005; Mertens et al., 2006), organophosphorus insecticides (Tehara and Keasling, 2003), carbamate (Mulbry and Eaton, 1991), atrazine (Aislabie et al., 2005; Martinez et al., 2001), Trifluralin (Bellinaso et al., 2003) and phenoxy acid herbicides (De Lipthay et al., 2001), there is very little knowledge genes and enzymes involved in biodegradation of phenylurea herbicides including isoproturon. Recently, phenylurea hydrolase genes *puhA* and *puhB* have been identified to be involved in biodegradation of phenylurea herbicide diuron (Turnbull et al., 2001; Khurana et al., 2009). As the genes and enzymes involved in isoproturon degradation still remain unknown, so, this part of the study was carried out to identify the genes involved in biodegradation of isoproturon.

In order to achieve the goal of identifying the genes involved in biodegradation of isoproturon, a BAC clone library was established from the size selected partially digested genomic DNA of the bacterial culture having the ability to mineralize isoproturon (Hussain et al., 2009b). 3000 clones were selected for functional screening based on estimating their ability to degrade isoproturon as well as its known metabolites i.e. MDIPU, DDIPU and 4-IA. In addition, a molecular screening was also performed by targeting the catA sequence which was recently suggested to be involved in the transformation of aniline by *Sphingobium* sp. YBL2, an isoproturon mineralizing bacterial strain (Sun et al., 2009). *catA* PCR led to the identification of three BACs which were pyrosequenced and analysed using bioinformatic tools.

Article under process:

Genomic characterization of a catechol-degrading gene cluster isolated through BAC library prepared from an isoproturon mineralizing bacterial culture

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Abstract

catA gene coding for catechol 1,2-dioxygenase, involved in the degradation of catechol, was amplified from an isoproturon mineralizing enriched bacterial culture. In order to search for the genes coding for IPU degrading enzymes, a BAC clone library, consisting of 3000 clones, was established from the genomic DNA of the strain SH. PCR based genomic screening led to the identification of three positive BAC clones harbouring *catA* sequence. Identified BAC clones were pyrosequenced. Multiple alignment and aggregation of the sequences of three BAC clones yielded in a DNA sequence of 33.1 kb. Annotation of the sequence using the bioinformatics tools indicated the presence of 32 open reading frames (ORF) including a *cat* gene cluster consisting of *catA*, *catB*, *catC*, *catD* and *catI* genes placed under the regulation of *LysR* family transcriptional regulator. Further sequence analysis revealed that overall gene organization of the *cat* gene cluster in this bacterial culture differed from that of identified in other bacterial strains. This work is considered as a first study describing the sequence and genetic organization of a *cat* gene cluster in an isoproturon mineralizing bacterial culture.

Key words: Isoproturon, catechol, BAC library, *cat* gene cluster, organization

1. Introduction

As a result of rapid industrialization and intensive agriculture, a wide variety of xenobiotics are contaminating the environment. Contamination with xenobiotics has been reported to influence the functioning of the overall ecosystem not only by affecting the quality of the natural resources including land and water but also by entering the food chain and having the adverse effect on humans, plants, animals and microorganisms (Mrozic et al., 2003; Sweetman et al., 2005; Vallotton et al., 2009; Widenfalk et al., 2008; El-Azhari et al., 2010). Among the xenobiotics, often contaminating the environment, pesticides are widespread in nature because of their repeated widespread use in agriculture sector to control the development of pests and diseases. Keeping the hazardous environmental impacts of the pesticides in view, various ways have been devised to reduce their use, decrease pesticide wastes and eliminate or reduce their dispersal and persistence in the environment. Based on recent studies indicating that that microbial biodegradation is the principal mechanism for the dissipation of pesticides from the soil environment, a lot of emphasis is done on promoting natural attenuation of pesticides (Fournier et al., 1975; Cox et al., 1996; Pieuchot et al., 1996; Arbeli and Fuentes, 2007; El-Sebai et al., 2007).

Isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea], IPU, a herbicide belonging to the phenylurea family, is one of the most extensively used herbicide for pre-and post-emergence control of many broadleaf weeds in cereal cultures. As a result of its intensive and repeated use and physico-chemical properties, IPU was dispersed from agricultural fields to the environment where it is often detected in surface and ground water in Europe at levels exceeding the EU drinking water limit fixed to 0.1 µg L^{-1} (Müller et al., 2002). In addition to the detection in water resources, ecotoxicological data suggest that IPU and its metabolites are harmful not only for animals, plants and microorganisms but also for human beings (Behera and Bhunya, 1990; Hoshiya et al., 1993; Mansour et al., 1999; Widenfalk et al., 2008; Vallotton et al., 2009). So, there is need to understand the biodegradation processes achieving the filter capacity of the soil which leads to its dissipation from the environment.

Recently, enhanced biodegradation was reported in agricultural soils often treated with this herbicide (Bending et al., 2003; Sorensen and Aamnad, 2001; El-Sebai et al., 2005). Observation of enhanced IPU degradation has stimulated the research aiming at isolating the microbial strains involved in IPU degradation and characterizing the genes coding for IPU degradation enzymes. As a result, the potential of IPU degradation has been reported in a number of bacterial and fungal strains isolated from the geographically different regions of

the world (Khadrani et al., 1999; Sørensen et al., 2001; Tixier et al., 2002; Bending et al., 2003; El-Sebai et al., 2004; Badawi et al., 2009; Hussain et al., 2009; Sun et al., 2009).

Although IPU degrading potential has been reported in a number of bacterial and fungal strains, the complete metabolic pathway and the genes involved in its degradation still remain poorly understood. However, it has to be noticed that Turnbull et al. (2001) have identified *puhA*, a gene coding for the phenylurea hydrolase involved in biodegradation of diuron by *Arthrobacter globiformis* D47. This bacterial strain initially isolated for its diuron-degrading ability is also able to degrade different phenylurea herbicides including IPU to their aniline derivatives by hydrolysis of the urea side chain. Although this gene was described in *Arthrobacter globiformis* D47, it could not be observed in *Sphingomonas* sp. SRS2 and *Methylopila* sp. TES, two isolates able to mineralize IPU (Sorensen et al., 2001; El-Sebai et al., 2004). Further studies have suggested that the initial steps of IPU degradation involved in upper metabolic pathway lead to 4-isopropylaniline (4-IA) accumulation. This key intermediate could be mineralized by phenyl ring cleavage (Sorensen et al., 2001; Sorensen et al., 2003). Based on detection and identification of metabolites, transitory accumulated during IPU mineralization, Sorensen et al. (2001) and Hussain et al. (2009b) proposed a metabolic pathway for IPU degradation. The upper metabolic pathway initiated by two successive N-demethylations followed by cleavage of the urea side chain results in the transitory accumulation of 4-IA. The lower pathway leads to the mineralization of the phenyl structure. Catechol which is considered as a key intermediate during the degradation of many aromatic compounds as well as its corresponding aliphatic metabolite i.e. cis,cis-muconate have also been also detected during the degradation of aniline by an isoproturon (IPU) mineralizing bacterial strain YBL2 (Sun et al., 2009). Although the steps involved in upper metabolic pathway have been proposed, until now, there are no reports about the lower metabolic pathway involved in the degradation of the phenyl ring found in 4-IA.

Catechol is not only a key intermediate of natural phenolic compounds but also numerous aromatic compounds including benzene, aniline, phenol, benzoate, naphthalein and resorcinol etc (Borraccino et al., 2001; Caposio et al., 2002; Murakami et al., 2003; Vesely et al., 2007). It is well known that the subsequent metabolism of the catechol into aliphatic compounds takes place either by *ortho*- or *meta*-cleavage of the phenyl-ring (Ngai et al., 1990; Caposio et al., 2002; Murakami et al., 2004; Tsai and Li, 2007; Vesely et al., 2007). *Ortho*-cleavage of catechol phenyl-ring takes place by the involvement of different *cat* genes (*catA, catB, catC, catD, catI, catJ, catF*) already reported in a number of bacterial strains

(Shanley et al., 1994; Harwood and Parales, 1996; Kim et al., 1999; Murakami et al., 1999; Caposio et al., 2002; Vesely et al., 2007; Yoon et al., 2007).

Recently, *catA* gene coding for catechol 1,2-dioxygenase, transforming catechol into cis,cis-muconate, has been reported to be amplified from the IPU mineralizing strains *Sphingobium* sp. YBL1, YBL2 and YBL3 (Sun et al., 2009). Based on this observation, Sun et al. (2009) proposed that the lower pathway of IPU could rely on the *ortho*-cleavage of catechol formed after the transformation of 4-IA. Although *cat* gene clusters have been identified in several bacterial strains involved in degradation of different aromatic compounds (Murakami et al., 1999; Nojiri et al., 2002; Murakami et al., 2004; Shen et al., 2004; Vesely et al., 2007; Yoon et al., 2007), to our knowledge, there is yet no report about their presence in IPU mineralizing bacteria although the skeleton of this herbicide is made of an aromatic ring. Therefore, in order to provide a better insight in the genetic basis of the IPU metabolic pathway, the present study reports the development of a molecular approach aiming at identifying gene cluster coding for *cat* operon from an IPU mineralizing bacterial culture (Hussain et al., 2009b) enriched from a French agricultural soil adopted to enhanced mineralization of IPU (El-Sebai et al., 2005).

2. Materials and methods

2.1. Bacterial culture

The mixed bacterial culture characterized for isoproturon mineralization in chapter 4 (Hussain et al., 2009b) was used for constructing a genomic bacterial artificial chromosome (BAC) clone library.

2.2. Cultural media and growth conditions

IPU mineralizing bacterial culture was grown at 28°C in liquid mineral salt (MS) medium (Rousseaux et al., 2001) containing 50 mg L^{-1} of IPU used as a sole source of carbon and nitrogen. *E. coli* were grown in Luria-Bertani medium (LB) [tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (5 g L^{-1})] at 37°C.

2.3. Genomic DNA extraction

The genomic DNA was extracted at the late exponential stage of growth of the IPU mineralizing bacterial culture using QIAGEN Genomic DNA Isolation Kit (QIAGEN, France) according to the provider's recommendation.

2.4. Amplification of partial *catA* gene

catA gene was amplified from the genomic DNA of the bacterial culture using the catAf (5'-CCATTGAAGGGCCGCTCTATGT-3') and catAr (5'-ACCGAARTTGATCTGCGT(G,C)GTCA-3') primers designed by Sun et al. (2009). The amplification reaction was carried out in a final 25 µL volume containing 2.5 µL of 10X Taq polymerase buffer containing 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, , 0.5 µM of each primer and 0.625 U of Taq polymerase. 25 ng of the genomic DNA was used as template for the PCR reactions. PCR was performed in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Mass) according to the following program: 30 cycles of 1 min of denaturation at 95 °C, 1 min for annealing at 55 °C and 30 seconds of extension at 72 °C with a final extension step at 72 °C for 5 min after cycling was compete

2.5. BAC cloning and sequencing of the genomic DNA

The genomic DNA extracted from the IPU mineralizing bacterial culture was partially digested with *Sau3A1* (0.125 U/µg) for 1.5 hours at 37°C. The partially digested genomic DNA was separated by electrophoresis on 0.6% agarose gel. Size selection was carried out by cutting out the DNA fragments between 20-30 kb from the gel. The DNA fragments were recovered by an enzymatic reaction catalyzed by GELase™ allowing the digestion of agarose gel according to provider's recommendations (Epicentre, France). This step was repeated twice to get a sharp size selection. The partially digested size selected DNA was ligated into CopyControl pCC1BAC™ Vector (EPICENTRE, France) and then transformed into TransforMax EP1300 electrocompetent *E. coli* (EPICENTRE, France) using the CopyControl™ BAC Cloning kit (EPICENTRE, France). The recombinant clones, white in colour, were picked, inoculated on new petri dishes and preserved at 4°C until their use.

