TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Bodenökologie

Enhanced degradation of isoproturon in soils: sustainability of inoculated, microbial herbicide degraders, and adaptation of native microbes

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. Siegfried Scherer
Prüfer der Dissertation:	1. Univ.-Prof. Dr. Jean Charles Munch
	2. Univ.-Prof. Jens Aamand, Ph.D. (Universität Kopenhagen, Dänemark)

Die Dissertation wurde am 03.04.2014 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 08.07.2014 angenommen

Acknowledgements

The present thesis was done at the Institute of Soil Ecology, Helmholtz Center Munich. Furthermore, some liquid culture studies and isolation tests within the thesis were conducted at the Department of Geochemistry, Geological Survey of Denmark and Greenland (GEUS) in Copenhagen, Denmark. A lot of people have been supporting me and my research in a variety of ways. I would like to thank all of them for their contributions:

I am grateful to Prof. Dr. Jean Charles Munch for giving me the opportunity to work on this topic, for the kind support of the thesis and the helpful review of the manuscript.

I thank Prof. Dr. J. Aamand for agreeing to be a co-examiner of my thesis and Prof. Dr. S. Scherer for agreeing to be chairman of the examination committee.

Special thanks go to Dr. Reiner Schroll, head of the working group, for the supervision of my thesis. Many thanks for your continuous interest in scientific discussions and new approaches and your inspiring enthusiasm on science. Many thanks also for the review of the thesis.

Dr. Ulrike Dörfler supported me in a lot of ways, especially with residue analysis and when the HPLC system was once again not working. Thanks also for the very useful comments on the manuscript.

Nghia Nguyen, who was a PhD student at the working group as well. Nghia, I enjoyed very much having you as a dear friend and colleague sharing with me a lot of happy but also difficult times during our PhDs. Thanks for all. You also acquainted me with vietnamesian food – hope you get your first Michelin star soon;-)

Fredrick Kengara – thanks for all your advice on lab and PhD life, and especially your calmness that outshined everything.

A special thank goes to Patrick Weiss for his dedicated support of some experiments. I always appreciated your indestructible good temper and enjoyed very much our coffee and cookie breaks.

Many thanks go to Jana Arthofer for help and advice in the lab and I thank all the “Zivis” and especially the “Hiwis” Karlo Behler, Christoph Maier and Christian Aigner who helped me with sampling and soil analysis. Without you it would not have been possible to cope with this vast amount of samples.

I especially thank Dr. Bernhard Ruth – I enjoyed our daily lunch times and discussions about our shared love for hiking and all kinds of other topics a lot! Is the boring bit that I borrowed from you still sticking on your cupboard? ;-)

I thank Dr. Michael Schmid for support on questions in microbiology and molecular biology and especially for help on the sequencing results. Many thanks also go to Barbara Pfitzner for her support and help on cloning and sequencing.

Many thanks go to Dr. Roland Fuß for valuable advice on study design and lab work.

Sabine Sichert: It was a great pleasure to share lab, office and leisure time with you. Your laughter + my laughter were an excellent hint to any other group member that was looking for one of us, I guess.

Tina Sieper, Doreen Fischer, Linda Kinzel and Christian Klein – I enjoyed a lot the time that I have spent with you, filled with swimming, beer garden, cooking, magic, concerts and much more. One could not wish for better friends and colleagues.

Thanks to all colleagues at the Institute for providing a pleasant working environment.

At GEUS

Special thank go to Prof. Jens Aamand and Dr. Sebastian Reinhold Sørensen, for supervision of my experiments at GEUS and many valuable and inspiring discussions on study design and practical lab work.

Spire Kiersgaard and Nora Badawi – thank you for the support especially with the UPLC system. Thanks go to to Pia Bach Jakobsen for help in the lab and especially on molecular biology.

Berith Knudsen, Malene Balleby Jensen, Meric Batioglu-Pazarbasi – it was a great pleasure having you as colleagues, thanks for discovering Copenhagen, making delicious flødeboller and many other things that made my time in Copenhagen so special for me.

Thanks to the whole team of the Department of Geochemistry at GEUS for a very good time in Copenhagen.

Meine Schwester und meine Eltern haben mich auf meinem Weg immer unterstützt, sich mit mir gefreut und mich durch Tiefen begleitet, nicht nur während meiner Doktorarbeit. Es ist schön, so eine Familie zu haben.

Nikolai, ohne Deine Unterstützung und Deinen Zuspruch würde es diese Doktorarbeit vielleicht nicht geben. Danke dafür. Und für die vielen anderen Dinge mit denen Du mein Leben bereicherst.

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1,2,4-TCB	1,2,4-Trichlorobenzene
2,4-D	2,4-dichlorophenoxyacetic acid
2-OH-MD-IPU	1-[4-(2-hydroxypropan-2-yl)phenyl]-3-methylurea
4IA	4-isopropylaniline
ASE	Accelerated Solvent Extraction
C	Carbon
CI	confidence interval
CFU(s)	Colony Forming Units(s)
Cum. $^{14}CO_2$	sum of evolved $^{14}CO_2$ (= mineralization)
DD-IPU	1-[4-(propan-2-yl)phenyl]urea (didesmethyl-isoprouron)
DNA	Deoxyribonucleic acid
dpm	radioactive decays per minute
GEMs	Genetically engineered microorganisms
H	Hydrogen
HGT	Horizontal Gene Transfer
HPLC	High-performance liquid chromatography
IMMC	IPU mineralizing microbial community
IMBS	IPU mineralizing bacterial strain
IPU	Isoproturon
LB plates	Luria Bertani plates
LOD	Level of detection
LOQ	Level of quantification
MD-IPU	1-methyl-3-[4-(propan-2-yl)phenyl]urea (monodesmethyl-isoproturon)
MEA	Malt Extract Agar
MeOH	Methanol
MER	Methanol-Extractable Residues
min	minute
MPN	Most Probable Number
MS-medium	Mineral salt medium
MS-IPU plates	Plates based on MS-medium
NaN_3	sodium azide
NB plates	Nutrient Broth plates
NER	Non-Extractable Residues
N	Nitrogen
O	Oxygen
PCP	Pentachlorophenol
PCR	Polymerase Chain Reaction
PW	Pore water
rDNA	ribosomal Deoxyribonucleic acid
rpm	rounds per minute
rRNA	ribosomal Ribonucleic acid
sec	second
SOM	Soil Organic Matter
SPE	Solid Phase Extraction
TOC	Total Organic Carbon
UPLC	Ultra Performance Liquid Chromatography

1 Introduction

1.1 Use of plant protection products

In the course of world population growth and changing food patterns (e.g. a higher consumption of meat), the global demand for food is rising rapidly. Extrapolations of the global demand for crop production predict an increase between 50 % (The World Bank, 2008; Davies et al., 2009) and 110 % (Tilman et al., 2011) from 2005 to 2050. In parallel, global climate change will aggravate the effect of stresses like heat and drought on crops (Parry et al., 2007). To guarantee sufficiently high and reliable harvests, appropriate measures have to be taken like efficient pest control. Yet, no efficient and large-scale alternative to chemical pest control is available in agriculture up to now (Soulas and Lagacherie, 2001; Hallmann et al., 2009).

Pesticides are applied in high amounts in agriculture worldwide, aiming to protect crops against diseases and pests (Hallmann et al., 2009). According to the target organism, pesticides are classified in groups, whereupon the most important groups worldwide are herbicides (against weeds), fungicides (against fungi) and insecticides (against insects). Within the European Union, about 220.000 tons of pesticides were deployed in 2003. Fungicides accounted for the highest consumption with 49 % of total pesticides applied, followed by herbicides with 38 %. However, data on usage in time series revealed the decreasing importance of fungicides while herbicides become increasingly important. In Germany, herbicides represent the most important pesticide group, amounting to 54 % of total pesticide consumption. This is attributed to the widespread cultivation of cereals, which are by far the most pesticide-consuming crops, and the outstanding importance of herbicides in cereal production (European Commission, 2007).

Within the group of herbicides, Isoproturon (IPU) is one of the most extensively used active substances applied in conventional agriculture in Europe and accounts for 14.3 % of total herbicides used there (European Commission, 2007). IPU as a representative of phenylurea herbicides is used for pre- and post- emergence control of annual grasses and broad-leaved weeds in the cultivation of cereals. IPU is taken up into plants by both plant roots and leaves and inhibits the transport of electrons within the photosystem II (Hock et al., 1995). In this study, the pesticide IPU was selected for several reasons: (1) it is degraded mainly by microbial processes (Cox et al., 1996; Johannesen et al., 2003; Sørensen and Aamand, 2003; Hussain et al., 2009) and (2) it is of high concern for humans and the environment. IPU was frequently detected in surface and ground waters in concentrations exceeding the limit level of 0.1 µg L^{-1} for pesticides set by the European Commission (Nitschke and Schüssler, 1998; Sørensen and Aamand, 2001; Johannesen et al., 2003; El-Sebai et al., 2004; Hussain et al., 2009). Ecotoxicological data indicate harmful effects of IPU

and some of its metabolites on aquatic invertebrates (Mansour et al., 1999), freshwater algae (Vallotton et al., 2009) and microbial communities (Widenfalk et al., 2008). Further, IPU is suspected to be carcinogenic to humans and animals (Behera and Bhunya, 1990).