2.6. *catA* PCR Screening of the BAC clone library

The screening of the BAC clone library was conducted by PCR as described above. Briefly, the first PCR screening was performed on 30 pools made of 100 clones each. The pools producing *catA* PCR signal were then analyzed by PCR in order to identify the BAC clone containing *catA* sequence.

The BAC clones harboring the *catA* sequence were then grown overnight in liquid LB media at 37°C. Recombinant plasmid DNA of the *catA* harboring BAC clones was extracted using the Qiafilter Plasmid Midi Kit (QIAGEN, France) according to manufacturer's recommendations. The genomic DNA inserts were fully sequenced by pyrosequencing using

the Genome Sequencer FLX Titanium System™ (Beckman Coulter Genomic, USA). A 33 kb genomic DNA sequence was deposited in the GenBank database under the accession number ……..

2.7. Phylogenetic analysis and annotation of the sequences

Annotation of the 33 kb BAC sequence was performed using different tools available online. Open reading frames (ORFs) in the sequence were identified by using the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The ORFs were checked using GeneMark (http://exon.biology.gatech.edu). GC content of the DNA fragment was estimated by using an applet (http://www.sciencebuddies.org/mentoring/project_ideas/Genom_GC_Calculator.shtml). Insertion sequences were searched using IS finder. To search for homology of each identified ORF with known sequences, blastN and blastX analyses were performed. To polish this first analysis, multiple alignments were performed with closest relative identified.

*cat*A sequence was compared to the known nucleotide sequences using BlastX (http://www.ncbi.nlm.nih.gov/BLAST). To construct a phylogenetic trees based on *cat*A sequences, multiple alignments were carried out using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) and the data obtained was processed using NJ Plot (http://pbil.univ-lyon1.fr/software/njplot.html) using the neighbor joining method.

3. Results and discussion

In a previous study, we have reported the isolation and characterization of a bacterial culture mineralizing IPU (Hussain et al., 2009b). We showed that this bacterial culture was able to degrade IPU through two successive N-demethylations followed by cleavage of the urea side chain leading to the formation of 4-IA. This metabolite was then entirely mineralized. A recent study has suggested that 4-IA could be transformed to catechol which is further degraded by *ortho*-cleavage of phenyl ring catalyzed by catechol 1,2-dioxygenase (*catA*) (Sun et al., 2009). This hypothesis was reinforced by the detection of *catA* sequences in IPU mineralizing *Sphingobium* sp. strains YBL1, YBL2 and YBL3 (Sun et al., 2009). In order to check this hypothesis, we performed UPLC analysis to detect the formation of catechol during IPU biodegradation (results will be obtained in August 2010 in collaboration with GEUS, Denmark). In addition, we searched for the presence of *catA* sequence by PCR using the *catA* primers designed by Sun et al. (2009). A DNA fragment of the expected size

Table VI-1. Localization and predicted functions of ORFs in the 33 kb DNA sequence isolated from the bacterial culture

Orf No.	Position (bp)[a]	Putative conserved domains (superfamily)[b]	Function of closest relative	Source organism	No. of amino acids /relative[c]	% Amino acid identity[d]	GenBank accession number	E value
1	50-976 d	DUF849	Hypothetical protein BAL199_26152	*Alpha proteobacterium* BAL199	308/312	75% (233/307)	EDP63681.1	2e-139
2	1050-2054d	AdoMet-MTase	Oxidoreductase, zinc-binding protein	*Pseudomonas syringae* pv. oryzae str. 1_6	334/335	45% (154/341)	ZP_04586095.1	4e-80
3	2051-2335d	COG3293	Transposase	*Mesorhizobium loti* MAFF303099	94/103	92% (86/93)	BAB54834.1	5e-44
4	2398-2817c	COG3293	Putative insertion element transposase protein	*Mesorhizobium loti*	138/139	83% (115/138)	CAD31638.1	1e-62
5	3453-4223d	metallo-dependent hydrolases	Guanine deaminase, putative	*Aspergillus flavus* NRRL3357	257/479	42% (82/191)	EED48226.1	2e-36
6	4274-4900d	metallo-dependent hydrolases	Hypothetical protein BpOF4_18180	*Bacillus pseudofirmus* OF4	208/449	37% (72/191)	YP_003428569.1	3e-30
7	4941-5675d	NADB_Rossmann	Short-chain dehydrogenase/reductase SDR	*Kribbella flavida* DSM 17836	244/247	46% (113/242)	ADB29804.1	2e-49
8	5924-6841c	FGE-sulfatase	Protein of unknown function DUF323	*Hyphomicrobium denitrificans* ATCC 51888	305/333	62% (188/302)	EET65645.1	2e-112
9	6857-9217c	Sulfatase	Sulfatase	*Novosphingobium aromaticivorans* DSM 12444	786/786	70% (554/782)	ABD26906.1	0.0
10	9301-11838c	OM channels	TonB-dependent receptor	*Novosphingobium aromaticivorans* DSM 12444	845/843	86% (703/811)	ABD26905.1	0.0
11	11952-12320d	TetR N	TetR family transcriptional regulator	*Novosphingobium aromaticivorans* DSM 12444	122/255	57% (48/83)	ABD26904.1	4e-18
12	12423-13151c	P-loop NTPase domain	IstB ATP binding domain-containing protein	*Sphingomonas wittichii* RW1	242/242	99% (241/242)	ABQ71598.1	2e-137
13	13138-14652c	Rve	Integrase catalytic subunit	*Sphingomonas wittichii* RW1	504/501	96% (482/501)	YP_001260008.1	0.0
14	14748-15143d	No putative conserved domain	TetR family transcriptional regulator	*Novosphingobium aromaticivorans* DSM 12444	138/255	31% (37/118)	ABD26904.1	4e-06
15	17625-18413c	Esterase lipase	3-oxoadipate enol-lactone hydrolase (CatD)	*Sphingomonas* sp. KA1	262/271	57% (150/260)	BAF03259.1	2e-81
16	18497-19210c	coA trans	3-oxoacid CoA-transferase, A subunit (CatI)	*Novosphingobium aromaticivorans* DSM 12444	237/242	84% (194/230)	ABP64682.1	2e-111
17	19220-20116c	Intradiol dioxygenase	Catechol 1,2-dioxygenase (CatA)	*Sphingobium japonicum* UT26S	298/296	73% (219/297)	BAI97352.1	2e-129

Orf No.	Position (bp)[a]	Putative conserved domains (superfamily)[b]	Function of closest relative	Source organism	No. of amino acids /relative[c]	% Amino acid identity[d]	GenBank accession number	E value
18	20142-20432c	MIase	Muconolactone delta-isomerase (CatC)	*Sphingobium japonicum* UT26S	96/96	75% (72/96)	BAI97353.1	3e-37
19	20437-21603c	TIM_phosphate_binding	Muconate cycloisomerase (CatB)	*Sphingomonas* sp. KA1	388/391	75% (288/384)	BAF03256.1	4e-166
20	21696-22580d	PBP2 LTTR substrate	LysR-family transcriptional regulator	*Sphingobium japonicum* UT26S	294/303	58% (173/298)	BAI97355.1	1e-93
21	22586-23059c	WHTH GntR	Transcriptional regulator, MarR family	*Sphingomonas wittichii* RW1	157/158	66% (96/145)	ABQ67114.1	7e-50
22	23171-24304d	GGCT like	Amidase	*Sphingomonas wittichii* RW1	377/382	64% (238/368)	ABQ67113.1	2e-107
23	24301-24831	No putative conserved domain	Hypothetical protein Swit_0745	*Sphingomonas wittichii* RW1	176/183	69% (126/181)	ABQ67112.1	6e-64
24	24726-26090d	Ring hydroxylating alpha and rieske	Rieske (2Fe-2S) domain protein	*Sphingomonas wittichii* RW1	418/418	82% (346/417)	ABQ67111.1	0.0
25	26087-26599d	NTF2 like	Aromatic-ring-hydroxylating dioxygenase	*Sphingomonas wittichii* RW1	170/171	64% (110/171)	ABQ67110.1	2e-58
26	26596-27378 d	FAA hydrolase	2-keto-4-pentenoate hydratase-like protein	*Sphingomonas wittichii* RW1	260/281	50% (128/255)	ABQ67109.1	1e-58
27	27697-28851d	ACAD	Acyl-CoA dehydrogenase	*Sphingomonas wittichii* RW1	384/386	77% (291/374)	ABQ67105.1	1e-172
28	28869-29231d	No putative conserved domain	Hypothetical protein Swit_0739	*Sphingomonas wittichii* RW1	120/119	87% (104/119)	ABQ67106.1	8e-55
29	29228-29968d	NADB_Rossmann	Short-chain dehydrogenase/reductase SDR	*Novosphingobium aromaticivorans* DSM 12444	246/246	67% (167/246)	ABP64666.1	4e-90
30	29978-30442c	WHTH GntR	Transcriptional regulator, MarR family	*Sphingomonas wittichii* RW1	154/157	58% (81/139)	ABQ67108.1	1e-38
31	30706-31617c	HTH AraC	Transcriptional regulator, AraC family	*Sphingomonas wittichii* RW1	303/307	50% (149/293)	ABQ70687.1	2e-76
32	31915-33099d	OM channels	TonB-dependent receptor	*Sphingomonas wittichii* RW1	395/755	74% (297/399)	ABQ70688.1	3e-173

a Letters indicate coding strand: c, complementary strand; d, direct strand.
b The superfamily, to which the conserved domain belongs, is indicated.
c Number of amino acids in the seqeunced ORF/number in the closest relative.
d Values in parentheses refer to numbers of amino acids with identity per total number examined.

(approximately 420 bp) was amplified from the DNA of the bacterial culture. Following its cloning and sequencing, one could confirm that PCR amplicon was 96% homologous with *catA* of *Sphingobium* sp. YBL2 (Sun et al., 2009). *catA* phylogeny revealed that it clusters with the gram negative bacterial group (Fig. VI-1). Furthermore, the *catA* sequence gathered with *catA* sequences of family *Sphingomonadaceae* including *Sphingobium japonicum* UT26S (GenBank Ac. No. NC_014006), *Sphingomonas wittichii* RW1 (GenBank Ac. No. CP000699), *Sphingomonas sp.* KA1 (GenBank Ac. No. AB270530) and *Sphingomonas sp.* SRS2. Based on the *catA* phylogeny, one could infer that it might be harbored by a bacterial strain belonging to *Sphingomonadaceae* family which is in accordance with previous results showing that a non cultivable *Sphingomonas* strain was dominating the IPU mineralizing bacterial culture. The detection of the *catA* sequence in our IPU mineralizing bacterial culture reinforces the hypothesis that the phenyl ring of IPU might be degraded through *ortho*-cleavage of the catechol following the β-ketoadipate pathway.

In order to give further insight in the genetic basis of the IPU lower metabolic pathway, a molecular approach was settled down to fully characterize the genomic island containing *catA* sequence. To do so, a BAC clone library was constructed from partially digested genomic DNA. About 3000 BAC clones of 20-30 kb in length were picked up which represent in total 75 Mb. Considering 4 Mb as the medium size for a bacterial genome, this library was designed to cover approximately 7.5 genomes. By screening of 3000 BAC clones, 3 positive clones were detected and their BACs were fully sequenced by pyrosequencing. Analysis of the three sequences indicated that they were identical and could be merged in one sequence of 33100 bp. This sequence has 59.6% of G+C content. This value is in the range of those found in several genera of gram-negative soil bacteria (Holben and Harris, 1995; Nusslein and Tiedje, 1998). A physical map of the sequence showing the ORFs is presented in figure VI-2. A total of 32 ORFs were identified all along the sequence using GeneMark and NCBI ORF Finder. Interestingly, no ORF was found in a DNA region of 2213 bp. This DNA region had GC content of 51.4%. This region is made of a duplicated sequence of 933 pb showing 84% of homology between the duplicates. This observation suggests that genetic rearrangement might have occured in this DNA region. Unfortunately, no IS could be found in this area.

Based on BlastX alignment allowing to identify closest protein sequences, putative functions were assigned to the majority of the ORFs (Table. VI-1). About 56% of the ORFs were putatively predicted to be involved either in basal metabolism of the bacteria or related

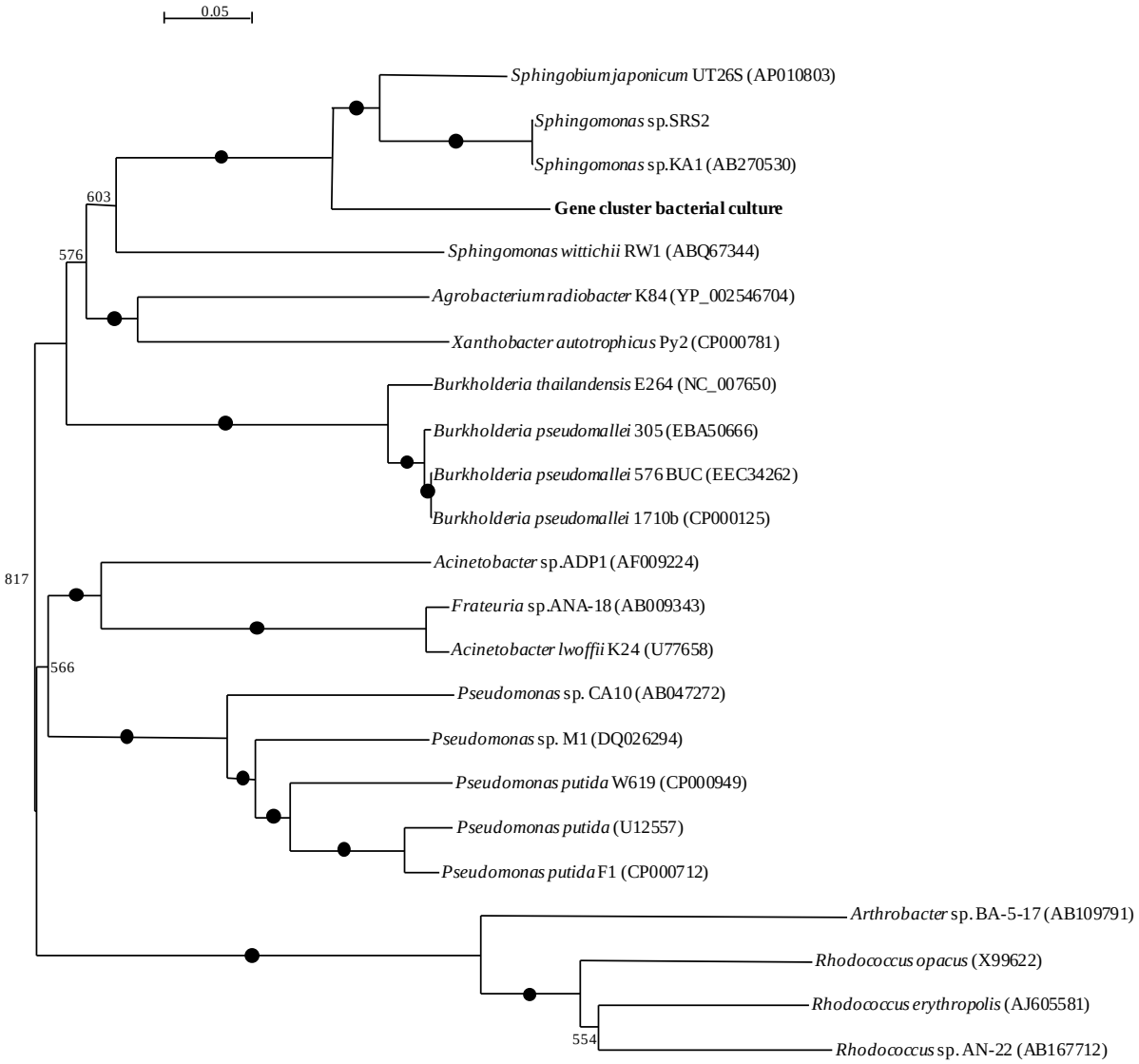

Fig. VI-1. Phylogenetic tree based on *catA* sequences of 23 bacterial strains. GenBank accession numbers of the *cat*A sequences are given in brackets. Bootstrap values greater than 900‰ are marked as black circles.

to catalyzing activities, 9% were predicted to be mobile elements, 18% encode putative transcriptional regulators and 6% were predicted to be involved in membrane sensing. Three ORFs representing up to 9% of the overall sequence showed high amino acid identity (>70%) with hypothetical proteins. It is noteworthy that about 70% of the ORFs (23/33) has maximum identity with that of found in different bacterial strains belonging to *Sphingomonadaceae* family including the genera *Novosphingobium*, *Sphingomonas and Sphingobium* (Table. VI-1). This observation is in agreement with the composition of the bacterial culture which was dominated by *Sphingomonas* strains representing up to 90% abundance of the members in bacterial culture.

About 9% of the identified ORFs (ORF3, ORF4, ORF12) had considerable identities with the proteins involved in DNA transposition. Two hot spots of genetic rearrangement could be found in the genomic sequence: one located between 2000-3000 (ORF3 and ORF4) and other one located between 12000-13000 (ORF12). ORF3 (nucleotides 2051-2335) and ORF4 (2398-2817) were found to have >80% amino acid identities with the putative transposases previously identified in *Mesorhizobium loti* (Kaneko et al., 2000). Analysis of this DNA region with IS Finder (http://www-is.biotoul.fr/is.html) revealed that a sequence of 780 bp (nucleotide 2054 to 2833) shared 81 % homology with IS*427*. This IS was previously described in *Agrobacterium tumefaciens* T37 (Demeirsman et al., 1990) and belongs to IS*5* family. However, this sequence is truncated at its right end suggesting that this IS element may not be functional anymore. Similarly, ORF12 (nucleotides 12423-13151) and ORF13 (nucleotides 13138-14652) showed high amino acid identity (>90%) with IstB ATP binding domain-containing protein and integrase catalytic subunit already identified in *Sphingomonas wittichii* RW1 (GenBank Ac. No. ABQ71598, GenBank Ac. No. YP_001260018). These ORFs are located on a DNA region (nucleotide 12 430 to 14 764) (2 335 bp) presenting 74% homology with IS*Ssp4*. However, again a close analysis of this sequence revealed that it was truncated at its left side 223 bp as compared to IS*Ssp4*. The deletion of the inverted repeat present at the left end side of the IS element may impair its functionality. However, the presence of these elements suggests that they play a key role in the evolution of the genome of the strain. This insertion sequence belongs to the IS*21* family and was first described on the plasmid CAR3 of *Sphingomonas* sp. KA1. Interestingly, this strain able to degrade carbazole through the β-ketoadipate pathway harbors IS*21* on pCAR3 at 29 kb from the *cat* (RABCDIJF) operon (Shintami et al., 2007). Insertion sequences are known to play a key role in the evolution of microbial degradation pathway of several xenobiotics including 4-

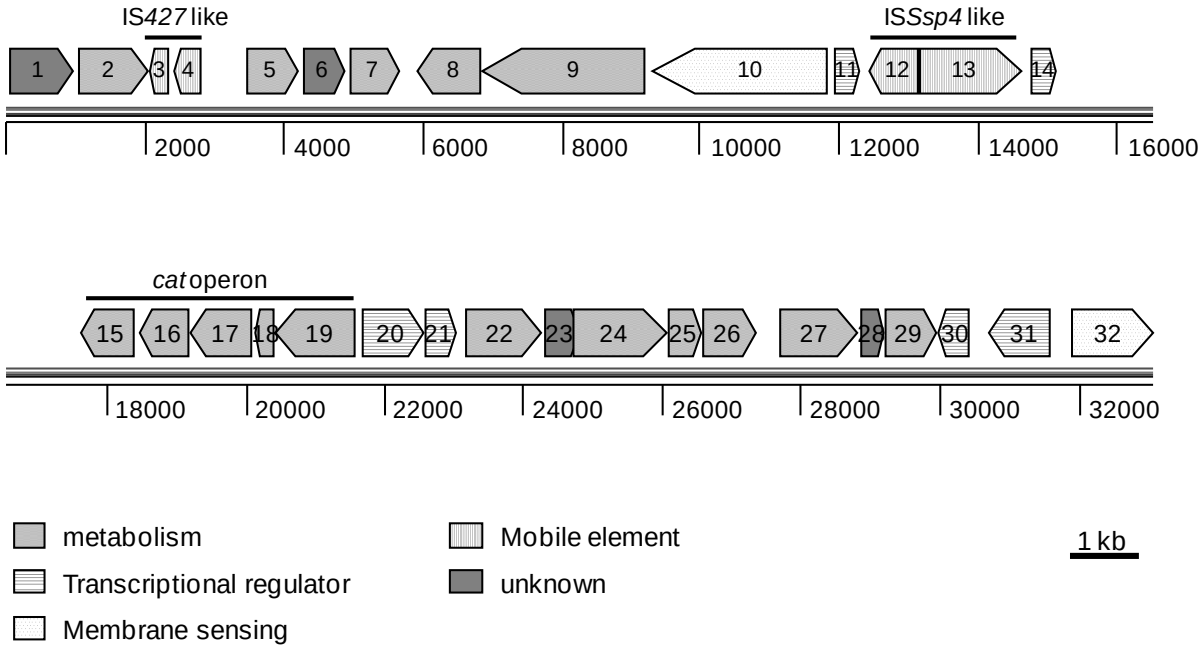

Fig. VI-2. Physical map of 33 kb DNA fragment highlighting the *catR-catBCAID* gene cluster

chlorobenzene (Layton et al., 1992), 2,4-D (Don and Pemberton, 1981), 3-chloroaniline (Boon et al., 2001), toluene (Tsuda et al., 1999), chlorocatechol (Van de Meer et al., 2001) and atrazine (Devers et al., 2008). These mobile genetic elements (MGE) are responsible for the recruitment and the rearrangement of catabolic genes increasing the diversity of the xenobiotic degrading community, thereby contributing to microbial adaptation to the degradation of xenobiotic (Top et al., 2002). Thus, the presence of transposases in the genome sequence isolated from the bacterial culture is a signature of its evolution which led to the adaptation for the IPU mineralization.