1.2 Fate of pesticides in the soil ecosystem

To impair the herbal metabolism, herbicides need to be taken up into the plant and be transported to the respective location of action. During pre-emergence control of weeds, herbicides are applied directly on the soil surface and enter the plants mainly via the roots. In post-emerge control, herbicides enter the plants mainly via the leaves (Hock et al., 1995; Sandmann and Böger, 2012). Nevertheless, the major part of applied herbicides reaches the soil directly or indirectly e.g. when plant surfaces are washed off by rainfall (White, 2006). Thus, the majority of herbicides applied ends up sooner or later within the soil matrix. This circumstance turns soil into a major sink for those compounds within the environment and proves the importance of soil as the main compartment for the removal of herbicides (Führ et al., 1998; Semple et al., 2003).

Typically, a vanishing low amount of 0.1 % of applied pesticides arrives at the target pest while the residual 99.9 % distribute in the environment (Pimentel and Levitan, 1986; Baxter and Cummings, 2008). Herbicides, as any organic contaminants, that enter the soil environment are subject to a variety of different dislocation and transformation processes. The former comprise processes like volatilization, leaching and sorption to soil particles. The latter processes comprise abiotic and biotic actions like photolysis, hydrolysis and microbial degradation (Pal et al., 2006; White, 2006). Microbial degradation usually constitutes the main transformation mechanism of organic pollutants in soil (Blume, 1990; White, 1997; Leung et al., 2007). As soil is a complex habitat, these processes can take place simultaneously (Müller et al., 2007). In the following, the most important mechanisms which determine the fate of pesticides in soil are described.

1.2.1 Microbial degradation of pollutants in soil

1.2.1.1 Microbial degradation processes

Microbial pesticide degradation is often carried out in sub-steps by different microbial species (Hock et al., 1995; Fomsgaard, 1997), typically involving bacteria, fungi, actinomycetes and algae (Blume, 1990; Scheunert, 1994; Hock et al., 1995; Müller et al., 2007). The activity of soil microbes depends mainly on the prevailing soil conditions such as soil temperature, water content, pH and the content of soil organic matter (SOM). However, the ability of microbes to finally degrade a contaminant is affected to a high degree also by the chemical itself: characteristics of the chemical like water solubility and sorption behavior play a decisive role, but also the pesticides´

concentration in soil, as toxic acting concentrations may have inhibiting effects on soil microbes (Sims et al., 1990; Gavrilescu, 2005; Müller et al., 2007).

Mineralization is defined as the complete breakdown of organic chemicals and thus constitutes the only route to completely remove a foreign substance from soil. As the resulting products CO_2, H_2O and inorganic molecules do not pose any risk to human health, mineralization is the most desirable method to remove organic chemicals from soil (Scheunert, 1994; Becker, 2010).

In general, the biodegradation of organic pollutants in soil can occur via cometabolic or metabolic degradation processes. During **metabolic degradation**, soil microbes are able to use the organic pollutant as a source of energy and carbon, which enables the synthesis of new microbial biomass (Blume, 1990; Scheunert, 1994; Fomsgaard, 1997). The size of the pollutant degrading population affects the metabolic degradation rate, i.e. the higher the population size, the higher the degradation rate of the pollutant (Becker, 2010). Metabolic degradation processes usually have higher degradation rates than those measured during cometabolic degradation (Blume, 1990; Scheunert, 1994). To distinguish a metabolic from a cometabolic degradation pattern, studies usually rely on the cumulative mineralization curves: As a response to the synthesis of new degrading cells, metabolic degradation processes typically exhibit sigmoid-shaped cumulative mineralization patterns. This pattern is characterized by consecutive lag, exponential and plateau phases (Levy et al., 2007; Folberth, 2008). During the lag phase, the degradation of the chemical is typically low. This period is often explained as the time needed for present degraders either (1) to induce and synthesize specific degradation enzymes not present or only present at low levels within the population, or (2) to proliferate to a critical threshold which enables effective pesticide degradation (Kearney and Kellogg, 1985; Aamand et al., 1989; Rensing et al., 2002). Often, the pesticide degrading microbes constitute only a small fraction of the biomass present in soil (Scheunert, 1994). During the exponential mineralization phase, the degrader population grows exponentially as it depletes the growth substrate, i.e. the pollutant. The plateau phase exhibits low degradation rates and is reached when the pollutants is decreasingly available due to its consumption (Racke, 1990). Studies with pure culture studies enable a reliable identification of the underlying degradation mechanism by setting up liquid cultures with the pollutant as the only source of energy and carbon (C). Often, the proliferation of degrading cells is examined as a second hint for the presence of a metabolic degradation mechanism. However, the degradation mechanism in soil might not be that clear detectable as metabolic, cometabolic and abiotic degradation processes typically run simultaneously. Metabolic degradation processes usually lead to the mineralization of the pollutant. Thus, metabolites are accumulating just temporarily before they are further transformed (Becker, 2010; Lebeau, 2011).

Cometabolic transformations are a well-known phenomenon in many soils (Criddle, 1993; Scheunert, 1994; Becker, 2010). During cometabolism, the organic contaminant does not serve as a

source of energy or C, thus the microbes involved cannot build up new biomass from this compound. The enzymes, which are produced by the soil microbes and which are responsible for the pesticides´ degradation, are induced by a primary substrate present in the soil solution and not by the pesticide itself (Harder, 1981; Fuchs, 2007; Leung et al., 2007). Thus, the contaminant is just acting as a co-substrate due to structural similarities between the actual microbial substrate and the contaminant. This leads to an undirected degradation of the chemical by enzymes with low specificity. Cometabolic degradation is usually characterized by more or less constant degradation rates at low level (Sims et al., 1990; Scheunert, 1994; Fuchs, 2007; Leung et al., 2007) as the contaminant and the primary substrate leading to microbial growth are competing for enzymes (Becker, 2010). Degradation typically starts more or less fast, a longer lag phase is not observed. Due to the low specificity of degradation enzymes, an incomplete degradation of the pesticide is frequently detected. The formed metabolites may either be further degraded by other microbial species and the pesticide might be finally mineralized (Harder, 1981; Madigan, 2003; Becker, 2010). Or, the metabolites can accumulate in soil. The chemical nature of the metabolite plays a decisive role for its environmental evaluation. Metabolites can be of lower, similar or higher toxicity than the original compound and might be also more stable (Madigan, 2003; Maier, 2009; Becker, 2010). Nevertheless, mineralization is definitively preferable compared to the formation of metabolites as mineralization of a pollutant is the only route to completely remove it from soil (Madigan, 2003; Becker, 2010).

1.2.1.2 Microbial degradation of IPU

Microbial degradation constitutes the main process eliminating IPU from agricultural soils (Cox et al., 1996; Johannesen et al., 2003; Sørensen and Aamand, 2003; Hussain et al., 2009). Up to now, both bacteria and fungi were shown to participate in the partial or complete microbial degradation of IPU. Primarily bacteria are of major importance while degradation by fungi is often characterized by a cometabolic degradation pattern (Sørensen et al., 2001; Bending et al., 2003; El-Sebai et al., 2004; Rønhede et al., 2005; Arbeli and Fuentes, 2007). In general, the complete degradation of IPU is considered as much more desirable than partial degradation as the latter may result in the accumulation of metabolites that might be more toxic to soil microbes than the parent compound (Remde and Traunspurger, 1994; Mansour et al., 1999; Tixier et al., 2002). The extent of microbial IPU degradation is controlled by various soil and environmental factors. Especially soil pH was shown to play a key role for biodegradation of IPU in isolated cultures and soils (El-Sebai et al., 2005; Hussain et al., 2009; Sun et al., 2009). Previous studies with IPU mineralizing strains suggest that neutral pH conditions provide optimal conditions for IPU degradation while the degradation rate is reduced as the pH shifts towards more acidic or alkaline conditions. For example, the strains *Sphingobium* sp. strains YBL1, YBL2 and YBL3 isolated by Sun et al. (2009) showed an optimum degradation activity at pH 7.0 with a considerably decreased degradation

performance below pH 6.0 or above pH 9.0. Similar results were presented by Hussain et al. (2011) for *Sphingomonas* sp. strain SH: optimum degradation activity was observed at pH 7.5 while degradation below pH 5.5 and above pH 8.5 was clearly reduced.

Previous studies in agricultural soils reported relatively low degradation rates for IPU, with mineralization amounting 10 - 34 % of applied IPU in a period of 67 – 135 days (Lehr et al., 1996b; Pieuchot et al., 1996; Perrin-Ganier et al., 2001). Recently, laboratory measurements showed considerable mineralization of IPU in various agricultural soils previously treated with IPU, with 40 - 53 % (Sørensen et al., 2001) and 35 – 40 % (Bending et al., 2001) of applied ^{14}C-IPU mineralized within 40 and 65 days, respectively. Similar amounts of IPU were mineralized in a lysimeter study by Grundmann et al. (2007) (44,0 % of applied ^{14}C-IPU within 46 days). These findings stimulated research targeting the isolation and characterization of microbial strains that mineralize IPU. So far, several bacterial strains able to mineralize IPU were isolated and characterized: *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), *Sphingobium* strains YBL1, YBL2 and YBL3 (Sun et al., 2009) as well as *Sphingomonas* sp. strain SH (Hussain et al., 2011). Those bacterial strains were isolated from different geographical locations in France, the UK and China. Furthermore, also bacterial consortia may be involved in the degradation of IPU by cooperative metabolic activities (Sørensen et al., 2002).