Six ORFs (about 18% of total) located in different regions of the 33 kb genomic sequence showed high homology with transcriptional regulators. Two ORFs, ORF11 (nucleotides 11952-12320) and ORF14 (nucleotides 14748-15143) shared 57% and 31% homology with TetR family transcriptional regulator identified in *Novosphingobium aromaticivorans* DSM 12444 (GenBank Ac. No. ABD26904). It is noteworthy that ORF11 and ORF14 are showing homology with TetR from amino acids 36 to 118 and 117 to 232 respectively. Since the two sequences found between these ORF11 and 14 are IS sequences, one suggests that TetR gene was first present in the strain but was interrupted by the insertion of Ssp4-like IS during evolution process. ORF21 (22586-23059) and ORF30 (29978-30544) were also identified as transcriptional regulators having 64 and 58% amino acid homology with two transcriptional regulators belonging to MarR family. These transcriptional regulators identified in *Sphingomonas wittichii* RW1 regulate the efflux and influx of phenolic compounds through bacterial membrane (Saridakis et al., 2008). ORF31 (30706-31617) showed 50% amino acid identity to the transcriptional regulator belonging to AraC family identified in *Sphingomonas wittichii* RW1. This transcriptional factor is known to regulate transcription operons involved in arabinose catabolism. Interestingly, ORF20 showed high similarity to LysR-type transcriptional regulator. This point will be discussed in detail along with the description of the *cat* gene clusters placed under the regulation of this transcriptional factor.

ORF10 and ORF32 showed high homology (68% and 74%) with TonB-dependent receptor identified in *Novosphingobium aromaticivorans* DSM 12444 (GenBank Ac. No. ABD26905) and *Sphingomonas wittichii* RW1 (GenBank Ac. No. ABQ70688), respectively. Three ORFs (ORF1, ORF2 and ORF29) showed up to >70% homology to the conserved

hypothetical proteins identified in the genomes of *Alpha proteobacterium* BAL199(GenBank Ac. No. EDP63681), *Rhodobacter sphaeroides* KD131 (Lim et al., 2009) or *Sphingomonas wittichii* RW1 (GenBank Ac. No. ABQ67106). The function of these proteins still remain to be described.

Half of the ORFs (18/33) showed similarities with different types of catalytic enzymes including hydrolase (ORF5 and ORF6), sulfatase (ORF9), amidase (ORF22), aromatic ring hydroxylating dioxygenase (ORF25) and short chain hydrogenase/reductase SDR (ORF29) (Table VI-1). It is noteworthy that several ORFs are highly similar to enzymes catalyzing the *ortho*-cleavage of catechol and further transformation to CO_2 with: catechol 1,2-dioxygenase (ORF17), muconate cycloisomerase (ORF19), muconolactone delta-isomerase (ORF18), 3-oxoadipate enol-lactone hydrolase (ORF15) and 3-oxoacid CoA-transferase (ORF16). This observation suggests that the bacterial culture harbors the genetic potential required to transform catechol formed during IPU mineralization following 4-IA transformation.

3.1. Sequence analysis of the *cat* gene cluster

Sequence analysis of the genomic island purified from the IPU mineralizing bacterial culture revealed the presence of a *cat* gene cluster of about 4 kb. This *cat* cluster consists of five open reading frames ORF16 (17625-18413), ORF17 (18497-19210), ORF18 (19220-20152), ORF19 (20142-20432) and ORF20 (20437-21603) corresponding to the *catD*, *catI*, *catA*, *catC* and *catB* genes, respectively (Table VI-1). ORF17 (933 bp) encoded a protein sharing 73% homology with catechol 1,2-dioxygenase (*catA*) of *Sphingobium japonicum* UT26S (GenBank Ac. No. BAI97352). ORF19 encoded a protein sharing 77% amino acid identity with the muconate cycloisomerase (*catB*) of *Sphingobium japonicum* UT26S (GenBank Ac. No. BAI97354). ORF18 located downstream of ORF19 homologous to *catB* encoded a protein sharing 75% homology with muconolactone delta-isomerase (*catC*) of *Sphingobium japonicum* UT26S (GenBank Ac. No. BAI97353). Similarly, ORF15 and ORF17 located downstream of ORF17 homologous to *catA* encoded proteins which had 57% homology with 3-oxoadipate enol-lactone hydrolase (*catD*) of *Sphingomonas* sp. KA1 (GenBank Ac. No. BAF03259) and 87% homology with a subunit 3-oxoacid CoA-transferase (*catI*) of *Sphingomonas wittichii* RW1 (GenBank Ac. No. ABQ71636), respectively. It is noteworthy that most of the genes found in the *cat* gene cluster showed highest homologies with sequences belonging to *Sphingomonadaceae* family. Interestingly, ORF21 (21696-22580) which was located upstream of ORF19 (*catB*) in the reverse sense , was found to have 58% and 53% amino acid identity with the *LysR* family transcriptional regulators of

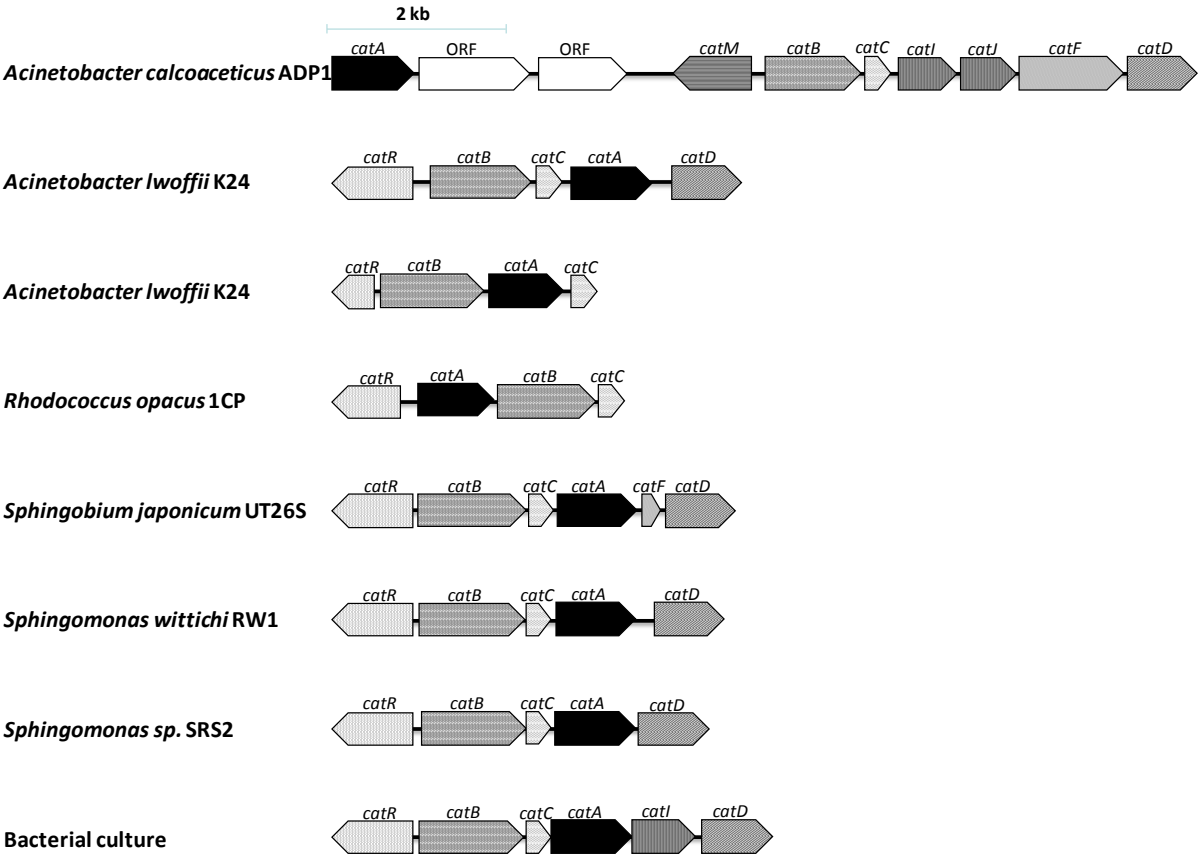

Fig. VI-3. Organization of *cat* gene cluster in various bacterial strains. Similar genes are shaded in the same manner.

Sphingobium japonicum UT26S (GenBank Ac. No. BAI97355) and *Sphingomonas wittichii* RW1 (ABQ67341), respectively. In several previous studies, *cat* genes have been reported to form an operon with regulatory *catR* proteins coded by divergently transcribed genes (Eulberg et al., 1997; Kim et al., 1999; Vesely et al., 2007). For most of the *cat* operon studies in different bacterial strains, the expression of the catechol degrading genes was shown to be placed under the regulator of LysR-type transcriptional regulators (Neidel et al., 1989; An et al., 2001; Murakami et al., 2004; Vesely et al., 2007). In addition, catechol intermediate such as cis,cis-muconate may act as inducer for LysR (Vesely et al., 2007). Therefore, the presence of the *LysR* transcriptional regulator in the antisense orientation is in accordance with most of the studies describing this type of organization for *cat* operon. The β-ketoadipate pathway is widely distributed in soil fungal and bacterial strains (Harwood and Parales, 1996) and *cat* genes, involved in β-ketoadipate pathway, have been identified in different types of the bacterial strains involved in the degradation of a variety of aromatic compounds including anilines (Murakami et al., 1999; Matsumura et al., 2006; Yoon et al., 2007), carbazole (Nojiri et al., 2002), benzamide (Murakami et al., 2004), phenol (An et al., 2001; Caposio et al., 2002; Shen et al., 2004) and benzoate (An et al., 2001; Yoon et al., 2007). However, the present study is considered as the first report indicating the presence of a catechol degrading *cat* cluster in a bacterial culture mineralizing isoproturon. Based on IPU degradation pathway drawn for the bacterial culture, one could hypothesize that *cat* cluster constitutes the lower pathway of IPU degradation.