Sørensen et al. (2003) proposed various **pathways for the microbial degradation of IPU**. The suggested scheme is presented in Figure 1. Compounds that are shown in boxes represent dead-end metabolites which cannot become further degraded. According to the scheme, the metabolism of IPU can be initiated by N-demethylation of the N,N-dimethylurea side chain, resulting in the formation of the metabolite MDIPU (step 1). MDIPU was frequently detected as main metabolite during IPU degradation in agricultural soils (Gaillardon and Sabar, 1994; Cox et al., 1996; Lehr et al., 1996a; Pieuchot et al., 1996). After successive N-demethylations of IPU to MDIPU (step 1) and further to DDIPU (step 2) as well as the cleavage of the urea side chain (step 3), the metabolite 4-isopropylaniline (4IA) is formed. 4IA might be metabolized to CO_2 and biomass. In addition, a different metabolic pathway is reported, involving the direct transformation of IPU to 4IA in a single step by hydrolytic cleavage of the dimethylurea side chain (step 4) (Turnbull, Ousley, et al., 2001). Two alternative degradation pathways for IPU involve initial hydroxylation of the isopropyl side chain, leading to 1-OH-IPU or 2-OH-IPU (steps 6 and 7) (Sørensen et al., 2003).

IPU belongs to the group of phenylurea herbicides which can be classified into two groups according to their molecular structure: (1) the N,N-dimethyl phenylureas like IPU and Diuron as well as (2) the N-methoxy-N-methyl phenylureas like Linuron. The metabolic pathways engaged in

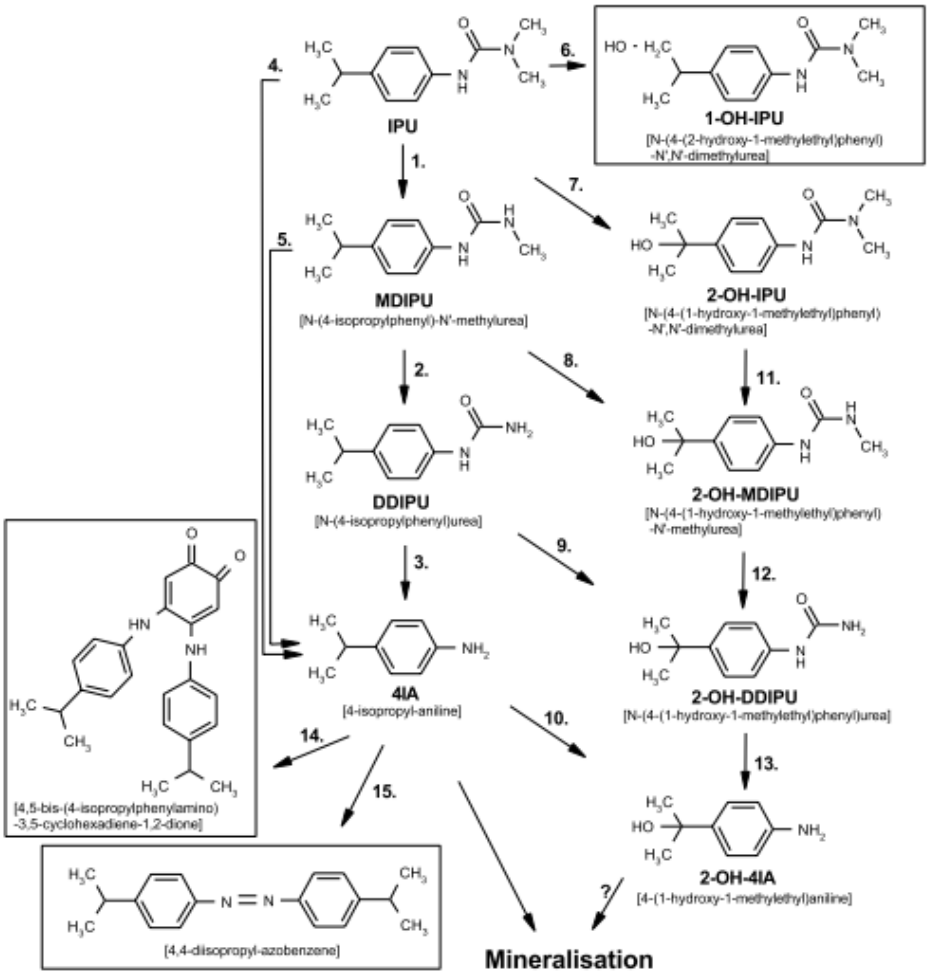


Figure 1: Degradation pathways of IPU in soil as proposed by Sørensen et al. (2003). *Compounds shown in boxes are dead-end metabolites which cannot become further degraded.*

the degradation of different phenylurea herbicides are characterized by great similarities. After successive N-dealkylations, the metabolites are hydrolysed to their aniline-derivates which may be further metabolized to CO_2 and biomass, depending on the present microbes (Sørensen et al., 2003). Several studies with phenylurea-degrading bacteria and consortia suggest specificity of degradation enzymes towards the group of N,N-dimethyl phenylureas (Sørensen et al., 2001; Sun et al., 2009) or N-methoxy-N-methyl phenylureas (Engelhardt et al., 1973; El-Fantroussi, 2000). However, *Arthrobacter globiformis* strain D47 exhibits a much broader substrate specificity, degrading representatives of both groups to their aniline derivatives (Turnbull, Cullington, et al., 2001; Turnbull, Ousley, et al., 2001). In contrast, the bacterial isolates *Sphingomonas* sp. SH and *Methylopila* sp. TES were reported to mineralize exclusively IPU, showing no activity towards other phenylurea herbicides (El-Sebai et al., 2004; Hussain et al., 2011).

Knowledge on the genes and enzymes involved in the degradation of phenylurea herbicides is rare as only a few isolates degrading phenylurea herbicides were characterized. Up to now, no

degradation genes and enzymes that result in effective IPU degradation were identified. All enzymes identified so far exhibited only a low activity for IPU (Turnbull, Cullington, et al., 2001; Turnbull, Ousley, et al., 2001; Khurana et al., 2009; Bers et al., 2011).

1.2.2 Sorption of IPU to the soil matrix

One of the fundamental factors that control the biodegradation of pollutants in soil is their bioavailability which is in turn depending on the pollutants´ sorption behavior (Nemeth-Konda et al., 2002; Kah and Brown, 2007). **Sorption** is characterized by the sub-processes adsorption and desorption. Adsorption is specified as the process of adhesion of a compound to the surface of soil particles while desorption denotes the process of getting dissolved in the soil solution (Hock et al., 1995). When adsorption and desorption rates of the chemical between the adsorbed and aqueous phase are equal, the equilibrium state is reached and the extent of adsorption may be quantified by the adsorption coefficient K_d, which gives the ration between the pesticide concentration that is adsorbed to soil particles and the concentration that is dissolved in soil pore water (Hock et al., 1995). The K_d–value is typically determined by batch experiments which include an excess of water and vigorous shaking (OECD, 2000; Cooke et al., 2004; de Wilde et al., 2008). When a pollutant is degraded, the sorption equilibrium gets disturbed and a portion of the adsorbed pollutant is subsequently delivered into the soil solution in order to reach the equilibrium state again. As soon as a pesticide enters the soil, it tends to adsorb to soil particles by physical and chemical bonds like hydrogen bondings, van der Waals forces and ligand exchanges. Several of these mechanisms can act simultaneously. The type of binding as well as the extent of adsorption to the soil matrix depends on chemical and physical soil characteristics as well as the chemicals´ properties (Hock et al., 1995; Gevao et al., 2000; Nemeth-Konda et al., 2002; Ertli et al., 2004; White, 2006).

IPU is adsorbed to the soil matrix by relatively weak types of bindings like van der Waals forces (Ertli et al., 2004) and is thus adsorbed to a low extent compared to other herbicides (Cox et al., 1996; Johannesen et al., 2003). The principal sorbent for IPU in the soil environment is soil organic matter, which exhibits large surface areas (Worall et al., 1997; Ertli et al., 2004). Sorption measurements showed a similar sorption behavior for IPU and its metabolite MD-IPU (Johannesen et al., 2003). As a result, both compounds were frequently detected in soils respectively ground and surface waters (Cox et al., 1996; Pieuchot et al., 1996; Johannesen et al., 2003; Hussain et al., 2009). In contrast, the metabolite 4IA was detected only in traces in IPU-treated agricultural soils (Mudd et al., 1983; Lehr et al., 1996a; Benoit et al., 1999; Sørensen and Aamand, 2001). This is attributed to the binding behavior of 4IA to soil: 4IA can, in contrast to IPU, MD-IPU and DD-IPU, bind to precursors of humic substances by covalent bonds and form dead-end metabolites such as 4,4-diisopropyl-azobenzene (Scheunert and Reuter, 2000; Sørensen and Aamand, 2003). These

reactions are considered as possible initial steps in the formation of non-extractable residues (NER) with SOM and thus document the importance of 4IA in the formation of NER of IPU (Scheunert and Reuter, 2000). The fast and strong binding of 4IA was demonstrated to considerably reduce the availability of 4IA to degrading soil microbes and thus biodegradation (Bollag et al., 1978; Scheunert and Reuter, 2000; Johannesen et al., 2003).