3.2. Organization of the *cat* gene cluster

cat gene clusters have already been reported in a number of bacterial strains including *Acinetobacter calcoaceticus* ADP1 (Neidle et al., 1988), *Acinetobacter lwoffii* K24 (Kim et al., 1999), *Rhodococcus opacus* 1CP (Eulberg et al., 1997). Bioinformatics analysis also allowed to find the *cat* operon in the genome of fully sequenced bacteria such as *Sphingobium japonicum* UT26S (GenBank Ac. No. NC_014006), *Sphingomonas wittichii* RW1 (GenBank Ac. No. CP00069) and *Sphingomonas sp.* SRS2 (Personal data, Sorensen). Although these genes code for enzymes having the same functions in different bacterial strains, the organization of *cat* clusters may differ with respect to the hosted bacteria (Fig. VI-3). The position of the *catA* gene differed significantly among the *cat* gene clusters (Fig. VI-3), specifically in *Acinetobacter calcoaceticus* ADP1 (Neidle et al., 1988) where it was found about 3.8 kb away upstream of *catB* gene. In *Rhodococcus opacus* 1CP (Eulberg and

Schlomann, 1998), *Rhodococcus erythropolis* AN-13 (GenBank Ac. No. D83237) and *Rhodococcus erythropolis* CCM2595 (Vesely et al., 2007), *catA* gene was found to be located upstream of *catB* in between the *catB* and the transcriptional regulator *catR*. On the contrary, *catB* and *catC* genes are adjacent and cotranscribed in most of the *cat* gene clusters observed in different bacteria (Aldrich et al., 1987; Houghton et al., 1995; Eulberg et al., 1997; Vesely et al., 2007). However, for *Acinetobacter lwoffii* K24 (Kim et al., 1999), *catA* gene was intervened between *catB* and *catC* genes and in this case, the *catC* gene was cotranscribed with *catA*. The IPU degrading bacterial culture showed a cat BCA operon similarly organized to *Pseudomonas putida* (McFall et al., 1998), *Pseudomonas resinovorans* (Nojiri et al., 2002), *Frateuria* sp. ANA-18 (Murakami et al., 1999) *Sphingobium japonicum* UT26S (GenBank Ac. No. NC_014006) and *Sphingomonas wittichii* RW1 (GenBank Ac. No. CP000699). In addition, it has to be noticed that *catI* was intervened between *catA* and *catD* in contrary to what was observed for *Acinetobacter lwoffii* K24 (Kim et al., 1999), *Sphingomonas wittichii* RW1, *Sphingomonas sp.* SRS2 (Personal data, Sorensen) and *Acinetobacter calcoaceticus* ADP1 (Neidle et al., 1988). The presence of *catI* between *catA* and *catD* makes the organization of *cat* gene cluster in this bacterial culture different from those described in other strains. The *cat* operon of IPU degrading culture could be placed under the regulation of the LysR-type transcriptional regulator located upstream of *catBCAID* in opposite orientation as previously shown in different bacterial strains (McFall et al., 1998, Nojiri et al., 2002, Vesely et al., 2007). In conclusion, the IPU degrading bacterial culture harbors a *catRBCAID* gene cluster showing the organization almost similar to that described in different strains belonging to family *Sphingomonadaceae* such as *Sphingobium japonicum* UT26S, *Sphingomonas wittichii* RW1 and *Sphingomonas sp.* SRS2. One could hypothesize that following IPU exposure, the *cat* operon found in *Sphingomonadaceae* strains has been recruited to form the lower pathway of IPU degradation, and that following the adaptation process, changes in genome organization might have occurred to promote catechol degradation.

In recent years, a number of researches have been carried out to study the biodegradation of isoproturon and a potential of isoproturon mineralization has been reported in several bacterial strains including *Sphingomonas* sp. SRS2 (Sorensen et al., 2001), *Methylopila* sp. TES (El-Sebai et al., 2004), *Sphingobium* sp. Strain YBL1, YBL2, YBL3 (Sun et al., 2009). Recently, Hussain et al. (2009) have also reported a mixed bacterial culture having the ability to mineralize isoproturon. Although the initial steps of isoproturon metabolic pathway have been proposed (Sorensen et al., 2001; Sorensen et al., 2003; Hussain

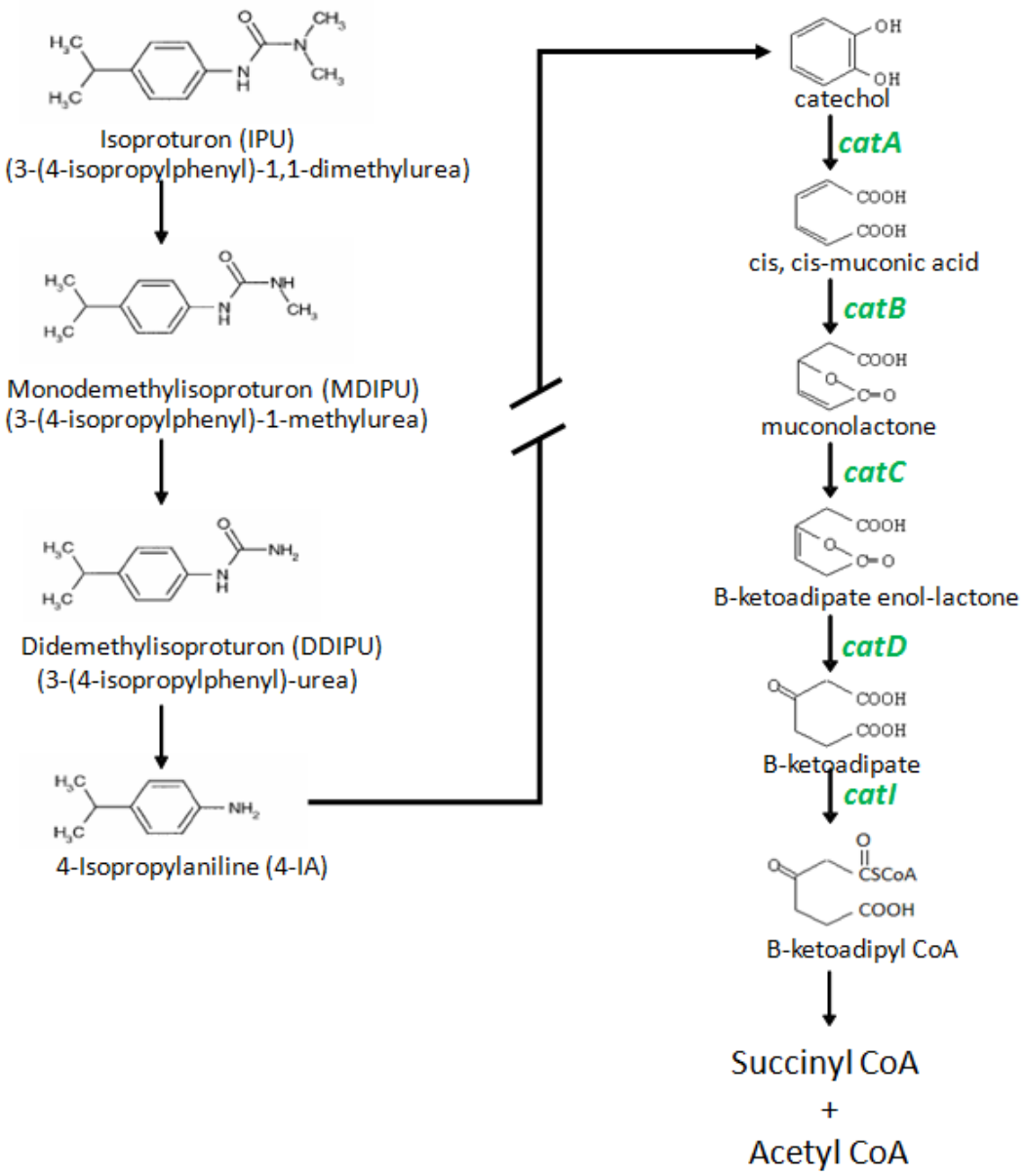

Fig. VI-4. Proposed metabolic pathway for IPU mineralization indicating the *cat* genes involved in catechol degradation

et al., 2009b), the lower steps of isoproturon metabolic pathway and the genes coding the enzymes involved in its metabolism have not yet been described. Recently, Sun et al. (2009) has reported the detection of *catA* sequence from IPU mineralizing bacterial strains *Sphingobium* sp. strain YBL1, YBL2, YBL3 as well as catechol production during aniline degradation by *Sphingobium* sp. YBL2. Therefore, based on the identification of catechol during IPU degradation by this bacterial culture and the characterization of *cat* gene cluster, one could propose that the lower pathway for IPU transformation takes place through *ortho*-cleavage of the catechol putatively formed after 4-isopropylaniline formation (Fig. VI-4).

Conclusion and perspecives

As primary objectives of this study, the genomic approach developed here did not yield in the identification of the genes coding the catabolic enzymes responsible for the upper pathway of isoproturon degradation. However, following this approach, we were at least able to identify a *cat* gene cluster putatively involved in the degradation of catechol through *ortho*-cleavage of the phenyl ring following the well known β-ketoadipate pathway. Catechol which is considered as a key intermediate during the degradation of aromatic compounds has not yet been detected as a metabolite during the isoproturon mineralization. Nevertheless, catechol as well as its first metabolite, cis,cis-muconic acid, after phenyl-ring cleavage have been detected during the degradation of aniline by an isoproturon mineralizing bacterial strain YBL2 (Sun et al., 2009). Similarly, *catA* gene coding for 1,2-dioxygenase, involved in catechol degradation, has also been found to be amplified not only in the strain YBL2 but also in other isoproturon mineralizing bacterial cultures including *Sphingomonas* sp. SH and the present enriched bacterial culture. So, based on this finding, we hypothesized that phenyl ring cleavage in isoproturon might also take place through *ortho*-cleavage of catechol putatively formed during isoproturon mineralization. So, we proposed, for the first time, the description of the lower pathway of IPU mineralization. However, to confirm this hypothesis, there is need to identify the formation of catechol and other metabolites during IPU mineralization using some more sensitive technique like ultra performance liquid chromatography (UPLC). Research will also be performed to detect the expression of cat genes during IPU mineralization.

GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

General Discussion

Phenylurea herbicides are one of the most important class of pesticides used worldwide for pre- or post-emergence control of broad leaf weeds in cotton, fruit or cereal production. They are systemic herbicides which inhibit the photosynthesis of the target weeds by disrupting the electron transfer in the photosystem II (Ducruet, 1991). Because of relatively higher water solubilities and moderate sorptions, phenylurea herbicides are fairly mobile in the soil compartment. As a result, they are often detected world widely in surface and ground water bodies (Field et al., 1997; Thurman et al., 2000, Gerecke et al., 2001; Johnson et al., 2001; Gooddy et al., 2002; Sorensen et al., 2003; Claver et al., 2006; Batisson et al., 2007). Isoproturon (IPU) is one of the most extensively used phenylurea herbicides in conventional agriculture in Europe (Nitchke and Schussler, 1998). Like for other phenylureas, IPU is often detected in surface and ground water bodies in Europe at levels exceeding the EU drinking water limit fixed to 0.1 µg l^{-1} (Nitchke and Schussler, 1998; Spliid and Koppen, 1998; Stangroom et al., 1998). In France, according to the French Institute for the Environment (IFEN), IPU was detected in 87% of the surface and 86% of the ground water samples tested in 2005 (IFEN, 2007). IPU and some of its known metabolites were reported to be harmful for aquatic invertebrates, fresh water algae and microbial activities as well as for plants, animals and human beings. Microbial biodegradation was reported as a primary mechanism of the dissipation of phenylureas including isoproturon in the soil environment (Fournier et al., 1975; Cox et al., 1996; Pieuchot et al., 1996). A number of bacterial strains have been shown to harbour the potential for isoproturon degradation (Sorensen et al., 2001, Bending et al., 2003; El-Sebai et al., 2004, Sun et al., 2009). Although a number of bacterial and fungal strains have been isolated and characterized for isoproturon degradation, the genes coding the catabolic enzymes responsible for IPU biodegradation still remain unknown. So, keeping the harmful effects of isoproturon in view, there is an urgent need to understand the behaviour and the processes involved in the dissipation of phenylureas not only at field level but also at cellular and molecular level. In this context, I carried out my PhD work with the objective to characterize and understand the isoproturon degradation processes from the bacterial community in the field to the degrading genes.