Up to now, there is no widely accepted definition for the **bioavailability** of a pollutant (Semple et al., 2004, 2007). In this study, the definition by Semple et al. (2003) is applied: "The term "bioavailability" refers to the fraction of a chemical in a soil that can be taken up or transformed by living organisms". The bioavailability of contaminants depends essentially on two factors: (a) the rate at which the pollutants is transferred to the microbial cell (mass transfer) and (b) the rate at which the pollutant is taken up into the cells and is metabolized (the intrinsic activity of the cell) (Bosma, 1997; Semple et al., 2003). As bioavailability is depending on organism and even species, it is not possible to obtain a chemical test that exactly depicts the bioavailability to all receptors (Reid et al., 2000), but it is desirable to achieve a ranking of chemicals´ bioavailability in different soils, e.g. for risk assessment. To estimate the bioavailability of chemicals in soils, classical batch methods with an excess of water and vigorous shaking, like the K_d approach, were used intensively (Beulke et al., 2004; Tyess et al., 2006; de Lipthay et al., 2007). However, the predictive capability of the K_d approach was shown to be limited (Kah and Brown, 2007). This was attributed to the artificial experimental conditions, as e.g. soil aggregates can break down which results in a significant increase of the surface area in soil that interacts with molecules of the pesticide (Wauchope et al., 2002). The limited information value of the K_d approach was confirmed for IPU by Folberth et al. (2009): ^{14}C-IPU residues were extracted from soil with an excess of water while IPU mineralization served as a reference for the actual availability to microbes. The study revealed that there was no correlation between IPU mineralization and its extractability from soil when an excess of water was used. In contrast, a high significant correlation was detected when the soil was centrifuged and IPU extracted with pore water. Thus, the amount of bioavailable IPU in the present thesis was determined in pore water according to Folberth et al. (2009).

1.2.3 Formation of Non-Extractable Residues

The biodegradation of organic contaminants in soil results in general in the formation of metabolites, microbial biomass, mineralization products (CO_2 and H_2O) and NER (Scheunert et al., 1985; Kästner, 2000). Pesticide NER, which are often referred to as bound residues, were originally defined by the International Union of Pure and Applied Chemistry (IUPAC) as "chemical species originating from pesticides, […], that are unextracted by methods which do not significantly change the chemical nature of these residues" (Roberts, 1984; Gevao et al., 2000; Barraclough et al., 2005). Later on, a modified version became widely accepted, extending the area of application

also to pesticides´ metabolites (Gevao et al., 2000). In a further step, chemical fragments transformed to natural compounds were excluded from this definition ("These residues are considered to exclude fragments recycled through metabolic pathways leading to natural products") (Roberts, 1984; Gevao et al., 2000).

It is agreed that NER are formed during the degradation of pesticides as a result of various physico-chemical interactions between the parent compound or its metabolites and SOM as the main binding site (Verstraete and Devliegher, 1996; Alexander, 2000; Gevao et al., 2000; Loiseau and Barriuso, 2002; Mordaunt et al., 2005). In case of NER from IPU, the formation of NER was concluded to depend to a large extent on the previous degradation of IPU into the metabolite 4IA (Scheunert and Reuter, 2000). During IPU degradation, typically a high amount of NER is formed in soil: Pieuchot et al. (1996) reported for three soils NER levels of 44 – 65 % of applied ^{14}C-IPU (after 2 months of incubation), Kühn (2003) determined 39 – 70 % in four soils (after 46 days) and Scheunert and Reuter (2000) measured 64% NER (after 55 days).

The amount of NER in soil depends on the physico-chemical properties of both the pollutant (e.g. functional groups) and the soil (e.g. quality and quantity of SOM, biological activity, soil temperature, pH) (Roberts, 1984; Kästner, 2000; Barriuso et al., 2008). Due to the operational definition of NER as fraction which is typically not extractable by organic solvents, also the extraction method and its experimental conditions (organic solvent, duration of extraction etc.) affect the recordings of NER (Gevao et al., 2000; Mordaunt et al., 2005).

Most studies on the formation of NER were conducted with radio labeled compounds as those allow the detection and quantification of NER as well as the establishment of a ^{14}C mass balance. Generally, the parent compound and its metabolites are extracted from soil by using methods such as Soxhlet or Accelerated Solvent Extraction using aqueous or organic solvents (Hawthorne et al., 2000; Northcott and Jones, 2000). Subsequently, non-extracted ^{14}C-residues remaining in soil are usually quantified as $^{14}CO_2$ released by the combustion of extracted soil aliquots (Scheunert, 1998; Barriuso et al., 2008). However, due to the destructive nature of the approach this technique does not provide information about the chemical structure of NER contained (Kästner et al., 1999; Richnow et al., 1999; Kästner, 2000; Nowak, 2011). Thus, it is not possible to distinguish whether the detected residues originate from the parent compound, its metabolites or from microbial biomass (Kästner et al., 1999; Kästner, 2000; Richnow et al., 2000; Miltner et al., 2004; Nowak et al., 2011). As biogenic residues are explicitly excluded from the IUPAC definition of NER, but usually not analyzed separately, the actual amount of NER and thus their environmental hazard might be overestimated (Nowak, 2011).

In the form of NER, pollutants are generally reported to be less toxic, less bioavailable for biota and less mobile in soil than the free original compound (Sims et al., 1990; Wang et al., 1990;

Kästner, 2000; Northcott and Jones, 2000). Thus, several authors proposed the enhanced transformation of pollutants to NER as a desirable technique to reduce their toxic potential and bioavailability in polluted soils (Berry and Boyd, 1985; Bollag et al., 1992; Verstraete and Devliegher, 1996). However, studies on the stability of NER uncovered that these residues are not irreversibly bound to SOM under all circumstances and might be remobilized (Burauel and Führ, 2000; Boivin et al., 2005; Gevao et al., 2005; Lerch et al., 2009). In case of IPU, remobilization of NER can take place even years after IPU application (Johnson et al., 2001). Subsequently, the parent compound or its metabolites may distribute over various compartments of the environment and may ultimately end up in the food web, posing a risk for human health (Barraclough et al., 2005).

1.3 The phenomenon of accelerated pesticide degradation

1.3.1 Accelerated pesticide degradation

In agricultural practice, pesticides are typically applied to the same crop several times in intervals of a few years in the course of crop rotation or even within a growing season, particularly in tropical countries (Singh et al., 2002; Fang et al., 2008). Several studies reported that the repeated use of a pesticide on the same site might lead to a significant increase in pesticide degradation rates due to the development of microbial populations in soils that are capable to utilize this compound as a source of energy and carbon (Soulas and Lagacherie, 2001; Walker and Austin, 2004). This phenomenon was observed for a limited but growing number of pesticides. For a few pesticides, e.g. Carbofuran, enhanced degradation was already measured after a single application (Karpouzas et al., 1999). Others seem to respond slower, e.g. triazine and phenylurea herbicides (Walker and Austin, 2004). Nevertheless, after more than 40 years of application, enhanced degradation was also reported for contaminants from these groups, e.g. Diuron (Cullington and Walker, 1999; Rouchaud et al., 2000), Chlorotoluron (Rouchaud et al., 2000) and Atrazine (Pussemier et al., 1997). Several studies reported also an increased mineralization of IPU in agricultural soils previously treated with IPU (Cox et al., 1996; Bending et al., 2001; Sørensen et al., 2001; Walker et al., 2001).

In literature, this observation is designated usually as “soil acclimation”, “accelerated degradation”, “enhanced degradation” or “soil adaption” (Soulas and Lagacherie, 2001; Walker and Austin, 2004). In the following, the phenomenon is referred to as accelerated pesticide degradation.

1.3.2 Adaptation to pesticide degradation

Accelerated pesticide degradation in soil requires the previous adaptation of microbes to that pesticide (Spain and van Veld, 1983; Aelion et al., 1987; Nishino and Spain, 1993; Cox et al.,

1996). Spain and van Veld (1983) defined adaptation as “change in the microbial community that increases the rate of transformation of a test compound as a result of a prior exposure to the test compound”.

Microbes are in general very versatile concerning their metabolic abilities. Due to their high metabolic diversity and their ability for fast genetic evolution, native soil microbes are known to possess an enormous adaptation capacity (Leung et al., 2007; Pandey et al., 2009). In the following, the term ´native microbes´ refers to (1) microbes that are present in non-inoculated soil and (2) microbes that are present in inoculated soil but that were not introduced to soil by inoculation.

The **mechanisms** that lead to adaptation of microbial communities are not fully understood up to now and may involve (a) the induction of specific enzymes involved in pesticide metabolism, (b) the proliferation of naturally occurring microbial populations that are already capable to transform the target pollutant and (c) the development of new metabolic capabilities via genetic changes (Sørensen and Aamand, 2003; Top and Springael, 2003; Macleod and Semple, 2006). A combination of several mechanisms is also possible (Top and Springael, 2003).

New metabolic capabilities in bacteria might evolve either by alterations in the existing genetic material or by receiving external degradative genes. The main processes involved are mutation, DNA rearrangement and horizontal gene transfer (HGT) (Springael and Top, 2004; van der Meer, 2006): **mutation** characterizes alterations of genetic material that are random and undirected. Mutations are hereditary and may lead to new phenotypes, e.g. with the ability to degrade a new contaminant. Microbial communities in undisturbed soils are probably relatively stable, but mutation rates might be increased by external factors such as temperature or the presence of chemicals. Nevertheless, mechanisms of DNA repairing prevent most mutations (Scherf, 1997; Munk, 2000; Rensing et al., 2002). **DNA recombination** results in a new combination of the existing genetic material. The consequences might be quite drastic as genes can become deleted, replaced or duplicated etc. In comparison to mutation, recombination usually accounts for a much higher degree in genetic variability (Scherf, 1997; Munk, 2000; van der Meer, 2006). In **horizontal gene transfer**, mobile genetic elements (MGEs) such as plasmids are transferred between individual bacteria of the same or even of different species (Racke, 1990; McGowan et al., 1998; de la Cruz and Davies, 2000). However, the transfer of genetic information usually takes place more frequently between closely related organisms (Ottow, 2011). After transfer, the received DNA becomes integrated in the host-DNA (Rensing et al., 2002; Springael and Top, 2004). This is particularly relevant for adaptation because several studies indicate a high importance of MGEs in the *in situ* spread and construction of catabolic pathways in bacteria as pointed out by Top and Springael (2003). Repeatedly, degradation genes for pesticides were shown to be located on plasmids (Kearney and Kellogg, 1985; Racke, 1990; Neilson et al., 1994), which are the best studied catabolic MGEs (Springael and Top, 2004). This was also reported for IPU: the genes that

are involved in the degradation of IPU by the bacterial strains *Methylopila* sp. TES (El-Sebai et al., 2004) and *Arthrobacter globiformis* strain D47 (Turnbull, Ousley, et al., 2001) were also demonstrated to reside on plasmids.

The rate of **gene transfer** and thus adaptation depends on several **influencing factors** e.g. the size of the plasmid, the number of total bacteria in soil and the number of MGEs within a population. Particularly the selection pressure, which is in case of pesticide use the presence of the chemical, was reported to play a decisive role for the HGT rate (Rensing et al., 2002) and also for the survival and proliferation of donor cells, that are introduced to soil, and the recipient cells (Top et al., 2002). In order to attain a population size that is sufficient for pollutant degradation, receptor cells need - also as introduced, pollutant degrading microbes in general – a selective advantage compared to native microbes (Top et al., 2002). This seems to be the case when high contaminant concentrations are acting in soil: DiGiovanni et al. (1996) demonstrated the transfer of a plasmid from introduced bacteria to native microbes when a very high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) (1.000 mg kg^{-1}) was added to the soil as well as the subsequent proliferation and degradation of 2,4-D by those recipient cells. In contrast, no HGT was observed when a lower dose of 500 mg 2,4-D kg^{-1} was applied to soil. Similar results were reported for soils treated with 2.000 mg Naphthalene kg^{-1} (Gomes et al., 2005). Dejonghe et al. (2000) showed that HGT from inoculated donor microbes to native microbes can also take place at a medium level regarding the selection pressure (100 mg kg^{-1} 2,4-D), also enabling native microbes to degrade 2,4-D. Thus, HGT from introduced, pollutant degrading microbes to native ones seems probable when a medium or high contaminant concentration is present. Nevertheless, there is no evidence that gene transfer would also take place and result in accelerated pesticide degradation when lower, more realistic concentrations of the chemical would be present in soil (Top et al., 2002; Top and Springael, 2003). In the present thesis, a very low concentration of IPU of only 5 mg kg^{-1} was applied to soil, which is the dose recommended by the manufacturer for agricultural soils.

Several studies reported that also the length of the contact time in which the pollutant resides in soil plays a decisive role for the biodegradation rate of the pollutant (Spain and van Veld, 1983; Macleod and Semple, 2006).

1.3.3 Consequences of accelerated pesticide degradation

Accelerated pesticide degradation may play an important role in bioremediation as it reduces the amount of pesticides distributing in the environment and thus negative effects on the environment and human health. Typically, pesticides do not only affect the target pest but also non-target organisms like fish and birds (Seghers et al., 2003; Pal et al., 2006). Furthermore, many pesticides persist in the environment for a long time and may e.g. leach from the soil matrix to the ground water where they can enter drinking water wells. Once in the food chain, pesticides can accumulate

in the body tissue of organisms and cause health problems over time due to the increasing level of toxic compounds within the body (Gavrilescu, 2005; Arias-Estévez et al., 2008; Hallmann et al., 2009).

To estimate the degree of microbial adaptation to pesticide degradation, typically data was collected on (1) the duration of the lag phase, (2) the total amount of applied pesticide that was mineralized and (3) the mineralization rate. Adaptation of soil microbes is usually expected to result in a reduction of the lag phase, an increase in the total amount mineralized and a raise in the mineralization rate (Aelion et al., 1987; Aamand et al., 1989; Macleod and Semple, 2006). In fact, several studies demonstrated that repeated pesticide application to agricultural soils may result in a reduction of the lag phase (Sanyal and Kulshrestha, 1999; Macleod and Semple, 2006), an increase of the degradation rate (Wada et al., 1989) and an increase in the total amount of pesticide that was mineralized (Macleod and Semple, 2006). Nevertheless, there are also studies which suggest an inhibiting effect of repeated pesticide applications on microbial pesticide degradation: e.g. Singh et al. (2002) reported the extension of the lag phase with an increasing number of pesticide applications, also the reduction of the degradation rate (Thompson et al., 1999) and the total amount mineralized (Thompson et al., 1999; de Andréa et al., 2003) were shown.

Repeated applications of phenylurea herbicides to soil can also result in the adaptation of soil microbes to their degradation, i.e. a reduction of the lag phase, an increased mineralization rate and cumulative mineralization. This was already demonstrated for Diuron (Cullington and Walker, 1999) and IPU (Cox et al., 1996).

No information is available to the author´s knowledge on the effect of repeated applications on NER formation for IPU and other phenylurea herbicides.

1.4 Control of pesticide degradation in soil by bioaugmentation

Xenobiotics are defined as chemically synthesized compounds foreign to the biosphere (Hutzinger and Veerkamp, 1981). To remediate polluted soils, numerous thermal, mechanical, chemical and biological techniques were developed. Apart from the biological remediation approaches, all techniques have the drawback that the treated soil material is affected in e.g. its soil structure and quality of SOM and thus soil properties (Brauer, 1997). Therefore, a biological treatment is desirable especially when it is necessary to maintain the soil functions as it is for agricultural soils.

Due to the enormous metabolic capabilities of microbes in their entirety, most xenobiotics are attackable by microbes. The lack of suitable enzymes is a common reason for the persistence of organic pollutants in the environment (El-Fantroussi and Agathos, 2005; Fuchs, 2007; Maier, 2009). In the past few years, intensified research was done towards the **biological remediation of soils** contaminated with organic chemicals. Depending on the location of bioremediation, one refers

in principal to *ex situ* or *in situ* techniques. Both *ex situ* and *in situ* techniques enjoy wide public acceptance as they rely on natural microbial processes (Gavrilescu, 2005; Leung et al., 2007). In ***ex situ* approaches**, the polluted soil is excavated by machines and subsequently remediated (Gavrilescu, 2005; Leung et al., 2007). In ***in situ* approaches**, the polluted soil is treated without its previous excavation and transport (Brauer, 1997; Förtsch and Meinholz, 2011). As a result, these techniques are low priced compared to *ex situ* techniques and are primarily applied in those cases where the contaminated site is accessible only to a limited degree, e.g. due to a coverage by buildings and infrastructure (Leung et al., 2007; Pandey et al., 2009; Förtsch and Meinholz, 2011). The use of *in situ* techniques requires detailed information on the pollutant and the prevailing soil conditions like soil structure, water content etc. (Brauer, 1997; Förtsch and Meinholz, 2011).

Bioremediation aims to degrade a pollutant by microbes to concentrations that are no more detectable or below the boundary values set by law (Gavrilescu, 2005; Leung et al., 2007; Becker, 2010). Generally, the degradation of pollutants within an environmental matrix like soil can be increased either by stimulation of the indigenous microbial populations (biostimulation), e.g. by addition of nutrients or aeration, or by the introduction of specific non-indigenous microbial strains or consortia that are capable to breakdown the chemical (bioaugmentation) (El-Fantroussi and Agathos, 2005; Leung et al., 2007). Bioaugmentation offers advantages over biostimulation especially in those cases where the native microbes with the desired metabolic capabilities are not or not in a sufficient amount present in the polluted soil (Vogel, 1996; Leung et al., 2007).

Bioaugmentation has already been utilized in agricultural soils for several decades, e.g. by inoculation of symbiotic, nitrogen-fixing bacteria to legumes. More recently, this technique was implemented with the goal to remediate sites contaminated with pesticides (van Veen et al., 1997; Gentry et al., 2004). Several **bioaugmentation techniques** are common for the remediation of polluted soils: the technique used most frequently is (1) the application of living microbial cells which were extracted from a soil adapted to pollutant degradation. These cells might be applied as (a) isolated single strain, (b) a combination of various efficient strains, (c) as a microbial consortium and are typically applied either (d) in liquid culture or (e) attached to a carrier material (Gentry et al., 2004; El-Fantroussi and Agathos, 2005). An alternative procedure for bioaugmentation is (2) to add activated soil, i.e. soil that was previously exposed to the contaminant and that contains indigenous pollutant degrading microbes (Gentry et al., 2004; El-Fantroussi and Agathos, 2005). The use of activated soil has several advantages, e.g. that degraders that are not cultivable and might get lost during isolation and cultivation are not missed (Gentry et al., 2004). Enhanced biodegradation may also be achieved from (3) application of genetically engineered microorganisms (GEMs) into soil (Gentry et al., 2004; El-Fantroussi and Agathos, 2005). However, the introduction of GEMs to open land is discussed very controversial as there is concern about unknown risks such as the potential of their genes to be transferred to indigenous

microbes (Gentry et al., 2004). Additionally, regulatory approval for their release into the environment is difficult to obtain (Sayler and Ripp, 2000; Gentry et al., 2004). For these reasons, research on bioaugmentation is mainly focusing on the optimization and commercial development of non-engineered microbes (Sayler and Ripp, 2000; Gentry et al., 2004).