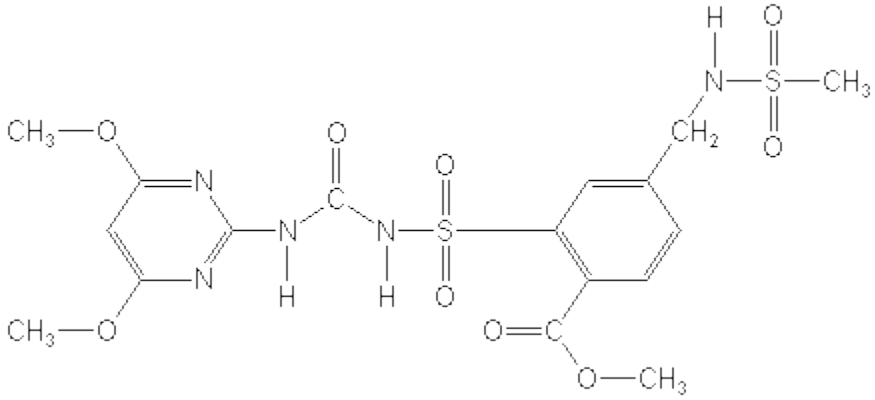

Fig. VII-1. Molecular structure of mesosulfuron-methyl (a sulfonylurea herbicide)

The field study carried out at the experimental farm of the INRA (Epoisses domain, Breteniere) showed that the field cropped with a winter wheat/barley/ rape seed rotation and periodically treated with IPU over the last decade showed the microflora adapted to isoproturon mineralization. This observation confirms the hypothesis that repeated exposure to IPU leads to the adaptation of the soil microflora to the degradation of IPU as already reported in a number of previous studies carried out in UK, Denmark and France (Soulas, 1993; Arrault, 2001; Sorensen and Aamand, 2003; El-Sebai et al., 2005). The mineralization potential observed in the soil of Breteniere was similar to that observed previously. Interestingly, although we showed that the IPU mineralization activity was varying within the field, the mean mineralization activity was shown to be higher the year when IPU or sulfonylurea were applied. This has already been described for 2,4-D (Soulas, 1993) for which it was shown that following a 2,4-D treatment, the size of the 2,4-D degrading community was increased and that, following a second treatment, the lag phase was reduced and the rate of mineralization was significantly increased. More recently, the similar observations were done for atrazine degrading community whose size increased in the maize rhizosphere following atrazine treatment (Piutti et al., 2002). This finding tends to demonstrate that in the soil environment, the pesticide degrading function is resilient being relatively lowered in the absence of the selection pressure and recovered in the presence of the selection pressure exerted by the pesticide application. The processes responsible for the resilience of this function of interest for the environment remain yet poorly understood. Interestingly, IPU degrading community seemed to react to sulfonylurea herbicide (Mesosulfuron methyl) application in the field. The sulfonylurea applied to the field of Breteniere showed homology with phenylurea herbicides (Fig. VII-1), and this observation tends to indicate that the soils adapted to phenylurea herbicide could also degrade sulfonylurea. This observation offers a new perspective of research which will aim at characterizing the ability of IPU degraders to degrade sulfonylurea at least in co-culture with soil microbial communities. The IPU mineralization activity was varying importantly at the field scale and it was found to be spatially structured. Interestingly, the range of variation of the IPU mineralization activity estimated by several parameters deduced from modeling IPU mineralization kinetics (A, μm, λ, ti) was considerably decreased when the field was treated with this herbicide. This further reinforced the hypothesis which was previously discussed and which suggests that repeated treatment increases the selection pressure leading to the increase of the IPU degrading genetic potential. This will, in consequence, diminish the variability of the IPU mineralizing activity due to the intrinsic parameters (physico-chemical properties of

soil). Therefore, this observation is of interest since it demonstrates that the filter (or purifying) capability of soil could be manipulated by extrinsic factors such as agricultural practices. The increase in the size of the degrading community is a very interesting goal which can be achieved to promote natural attenuation of pesticides. This could be through several ways and amendment with organic matter such as compost or farmyard manure is one of these ways which offers interesting opportunities to achieve this goal. However, these possibilities were not addressed here.

The use of geostatistics allowed to demonstrate that the variability of IPU mineralization activity was structurally spatialized. We showed that the spatial patterns observed for IPU mineralization were also shared with several soil physico-chemical parameters including C and N content. Based on geostatistical analysis and linear regreassion tested by the Pearson's coefficient of variation (r), variability in mineralization activity was found to be correlated with some of the physico-chemical properties of the soil including organic matter content, organic carbon content, C/N ratio and cation exchange capacity (CEC). It is noteworthy that the maps obtained with number of culturable heterotrophic bacteria as well as the global structure of the soil micro flora did not share any similarities with the map of IPU mineralization. This could be explained by the fact that the IPU degraders are forming a small community not detected by using these tools. It could be of interest to develop molecular marker of IPU degraders in order to be able to follow functional community at field level. These observations are in agreement with the previous studies reporting the variations of IPU mineralization in relation to the soil microbiological and physico-chemical properties (Walker et al., 2001; Bending et al., 2003; El-Sebai et al., 2007; Vieublé-Gonod et al., 2009). Spatial variability has also been observed while studying the degradation of different other pesticides including carbofuron, chlorotoluron, azoxystrobin, chlorsulfuron and diflufenican (Walker and Brown, 1983; Parkin and Shelton, 1992; Walker et al., 2001; Bending et al., 2006). Based on the results of the field experiment, we concluded (i) that repeated application of IPU in soil led to the adaptation of soil microflora to the mineralization of IPU, and (ii) that the physico-chemical properties and pesticide treatment history of the soil should be considered to better understand the fate of pesticides in the soil including their biodegradation.

In order to further understand the processes responsible for IPU dissipation in agricultural soils, this PhD work was further developed with the aim to isolate and characterize IPU degrading population from the soils adapted to IPU mineralization. To do so,

enrichment cultures were set up (Sorensen et al., 2001; El-Sebai et al., 2004; Sun et al., 2009). Enrichment cultures were performed from the soil samples showing a high ability to mineralize isoproturon in agricultural field of le Souiche (El-Sebai et al., 2005) and of Epoisses (described above). Through enrichment cultures, an IPU mineralizing mixed bacterial culture was isolated from the Le Souich soil and a pure bacterial strain, designated *Sphingomonas* sp. SH, was isolated from the field of Epoisses. Interestingly, we found that the bacterial culture was composed of several populations among which the strains belonging to genus *Sphingomonas* were dominating. Unfortunately, the dominant members could not be isolated although several trials were done using different cultural media. In addition, it is noteworthy that the bacterial strain isolated from the soil of Epoisses was also belonging to the *sphingomonadaceae* family. These observations are in accordance with the previous work showing that almost all the bacterial strains already isolated for their ability to degrade IPU belong to this family. Only *Methylopila* sp. TES isolated by El-Sebai et al. (2004) does not belong to this family but it belongs to the same class (i.e. alphaproteobacteria). This suggests that the ability of IPU degradation might be specifically related to this family. However, this observation is in contradiction with what was observed for s-triazine degrading populations. For example, atrazine degrading potential (*atz* and *trz* genes) has been shown to be widespread in both gram negative and gram positive bacterial strains (Devers et al., 2008). Similar observations have also been reported for the 2,4-D degrading potential which has been observed in several genera including *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, *Pseudomonas* and *Sphingomonas* (Vieuble-Gonod et al., 2006). It is most likely that this observation could be due to the relatively low number of isolates known to degrade IPU and other related phenylurea herbicides. As an example, *A. globiformis* isolated for diuron degradation is also able to degrade IPU (Turnbull et al., 2001). In addition, the strains isolated for IPU and diuron degradation were also shown to degrade linuron (Sorensen et al., 2001; Sorensen et al. 2008; Sun et al., 2009). In addition, a bacterial consortium made of *Variovorax* sp. strain WDL1, *Delftia acidovorans* WDL34, and *Pseudomonas* sp. strain WDL5 was shown to degrade linuron. Therefore, it is possible that the degradation of phenylurea herbicides including IPU is not restricted to family *Sphingomonadaceae*. Further work is required to confirm these observations and to understand the genetics behind, which may furnish explanation of this observation.

Although the enriched bacterial culture and *Sphingomonas* strain SH were isolated from two different agricultural soils, most of their degrading capabilities were found to be

similar with each other. Both the enriched bacterial culture and the strain SH were found to have a high ability only to mineralize isoproturon and its known metabolites but they did not have the ability to degrade any of related phenylurea herbicides included in this study. Based on this finding, it was hypothesized that the enzymes and genes involved in IPU metabolism by the enriched bacterial culture and the strain SH might be highly specific only for isoproturon and none of other phenylurea herbicides which is in accordance with a previous study reporting the same type of catabolic abilities in *Methylopila* sp. TES (El-Sebai et al., 2004). This observation seems to indicate the specificity of the French isolates and French soils for the degradation of a particular herbicide like IPU. Recently, we have also observed that a soil adapted to diuron mineralization in a vineyard was not able to mineralize IPU. It indicates that IPU and diuron metabolic pathways might be different although they are structurally close to each other. This observation in case of phenylurea herbicides also differs from other that of other pesticides. For example, atrazine degraders have also been found to be degrading other related s-triazine herbicides (Udikovic-Kolic et al., 2008). The question which arises here is to know if this observation is related to a genetic or biochemical limitations or it is due to methodological biases related to the isolation procedure. Indeed, it is well known that only 0.1 to 1% of the soil micro organisms are cultivable. Therefore, the possibility of preferential selection of a part of the IPU degraders more specifically related to this metabolism cannot be excluded. Based on their ability to degrade the known IPU metabolites i.e. MDIPU, DDIPU and 4-IA, and identification of these metabolites during the degradation kinetics of IPU by the enriched bacterial culture, it was hypothesized that IPU degradation might have initiated by two consecutive demethylations followed by the cleavage of the urea side chain and ultimately mineralization of the phenyl ring which is in accordance with the metabolic pathway already hypothesized for *Sphingomonas sp.* SRS2 (Sorensen et al., 2001). Both the enriched bacterial culture and the strain SH demonstrated the optimum IPU degrading activity at pH 7.5. IPU degrading activity was also shown to be considerably decreasing at pH values lower and higher than optimal pH. pH effect on IPU mineralization has already been shown both in soil microbial communities and in pure strains (Bending et al., 2003; El-Sebai et al., 2007). However, although this pH effect was often underlined, the processes responsible for this effect remain poorly understood. One possible explanation could rely on the modification of IPU availability to microorganisms at low pH. This was observed for s-triazines which are protonated at low pH and as a consequence become less available. One could also hypothesize that this change of the conformation could also modify the transport of pesticide through the bacterial membrane. As an example, although in most of

the cases pesticide is entering in the cell through simple diffusion, it is know that for 2,4-D, *tfd* is coding for a transporter responsible for the entrance of 2,4-D in degrading bacterial cells. Recently, the possible existence of such transporter for atrazine degrader was reported. Since the pH effect on pesticide degradation seems to be a general feature and is of interest from environmental point of view, further research must be conducted aiming at characterizing the processes involved in this phenomenon.

The proposed metabolic pathway for the bacterial culture and for *Sphingomonas* sp. SH was very similar to that proposed for *Sphingomonas* sp. SRS2 (Sorensen et al., 2001). However, based on the recent finding of Sun et al. (2009) reporting the possible involvement of *catA* in the degradation of the phenyl ring of the aniline by an IPU degrading bacterial strain, we studied the lower pathway of IPU degradation. Interestingly, *catA* gene coding for 1,2-dioxygenase degrading the catechol was amplified both from the enriched bacterial culture and the strain SH. However, there is yet no direct proof for the involvement of *catA* in the degradation of IPU. Analyses are currently ongoing with our colleagues from GEUS (Denmark) in order to check for the presence of catechol during the time course of IPU degradation. This observation will allow us to link the degradation of 4-IA to catechol and thereby identify the lower metabolic pathway of IPU. This observation has already been reported in several xenobiotics having a phenolic ring in their skeleton (Caposio et al., 2002; Murakami et al., 2003; Vesely et al., 2007). In order to get further insight in the IPU degradation pathway and to identify the genes involved in isoproturon mineralization, a BAC cloning library consisting of 3000 clones was established from the genomic DNA of the enriched bacterial culture. Functional screening of the clone library was carried out by testing the ability of the clones to degrade either IPU or its known metabolites i.e. MDIPU, DDIPU and 4-IA. By this approach, we were not able to identify any clone showing an ability to degrade IPU or its known metabolites. We carried out a genomic screening of the BAC library for *catA* sequences with idea of identifying the BAC containing not only the genomic sequence harboring the lower pathway but also, in the surrounding region, the genes possibly coding for upper pathway. This assumption was made since it was already shown that *cat* genes could be recruited in catabolic operon (Harwood and Parales, 1996). Genomic screening based on amplification of *catA* gene, led to identification of a *cat* gene cluster coding for the degradation of catechol through *ortho*-cleavage of the phenyl ring following the β-ketoadipate pathway. Catechol which is considered as a key intermediate during the degradation of aromatic compounds as well as its first metabolite, cis,cis-muconic acid, after

phenyl-ring cleavage have been detected during the degradation of aniline by an isoproturon mineralizing bacterial strain YBL2 (Sun et al., 2009). So, based on this finding, it was hypothesized that phenyl ring cleavage in isoproturon might also take place through *ortho*-cleavage of catechol putatively formed during isoproturon mineralization following the formation of 4-IA.

Functional significance, conclusions and perspectives of this study

We know that pesticide contamination constitutes major threats not only to the public health but also to the soil environment. Although incineration and other chemical methods are frequently used for contaminated soils, bioremediation method based on biodegradation of pesticides, using a diverse array of microbial communities in the soil, is gaining support because of being more efficient, less costly and environment friendly. In order to facilitate the process of biodegradation and bioremediation to take place actively in the soil environment, there is need to well understand the fate of pesticides after their application and the metabolic potential of the soil microbial community in relation to the physico-chemical and biological properties of the soil environment. As the conditions at each contaminated site vary, so all physico-chemical factors must be taken into account while studying the biodegradation of pesticides. There is also need to better understand the degrading capability at molecular level by characterizing the catabolic gene of the microorganisms to expand the use and successful application of biodegradation in bioremediating the contaminated soils.

In this context, the present study represents an example for the characterization of xenobiotic degradation from the field conditions to the pure cultures with the involvement of putative catabolic genes taking isoproturon and its metabolites as a target pesticide. Isoproturon was found to be representing an example demonstrating a high capacity of the adaptation of the soil microflora to the degradation of xenobiotic compounds. The first question which was generated by the results obtained in this study was the role of the soil ecosystem on the extent and intensity of biodegradation as a result of microbial adaptation. To tackle this question, the tools of geostatistical analysis were considered over the field exhibiting the mineralization activity. One of the key advantages of geostatistics over other spatial interpolation procedures is its ability to incorporate secondary information, which can be available at all locations where a prediction is sought (i.e. simple kriging with a local mean or external drift). Geostatistics also allows to create maps of the probability for a given variable over the field to know that a predefined threshold has been exceeded or not. Based on

our results, we conclude that variability in mineralization activity is correlated not only with the physico-chemical properties of the soil but also mainly with the pesticide application which exerts a selection pressure during the adaptation of microorganisms. We showed that repeated application of a pesticide would increase the potential of its mineralization in the soil by increasing the fraction of the soil microflora adapted to its degradation. However, there is need to understand the mechanism of enhanced pesticide biodegradation in response to their repeated use. There is need to testify whether adaptation is favored by the dispersion of catabolic genes through horizontal gene transfer or through the growth and activation (gene expression) of the opportunistic bacterial community already having the catabolic ability.

Although we demonstrated the role of soil properties, the reliability of the microcosm studies in the laboratory to interpret the field conditions is much debated because the microcosms, which are under constant controlled conditions in the laboratory, differ from the field situation concerning the influence of temperature and moisture dynamics, the influence of root presence and the compositions of the soil microbial communities. However, these uncertainties can be avoided by studying the pesticide biodegradation under semi-field or field conditions. Furthermore, we demonstrated the importance of pH in relation to degradation as already reported for different pesticides. Although a very weak effect of pH on biodegradation was observed in this field study, there are some recent studies reporting the effect of pH on the degradation activity in soil environment (Bending et al., 2003; El-Sebai et al., 2005). Thus, it was necessary to estimate the effect of pH on IPU degradation kinetics of the bacterial culture and the strain SH. pH was found to strongly affect the degradation activity in pure cultures. However, the question which arises here is to know whether the pH plays role on the physiological state of the degrading microorganism or on the physico-chemical state of the pesticide molecule? If the pH is influencing the physiological state of the degrading microorganisms, the putative transcriptional, translational and post-translational regulation of the degrading genes might be hypothesized to be involved in this process, however, it remains to be investigated by identifying the genes involved. Although we were not successful in achieving the objective of identifying the genes coding for the enzymes involved in isoproturon mineralization, the identification of the *cat* gene cluster is considered as a first report for the presence of such cluster in an isoproturon mineralizing bacterial community. We know that most of the times the degradation of the complex xenobiotics molecules takes place in two steps i.e. the upper metabolic pathway leading to some key intermediates and lower metabolic pathway involving the cleavage of the ring structure.

Although the upper metabolic pathway mostly differs depending upon the type of the molecule, the lower metabolic pathway might be similar in a variety of xenobiotic compounds (Borraccino et al., 2001; Caposio et al., 2002; Murakami et al., 2003; Vesely et al., 2007). So the identification of the *cat* gene cluster in this isoproturon mineralizing bacterial culture might be helpful not only in describing the lower metabolic pathway and phenyl ring cleavage of the isoproturon molecule but also in identifying the catabolic genes for the upper metabolic pathway which might be present in the regions flanking the genes of lower metabolic pathway. However, some of the questions arise from the results obtained in this study. Does isoproturon mineralization passes through accumulation of catechol as an intermediate metabolite? Is the activity of the catechol degrading enzymes regulated in relation to isoproturon mineralization kinetics? Why does a functional screening approach not work for identifying the catabolic genes?

In order to address all the above questions, further research will be carried out firstly to identify the metabolites formed during isoproturon mineralization using more sensitive detection methods like GC-MS and UPLC. The significance of formation of catechol will also be tested by enzymatic activity of the dioxygenase enzymes over the mineralization kinetics. For further elaboration, transcriptional regulation of the genes in the *cat* gene cluster will be analyzed on functional basis. Research will also be carried out to develop a suitable cloning and screening technique for the identification of the catabolic genes for the upper metabolic pathway through functional approach. One possible choice might be the construction of a cosmid vector based clone library which has already been used for the identification of *puhA* and *puhB* genes involved in degradation of diuron (Khurana et al., 2009). As the degradation ability is mostly found in the bacterial strains belonging to genus *Sphingomonas*, one of the possible reasons for the failure of functional screening might be the specificity of the genes to be expressed only in this genus. In this context, it will also be interesting to develop some genetic tools dedicated to the *Sphingomonas* sp. to study the expression of the catabolic genes.

In short, we can say that the results obtained in this study give an insight to understand the mechanisms for the adaptation and accelerated microbial biodegradation in order to use them to develop the advanced strategies for the prevention of xenobiotic pollution which presents a major health problem even in this century. However, further research will be needed to explore deeply and to apply these mechanisms.

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Appendices

Appendix-1

A. Mineral Salt (MS) Medium

Elements	Concentration	Volume for one Litre
K_2HPO_4	100 g/l	16 ml
KH_2PO_4	100 g/l	4 ml
$MgSO_4.7H_2O$	20 g/l	10 ml
NaCl	10 g/l	10 ml
$CaCl_2$	20 g/l	1 ml
Oligoelements	-------	1 ml
Vitamins	-------	1 ml
$FeSO_4.6H_2O$	5 g/l	1 ml

Vitamins and $FeSO_4.6H_2O$ are added after autoclaving. For solid medium, 15 grams of agar is added before autoclaving. Whenever needed, MS medium wsa supplemented with defined mixtures of amino acids (below)

Preparation of Oligoelement Solution (For one Litre)

Boric acid	2.0 g
$MnSO_4.H_2O$	1.8 g
$ZnSO_4$	0.2 g
$CuSO_4$	0.8 g
NaMo	0.25 g
$Co(NO_3)_2$	Traces

Preparation of Vitamins Solution (For one Litre)

Thiamine-HCl	100 mg
Biotine	40 mg

Amino Acid Mixtures

Amino Acid Mix-1 (For one Litre)

L-Glutamic acid	5g
L-Proline	5g
L-Lysine	5g
L-Valine	5g
L-Leucine	5g

Amino Acid Mix-2 (For one Litre)

L-Phenylalanine	5g
L-Asparagine	5g
L-alanine	5g
L-Threonine	5g

Amino Acid Mix-3 (For one Litre)

L-Arginine	5g
L-Histidine	5g
Glycine	5g
L-Methionine	5g

One millilitre (1ml) of any of these stock solutions of amino acids can added to one litre of the medium to have a final concentration of 5mg L^{-1}

Herbicides and their metabolites

Herbicides as well as their metabolites were dissolved in methanol and then added into the medium. For liquid media, 50 mg of the herbicide or its metabolite was added per one litre of MS medium or Knapp buffer. Solid media plates were prepared by adding 500 mg of IPU in one litre of the MS agarose medium.

B. Knapp buffer (For one litre)

K_2HPO_4	1 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	40 mg

$FeCl_3$	4 mg

C. Nutrient agar (NA) Medium (For one litre)

Beef extract	3 g
Peptone	5 g
Agar	15 g

D. TY Medium (For one litre)

Trypton	5 g
Yeast extract	3 g
Agar	15 g

After autoclaving the medium for 20 minutes at 120°C, 10 mL of 60 mM solution of $CaCl_2$ was added.