Although a large number of studies dealt with the bioremediation of polluted soils by introduction of specific microbes (e.g. Shapir and Mandelbaum, 1997; Struthers et al., 1998; Halden et al., 1999; Juhanson et al., 2009) this technique remains in large parts experimental because most studies were conducted at laboratory scale while well-documented field studies are rare (El-Fantroussi and Agathos, 2005; Owsianiak et al., 2010). Altogether, bioaugmentation continues to be a controversial subject within environmental microbiology due to its unreliable remediation success. Several studies in soil have demonstrated increasing degradation of various pesticides such as Atrazine (Silva et al., 2004; Getenga et al., 2009), 1,2,4-Trichlorobenzene (1,2,4-TCB) (Schroll et al., 2004; Wang et al., 2007), IPU (Grundmann et al., 2007) and Phenanthrene (Schwartz and Scow, 2001) by inoculation of specific microorganisms. However, numerous studies exist in which no enhanced biodegradation of the pollutant was observed despite bioaugmentation (Mueller et al., 1992; Bouchez et al., 2000). A key factor for a successful **bioaugmentation by inoculating microbial cells** into soil is the **sustainability of this approach**. Once applied, the success of bioaugmentation depends on the survival and catabolic activity of introduced microbes on a level sufficient for pollutant degradation (Cassidy et al., 1996; Leung et al., 2007; Juhanson et al., 2009). However, the size of microbial populations often declines after their inoculation into soil, while increases in population size are a seldom reported phenomenon (van Elsas and Heijnen, 1990; Pritchard, 1992; van Veen et al., 1997; Gentry et al., 2004). The survival rate of introduced microbes is influenced mainly by (1) abiotic and (2) biotic environmental factors as well as from (3) the contaminant concentration in soil. Abiotic stresses which affect survival and activity of inoculants in the new soil environment might be fluctuations or extremes e.g. in soil physico-chemical characteristics (e.g. pH, water content) or nutrient availability together with pollutant characteristics (e.g. bioavailability, microbial toxicity) (Vogel, 1996; Gentry et al., 2004). Biotic factors are often regarded as more relevant than abiotic ones as sterile soil is usually more hospitable to foreign microbes than a biologically active soil (El-Fantroussi et al., 1999). As biotic stresses, parameters like competition with indigenous organisms for limited nutrients or predation by protozoa were reported to impede the survival of external cells and thus bioremediation effectiveness (Gentry et al., 2004; Owsianiak et al., 2010). To improve the degradation performance of bacterial inocula in the new soil environment, it was proposed to immobilize the external microbes, e.g. on a porous matrix or in gels, to provide a protective surrounding area (El-Fantroussi and Agathos, 2005; Owsianiak et al., 2010). In fact, cells attached to a carrier material were shown to be characterized by a more efficient degradation performance than cells applied to

soil as a suspension (Moslemy et al., 2002, 2003; Wang et al., 2010). The survival rate of introduced microbes depends also upon a certain selection pressure caused by the contaminant concentration in soil as the contaminant provides a selective advantage for the introduced microbes towards native microbes that are not able to degrade it (Pritchard, 1992; van Veen et al., 1997). Successful inoculations of external microbes were shown to be feasible at both middle (50 – 1000 mg kg^{-1}) and high (> 1000 mg kg^{-1}) contaminant concentrations (Dejonghe et al., 2000; Goux et al., 2003; Gomes et al., 2005; Juhanson et al., 2009). At these conditions, even the transfer of degradative genes by HGT from inoculated microbes to native ones was observed (Dejonghe et al., 2000). Long-term studies with high (10 000 mg kg^{-1}) contaminant levels showed that introduced microbes were able to survive in soil and to maintain their capability to degrade the pollutant under such conditions even 9 months (Goux et al., 2003) after their inoculation into soil.

Several studies demonstrated that increased mineralization after inoculation of specific microbes is possible also in soils with **low pesticide concentrations** (< 50 mg kg^{-1}) as it is typical for agricultural soils, e.g. for Atrazine (Silva et al., 2004), 1,2,4-TCB (Schroll et al., 2004; Wang et al., 2007) and also phenylurea herbicides as IPU (Turnbull, Ousley, et al., 2001; Grundmann et al., 2007) and Diuron (Cullington and Walker, 1999; Turnbull, Ousley, et al., 2001). Grundmann et al. (2007) showed that bioaugmentation at low concentrations (IPU at 5 mg kg^{-1}) also works under outdoor conditions at a lysimeter station. However, the number of introduced cells is frequently decreasing with time (van Veen et al., 1997; Gentry et al., 2004; El-Fantroussi and Agathos, 2005) and thus the long-term survival of those microbes in soil is questionable, especially when the contaminant concentration is declining. Inoculation studies in soil at low pesticide concentrations typically encompassed a period of less than two months (Turnbull, Ousley, et al., 2001; Schroll et al., 2004; Silva et al., 2004; Grundmann et al., 2007; Wang et al., 2007).

1.5 Objectives of this thesis

The present work is based on previous studies within the working group (Kühn, 2003; Grundmann et al., 2007): An **I**PU **mi**neralizing **mi**crobial **c**ommunity (further referred to as “IMMC”) was isolated from an agricultural soil. The consortium was established on Seramis® clay particles as carrier material, providing a stable mineralization performance in liquid culture. By inoculation of these specific microbe-carrier-complexes into soil supplemented with IPU, it was demonstrated that the *in situ* mineralization of IPU could be considerably enhanced. The members of the microbial community were identified in a further step. This promising inoculation approach provided the opportunity for a detailed investigation of the microbial community and its capabilities.

However, new xenobiotics are developed and brought to the market continuously. But effective bioaugmentation requires the introduction and thus availability of microbes that are capable to degrade that pollutant. For several pesticides, e.g. Atrazine and 2,4-D, it took decades under

common agricultural practice until soil microbes adapted to the degradation of these pollutants, a period during which the pesticides may distribute and impair the environment (van der Meer, 2006; Leung et al., 2007). For a faster use of microbes in bioaugmentation, it is thus desirable to accelerate the adaptation of soil microbes to the degradation of new pesticides.

The goal of this thesis was to investigate the properties of IMMC and to isolate the microbial species therein that is responsible for the mineralization of IPU. It was also aimed to examine the long-term sustainability of IMMC after its inoculation in soil. Furthermore, it was investigated to what extent microbial communities, that inhabit soils which degrade IPU cometabolically, can adapt to accelerated IPU mineralization by repeatedly adding IPU to these soils.

The specific objectives were:

1. To isolate and identify the IPU mineralizing bacterial strain (IMBS) within IMMC.
2. To study the capability of IMBS and IMMC to degrade IPU and some of its metabolites. Furthermore, it was intended to analyze the metabolites which were formed during IPU degradation.
3. To investigate if IMMC is capable to degrade the phenylurea herbicide Diuron. This gives information on the specificity of IMMCs´ degradation enzymes.
4. To test how long the acceleration in IPU degradation, which is induced by inoculation of IMMC, is maintained in soil without any further delivery of IPU as a food source.
5. To investigate to what extent microbial communities, that inhabit agricultural soils which degrade IPU cometabolically, can adapt to accelerated IPU degradation. For this purpose, several soils were treated repeatedly with IPU under optimized conditions in the laboratory; IPU was applied in shorter intervals as it is commonly done in agricultural practice.

This study is mainly based on ^{14}C-technique. ^{14}C-ringlabeled IPU was used to be able to investigate the fate of IPU and its metabolites in soil. The use of this technique allowed the identification of IPU and its metabolites as well as the quantification of non-extractable residues and $^{14}CO_2$, originating from the mineralization of the compound. Further, a ^{14}C mass balance could be established as a quality check.

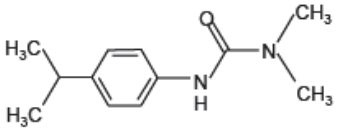
2 Materials and methods

2.1 Chemicals

2.1.1 Isoproturon and its metabolites

In this study, the herbicide Isoproturon {1,1-dimethyl-3-[4-(propan-2-yl)phenyl]urea} (IPU) was used. Its main chemical and physical properties are shown in Table 1.

Table 1: Chemical-physical properties of IPU (Schroll et al., 2006).

chemical formula	$C_{12}H_{18}N_2O$		
vapour pressure at 20 °C	2.8 – 8.1	Pa	
water solubility at 20 °C	70.2	mg L^{-1}	
molecular mass	206.3	g mol^{-1}	
half-life under field conditions	approx. 60 - 300	d	

To quantify mineralization, i.e. the total degradation of a molecule up to CO_2, ^{14}C-ringlabeled IPU was employed. The radioactive marker was placed at the most stable part of the molecule. This allowed the measurement of mineralization (= formation of $^{14}CO_2$), identification of metabolites, quantification of NER and the compilation of a mass balance for IPU as a quality check.

The ^{14}C-ring-labeled IPU had a radiochemical purity of > 98 %. To reduce costs, ^{14}C-IPU was further diluted with non-labeled IPU for soil and liquid culture experiments. The specific radioactivity of these application standards is given in the description of the respective experiments. In two experiments (chapters 2.4.1 & 2.5.1.1), ^{14}C-ring-labeled IPU and unlabeled IPU were applied separately. In these experiments, ^{14}C-IPU-standard I9 was utilized, which had a specific radioactivity of 4.43 MBq mg^{-1} and a radiochemical purity of > 97 %.