E. Luria Bretani (LB) Medium (For one litre)

Bacto-trypton	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g

Whenever needed, LB medium was added with 1 mL of Ampicilline (100 mg mL^{-1}), 1 mL of IPTG (100 mg mL^{-1}), 2 mL of X-Gal (40 mg mL^{-1}) and 12.5 mg of chloremphenicol.

Appendix-2

A. Extraction buffer

Tris (pH 8.0)	100mM
EDTA	100mM
NaCl	100 mM
Polyvinylpyrrolidone	1% (w/v)
Sodium dodecyl sulphate	2% (w/v)

B. Preparation of Polyvinyl polypyrrolidone (PVPP) column

- Place the column in 2 mL tube
- Fill about 1.2 cm of the column with PVPP
- Add 400 µL of water and centrifuge for 2 min at 1000xg at 10°C.
- Add 400 µL of water and centrifuge for 2 min at 1000xg at 10°C.
- Add 400 µL of water and preserve the column at 4°C.

C. Preparation of Sepharose 4B column

- Place the column in 2 mL tube
- Add 1 mL of sepharose 4B into the column and centrifuge for 2 min at 1100xg.
- Add 500µL of TE (pH 8.0) and place the column at 4°C in a clean 1.5 mL tube.

Appendix-3

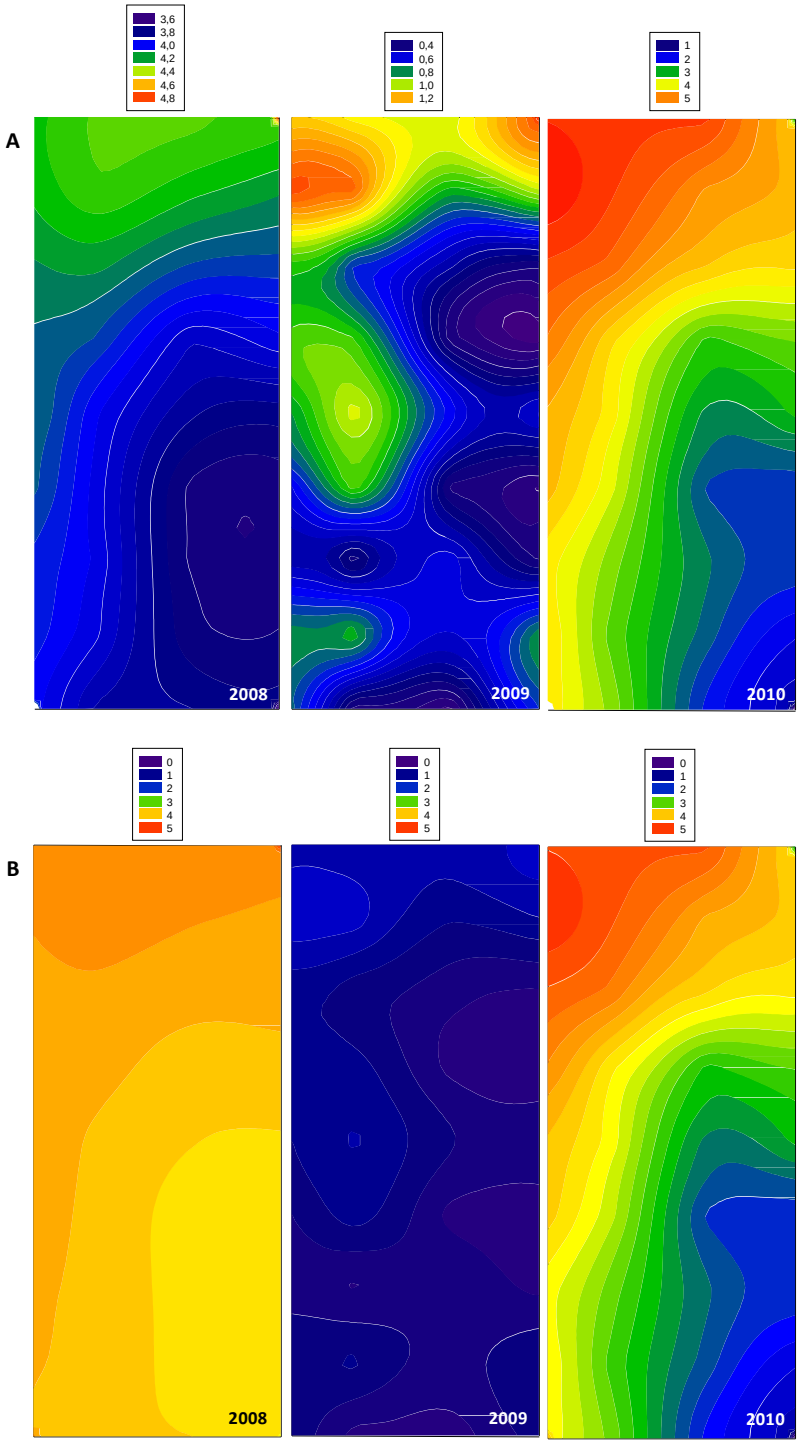

Fig. Kriged map of maximum mineralization rate (µm) of the field sampled at Epoisses in 2008, 2009 and 2010. A and B represent the same maps with the unequal and equalized axis respectively.

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ABSTRACT

Frequent use of phenylurea herbicide isoproturon (IPU) in agricultural fields has resulted not only in the contamination of the natural resources including soil and water but also in the adaptation of the soil microflora to its rapid degradation. However, up to now, the mechanisms underlying this microbial adaptation are not well elucidated. The aim of this study was to explore the processes and factors implicated in IPU degradation from the agricultural field to the genes coding for catabolic genes.

The study carried out at the experimental field of Epoisses cropped with a winter wheat / barley / rape seed crop rotation indicated that as a result of its periodically repeated use, the soil microflora adapted to IPU mineralization activity. Further analysis using exploratory and geostatistical tools demonstrated the existence of spatial variability in IPU mineralization activity at the field scale which was correlated not only with several soil physico-chemical parameters like organic matter content, CEC and C/N ratio but also with the pesticide application plan over a three year crop rotation.

In order to get further insight into underlying mechanisms, an IPU mineralizing bacterial culture and strain *Sphingomonas* sp. SH were isolated through enrichment cultures performed from two different adapted soils. Both had the catabolic activities highly specific for the mineralization of IPU and its metabolites but none of other structurally related phenylurea herbicides. IPU metabolic activity of both the mixed culture and the strain SH was found to be affected by pH with optimal activity taking place at pH 7.5. Based on the accumulation of different known metabolites during mineralization kinetics, IPU metabolic pathway was proposed to be initiated by two successive demethylations, followed by cleavage of the urea side chain resulting in the accumulation of 4-isopropylaniline, and ultimately the mineralization of the phenyl ring.

In order to identify the genes involved in IPU degradation, BAC clone library was established from the genomic DNA of the bacterial culture. Although, the functional screening did not yield in identifying any BAC clone able to degrade IPU or its known metabolites, the PCR based screening led us to identify a *cat* gene cluster involved in *ortho*-cleavage of the phenyl ring of catechol through β-ketoadipate pathway. Based on this finding, it was hypothesized that phenyl ring of 4-isopropylaniline formed during IPU transformation might be mineralized through *ortho*-cleavage of catechol. This finding allowed us to propose the lower IPU metabolic pathway which was not yet described.

Keywords: Soil microbiology, herbicides, isoproturon, spatial variability, biodegradation, metabolic pathway, 1,2-dioxygenase, BAC cloning

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Résumé

L'usage répété d'isoproturon (IPU) en agriculture pour contrôler développement de plantes adventices dans les cultures céréalières a non seulement abouti à la contamination du sol et des ressources en eaux mais également à l'adaptation de la microflore du sol à la dégradation accélérée de cet herbicide appartenant à la famille des phénylurées. A l'heure actuelle, les mécanismes microbiens impliqués dans cette adaptation ne sont pas encore parfaitement élucidés. Dans ce contexte, l'objectif de cette étude était d'explorer les processus et les facteurs impliqués dans la biodégradation de l'isoproturon, et ce, depuis l'échelle agricole de la parcelle jusqu'à celle des gènes codant cette fonction dans des populations microbienne dégradantes.

L'étude réalisée à partir d'une parcelle expérimentale du domaine d'Epoisses, cultivée selon une rotation blé d'hiver/ orge / colza, a montré que, suite à l'usage répété d'IPU, la microflore du sol s'était adaptée à sa minéralisation. Des analyses réalisées à l'aide d'outils statistiques et géostatistiques ont révélé l'existence d'une variabilité spatiale de la minéralisation de l'IPU au sein de la parcelle agricole. Celle-ci s'est révélée être non seulement corrélée avec différents caractéristiques physicochimiques du sol (C/N, CEC, …) mais également avec le plan d'épandage des pesticides au cours de la rotation culturale.

Afin de mieux étudier les mécanismes moléculaires responsables de la minéralisation de l'IPU, une culture bactérienne ainsi qu'une souche (*Sphingomonas* sp. SH) minéralisant l'IPU ont été isolées par enrichissement à partir de deux sols différents, tous deux adaptés à la biodégradation accélérée de l'IPU. La culture bactérienne et la souche pure ont toutes deux montré un métabolisme spécifique pour la dégradation de l'IPU, étant capables de dégrader l'IPU et ses principaux métabolites mais aucun des autres herbicides de la famille des phénylurées. La culture bactérienne et la souche présentaient une activité dégradante optimale à pH7,5 et étaient affectées par des pH inférieurs et supérieurs à cette valeur optimale. Sur la base des métabolites accumulés lors de la dégradation de l'IPU, nous avons proposé que l'IPU serait dégradé par deux déméthylations successives, suivi par la coupure de la chaine urée aboutissant à l'accumulation de 4-isopropylaniline, et finalement la minéralisation du cycle phényl.

Afin d'identifier les gènes impliqués dans la minéralisation de l'IPU, une banque de clones BAC a été réalisée à partir de l'ADN génomique purifié de la culture bactérienne. Bien que le crible fonctionnel réalisé n'a pas permis d'identifier de BAC capable de dégrader l'IPU ou l'un de ses métabolites, un criblage moléculaire par PCR ciblant la séquence *catA* codant la catéchol 1,2-dioxygénase, nous a permis d'identifier trois BACs. Le pyroséquençage des ces 3 BACs et l'agrégation des séquences correspondantes ont permis d'identifier un fragment génomique de 33 kb présentant notamment l'opéron *cat* impliqué dans le clivage ortho du cycle phényl du catéchol. De ce fait nous avons émis l'hypothèse selon laquelle la 4-isopropylaniline formée lors de la dégradation de l'IPU pourrait être minéralisée par le clivage ortho du catéchol, un intermédiaire clef de la voie des β-kétoadipates. Ceci nous a donc permis de proposer une voie métabolique pour la voie basse de la dégradation de l'IPU qui, jusqu'alors, n'avait pas encore été décrite.

Mots-clefs: Microbiologie du sol, herbicide, isoproturon, variabilité spatiale, biodégradation, voie métabolique, 1,2-dioxygénase, clonage BAC