For analysis with **H**igh-**P**erformance **L**iquid **C**hromatography (HPLC) as reference substances and also for soil and liquid culture experiments, non-labeled IPU, 1-methyl-3-[4-(propan-2-yl)phenyl]urea (MD-IPU), 1-[4-(propan-2-yl)phenyl]urea (DD-IPU), 1-[4-(2-hydroxypropan-2-yl)phenyl]-3-methylurea (2-OH-MD-IPU) and 4-isopropylaniline (4IA) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All compounds were solved in methanol (LiChrosolv, gradient grade for liquid chromatography, Merck, Darmstadt, Germany) and had a chemical purity of > 98 %.

Ring-labeled ^{14}C-MD-IPU was applied in one experiment to investigate the microbial degradation of IPU and its metabolites (chapter 2.5.1.1). This standard M had a radiochemical purity of > 98 %

and a specific radioactivity of 4.42 MBq mg^{-1}. To reduce costs, unlabeled MD-IPU standard was added separately to liquid culture flasks. Both standards were dissolved in acetonitrile (ROTISOLV HPLC, Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2.1.2 Diuron

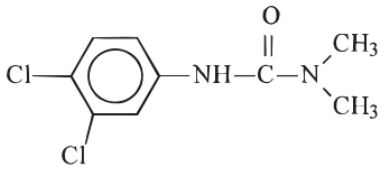
For one degradation experiment (chapter 2.5.2.2), the phenylurea herbicide Diuron ([N-(3,4-dichlorophenyl)-N,N-dimethylurea]; $C_9H_{10}Cl_2N_2O$) was applied ^{14}C-labeled. The compound was available both ring- and side chain-labeled. ^{14}C-labeled compounds were obtained from Dr. Sebastian R. Sørensen, GEUS, Copenhagen, Denmark.

The ring-labeled compound had a specific radioactivity of 3.10 MBq mg^{-1} and a radiochemical purity of > 95 %. The ^{14}C-labeled impurities were identified as 1% N-(3,4-dichlorophenyl)-N-methylurea and 4 % N-(3,4-dichlorophenyl)urea. The side chain-labeled ^{14}C-Diuron had a purity of 98 %.

To reduce costs, non-labeled Diuron (Dr. Ehrenstorfer Augsburg, Germany) was solved in methanol (LiChrosolv, gradient grade for liquid chromatography, Merck, Darmstadt, Germany) and additionally applied to the liquid cultures. The non-labeled Diuron had a chemical purity of > 98 %.

The chemical structure of Diuron is shown in Table 2.

Table 2: Chemical structure of Diuron (Field et al., 1997).

chemical formula	$C_9H_{10}Cl_2N_2O$
	

2.2 The IPU mineralizing microbial community IMMC

2.2.1 Origin of the consortium

The IPU mineralizing consortium IMMC, which is examined in the present study, was extracted from soil Feldkirchen in 2000/2001 by Kühn (2003). Subsequently, the enriched culture was established by Kühn (2003) on Seramis® clay particles (chapter 2.2.3) as carrier material by incubating the consortium in 25 mL MS-medium (chapter 2.3.1) that contained also autoclaved carrier material and 25 mg L^{-1} IPU as the only source of C and N. After three weeks of incubation, the carrier material was transferred with an autoclaved spoon to fresh MS-medium that contained

again 25 mg L^{-1} IPU. The presence of microbes on Seramis® clay particles was confirmed by Fluorescence *in situ* hybridization (FISH). After three transfer cycles, Kühn (2003) introduced an aliquot of Seramis® clay particles overgrown by IMMC to agricultural soils supplemented with 5 mg kg^{-1} ^{14}C-IPU and measured accelerated IPU mineralization, demonstrating that the carrier material was well overgrown with IMMC. Kühn (2003) identified also the members of the microbial community IMMC.

Since then, the complexes of IMMC and Seramis® clay particles were regularly transferred into fresh MS-medium (2.3.1) containing 25 mg L^{-1} IPU in the same way as Kühn (2003) had done previously. After regular time periods, complexes were frozen at -80 °C. Then, an equivalent amount of autoclaved Seramis® clay particles was added to the liquid cultures.

2.2.2 Overgrowing of carrier material by IMMC

In the present study, these complexes of IMMC and Seramis® clay particles were used for soil and liquid culture studies. Prior to the studies, it was necessary to increase the amount of complexes. This was done by adding sterilized Seramis® clay particles to liquid cultures that already contained such complexes. The respective liquid cultures (25 mL MS-medium, 25 mg L^{-1} IPU) typically contained one spoon of carrier material that was already overgrown with IMMC and in addition one spoon of freshly sterilized Seramis® clay particles was applied. The final two spoons of the carrier material per liquid culture corresponded to 3.3 g (dry weight) and 80 particles, respectively. Liquid cultures were typically incubated until the daily mineralization rate was lower than 0.1 µg d^{-1}, which took approximately a period of three to four weeks.

The sterilization of Seramis® clay particles was carried out in a steam high pressure autoclave (Systec D65/V65/50, Systec GmbH, Wettenberg, Germany) for 20 min at 121 °C. In the further thesis, this sterilization mode is named autoclaving.

2.2.3 Characteristics of the carrier material

The Seramis® clay particles with a diameter of 2-4 mm (approx. pH 7 (in H_2O), pore volume > 80 %, total N: 3-8 mg l^{-1}; P_2O_5: 5–10 mg l^{-1}; K_2O: 100–120 mg l^{-1}) consisted of fired clay material manufactured by Mars GmbH, Mogendorf, and with Kaolinite, Illite and Quartz as dominating minerals.

2.3 Culture medium and buffer for liquid culture experiments

The culture media, agar plates and buffer solutions mentioned in the following were prepared with double distilled water out of a high-purity water complex (Milli-Q PLUS, Millipore GmbH, Schwalbach, Germany). The pH was adjusted with NaOH- or HCl- solutions of different molarities using a pH-meter with integrated temperature probe (inoLab pH level 1, WTW, Weilheim,

Germany). The sterilization of prepared solutions as well as other consumable supplies was carried out in a steam high pressure autoclave (Systec D65/V65/50, Systec GmbH, Wettenberg, Germany) for 20 min at 121 °C. In the following, this sterilization mode is named autoclaving.

2.3.1 Mineral salt medium

For experiments on the degradation of IPU and its metabolites in liquid culture, the mineral salt medium (MS-medium) according to Sørensen et al. (2001) was used. Its composition is given in Table 3. After cooling, 5.14 mg L^{-1} of sterile filtered $FeCl_3$ * 6 H_2O were added.

Table 3: Mineral salt medium used for liquid culture experiments.

Compound	Concentration
KH_2PO_4	1.36 g L^{-1}
Na_2HPO_4*2 H_2O	1.78 g L^{-1}
$MgSO_4$*7 H_2O	0.05 g L^{-1}
$CaCl_2$	0.01 g L^{-1}
H_3BO_4	2.86 mg L^{-1}
$MnSO_4$* H_2O	1.54 mg L^{-1}
$CuSO_4$*5 H_2O	0.04 mg L^{-1}
$ZnCl_2$	0.02 mg L^{-1}
$CoCl_2$* 6 H_2O	0.04 mg L^{-1}
Na_2MoO_4* 2 H_2O	0.03 mg L^{-1}

In general, the application standard (either IPU, one of its metabolites or Diuron) was added as the only source of carbon (C) and nitrogen (N).

For liquid culture experiments conducted with the IPU mineralizing strain, 0.1 g L^{-1} of Casamino acids (US Biological, Casein acid and hydrolysate, molecular biology grade, Swampscott, MA, USA) were added to the liquid MS-media before autoclaving. The amino acids added within the mix were (in alphabetical order and according to the manufacturers´ leaflet) alanine, arginine, asparagines, cystine, glutamic acid, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

$MgSO_4$*7 H_2O, $CuSO_4$*5 H_2O and $CoCl_2$* 6 H_2O were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), while all other compounds of the MS- media were bought from Merck KGaA (Darmstadt, Germany).

2.3.2 PBS-buffer

The 10 x concentrated buffer contained the following components: 12 g L^{-1} NaH_2PO_4, 14.2 g L^{-1} Na_2HPO_4 and 75.97 g L^{-1} NaCl. All compounds were purchased from Merck (Darmstadt, Germany). The pH was adjusted to pH 7.2 using 10 N NaOH.

The 1x concentrated PBS-buffer was used for the application of dilution series (chapter 2.4.1) and the preparation of inocula of the IPU mineralizing strain (chapter 2.6.1).

2.4 Isolation and identification of the IPU mineralizing bacterial strain from the consortium IMMC

2.4.1 Isolation of an IPU mineralizing bacterial strain (IMBS)

To spatially isolate the various members of IMMC established on Seramis® clay particles and get a pure culture of the IPU mineralizing bacterial strain, microbial cells were (1) detached from the clay particles and (2) attained cells were individualized by creating a dilution series for detached cells and spreading solutions on agar plates. The exact procedure for these two steps is described in detail in the following.

Detach microbial cells from clay particles

As step one, two middle-sized Seramis® clay particles were removed from liquid culture by a spoon and placed on a sterile petri-dish (Greiner-Bio-One GmbH, Frickenhausen, Germany). Subsequently, the weight of these particles was determined gravimetrically, then particles crushed by tweezers and all single parts carried to tubes filled with 1000 µl 1x PBS (chapter 2.3.2). The mixture was homogenized on a shaker (MS2 Minishaker, IKA, Staufen, Germany).

Conduction of dilution series

As step two, an aliquot of 100 µl was removed and mixed with 900 µl of 1x PBS to attain a decimal dilution. Analogue to this procedure, dilutions -2 to -6 were conducted. Four dilution series were done. Subsequently, in each case 100 µl of dilutions -2 to -6 were spread with a drygalski spatula on agar plates. On average, only 0.1-10 % of all bacteria present in soil are cultivable in the laboratory (Ottow, 2011). To increase the chance to cultivate IMBS, three different types of agar plates were utilized. The prepared dilutions were spread on Luria-Bertani (LB) plates, R2A plates and MS-IPU plates (see below) with one plate per plate type and dilution. For incubation, plates were stored at + 20 ± 1 °C in the dark and checked regularly for growth of **C**olony **F**orming **U**nits (CFUs).

2.4.1.1 Solid culture media used for isolation of IMBS

LB plates were composed of 10 g Tryptone (enzymatic digested from Casein, Sigma-Aldrich, Steinheim, Germany), 5 g NaCl (Merck, Darmstadt, Germany), 5 g Yeast Extract (AppliChem, Darmstadt, Germany) and 15 g Agar Agar (Bernd Euler Biotechnologie-Mikrobiologie, Frankfurt am Main, Germany) per 1 L of distilled water. For cloning purposes, ampicillin (Ampicillin sodium salt, Sigma-Aldrich, Steinheim, Germany) was added after autoclaving to the media cooled to about 45 °C to a final concentration of 50 µg mL^{-1}.

R2A plates were prepared by using R2A agar for microbiology (Merck, Darmstadt, Germany) according to manufacturers´ instructions (15.2 g per 1 L of distilled water). 1 L of R2A agar consisted, according to the manufacturers´ technical information, typically of the following ingredients: yeast extract (0.5 g), proteose peptone (0.5 g), casein hydrolysate (0.5 g), glucose (0.5 g), soluble starch (0.5 g), sodium pyruvate (0.3 g), K_2HPO_4 (0.3 g), $MgSO_7$ (0.05 g) and agar-agar (12.0 g).

To prepare a solid mineral salt medium containing IPU as the only source of C and N (**MS-IPU plates**), a liquid mineral salt medium was set up (chapter 2.3.1) and supplemented with 15 g L^{-1} Agar-Agar (Bernd Euler Biotechnologie-Mikrobiologie, Frankfurt am Main, Germany) before autoclaving. After the sterilization process unlabeled IPU (chapter 2.1.1) was added to the liquid cooled to about 45 °C to a final concentration of 50 mg L^{-1}.

2.4.1.2 Test of colonies for ^{14}C-IPU-mineralization

Setup of liquid cultures

Eight, 14 and 36 days after spreading, the number of living microbial cells on agar plates was determined by counting the number of CFUs. Eight, 14 and 48 days after plating (= samplings 1-3), colonies of different shape and color were detached from plates by a sterile inoculation loop and single colonies respectively combinations of colonies were screened for their ability to mineralize IPU by using them as inoculum for liquid cultures. Two, 10 and 16 cells were detached for testing at samplings 1, 2 and 3, respectively. To be able to detect mineralization with this small amount of cells, liquid cultures contained just 5 ml of MS-medium. IPU was applied as the only source of C and N. Liquid cultures were prepared according to the following procedure: 15 µl of ^{14}C-IPU-standard I9 (specific radioactivity 4.43 MBq mg^{-1}) and 12.5 µl non-labeled IPU (10 mg mL^{-1}) (chapter 2.1.1), both dissolved in acetonitrile (ROTISOLV HPLC, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), were applied to the bottom of a sterile 20 ml glass vial (neoLab, Heidelberg, Germany) resulting in a final IPU-concentration of 25 mg L^{-1}. After evaporation of the solvent, 5 mL MS-medium (chapter 2.3.1) was added per flask. Glass vials were then placed on a horizontal shaker (IKA Labortechnik KS 250 Basic Orbital Shaker, Staufen, Germany) at 100 rounds per minute (rpm) at +20 ± 1 °C to guarantee the complete and homogeneous dissolving of the added

compound in the liquid medium. On the next day, an aliquot of 100 µl was taken and the exact amount of applied ^{14}C was measured by liquid scintillation counting (see below). Subsequently, liquid cultures were inoculated as mentioned above with single colonies respectively combinations of colonies. To trap the evolved $^{14}CO_2$, a glass test tube (Assistent, Sondheim, Germany) was placed within the glass vial (Figure 2) and 1 mL of sterile 0.1 N NaOH (Merck, Darmstadt, Germany) was filled with a pipette into the glass test tubes. The NaOH was regularly removed and the trapped ^{14}C quantified by liquid scintillation counting.

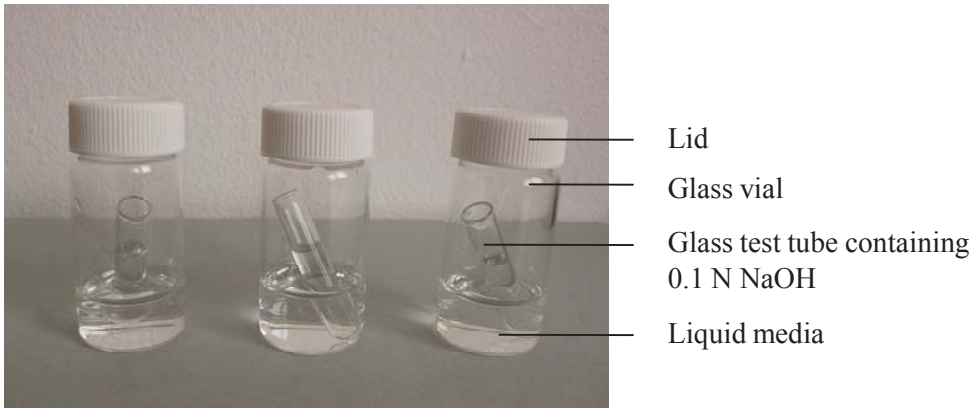


Figure 2: Liquid culture systems for isolation of the IPU mineralizing strain.

For an IPU mineralizing strain isolated from an agricultural soil, it was reported that IPU mineralization was considerably increased by supplementing the medium with a mixture of amino acids (Sørensen et al., 2001). Thus, to test the effect of amino acids on IPU mineralization by cells of IMMC, 48 days after plating (= sampling 3) 16 single colonies (= colonies C1-C16) were detached from R2A plates and each colony used to inoculate two liquid cultures: one was prepared as described above, the MS-medium of the other one was supplemented with 0.1 g L^{-1} Casamino acids (US Biological, Casein acid and hydrolysate, molecular biology grade, Swampscott, MA, USA).

Measurements in liquid medium showed that inoculation with colonies C1 and C2 resulted in good mineralization of ^{14}C-IPU in both types of medium (chapter 3.1.2.2). As cells of both colonies which were used for inoculation had shown up on agar after 4-5 weeks, were yellow pigmented and rod shaped, just one of the isolates (containing colony C1) was selected for purification, which should ensure that the isolate was a pure culture that contained solely microbes of the IPU mineralizing strain. Purification was done as recommended by Bast (2001) by repeatedly streaking serial dilutions of the isolate on agar plates: when colonies showed up on R2A plates, single colonies were detached with a sterile inoculation loop and used for inoculation of new liquid culture systems. Then, serial dilutions of this liquid culture were spread on R2A plates and the plates incubated. The streaking and inoculation procedure was repeated once more to verify the

purity of the isolate as well as its capability to mineralize ^{14}C-IPU. The obtained purified strain which originated from colony C1 was subsequently identified (chapter 2.4.2) and designated strain AK1.

Quantification of mineralization by liquid scintillation counting

Mineralization of ^{14}C-IPU was quantified by liquid scintillation counting. At sampling of NaOH-solution, which trapped evolved $^{14}CO_2$, the solution was collected in scintillation vials made of polyethylene (PerkinElmer, Shelton, USA). Vials were weighed before and after sampling to calculate the exact sample volume.

For measurement of evolved $^{14}CO_2$, 1 mL of NaOH solution was mixed with 10 ml of Wallac OptiPhase HiSafe 3 scintillation cocktail (Turku, Finland). The mixture was analyzed with a liquid scintillation analyzer (TriCarb 2800 TR, PerkinElmer, Überlingen, Germany). Customized quench curves were applied in order to correct results for quenching. Each sample was measured 3 times for 5 min. For data analysis, mean values were used.

To measure the applied amount of ^{14}C to a liquid culture, 100 µl liquid of the liquid culture was removed by a pipette and analyzed by liquid scintillation counting before microbial cells were applied to the liquid culture. This aliquot was then mixed with 5 ml Ultima Gold XR (Packard, Dreieich, Germany) and measured for contained ^{14}C by liquid scintillation counting. The radioactivity was then extrapolated to the total volume of the liquid culture.

2.4.2 Identification of the isolated IPU mineralizing strain by genetic approach

2.4.2.1 PCR amplification of 16S-rDNA coding genes

Four colonies of isolate AK1 were picked from R2A plate and bacterial 16S-rDNA genes were amplified by **p**olymerase **c**hain **r**eaction (PCR) using universal primer 1492r (5´-GGT TAC CTT GTT ACG ACT T-3´) and bacterial primer 27f (5´-AGA GTT TGA TCC TGG CTC AG-3´) (Lane, 1991). The PCR mixture contained 0.25 µl TopTaq DNA polymerase (Quiagen, Hilden, Germany), 5 µl TopTaq PCR buffer (10x) (Quiagen, Hilden, Germany), 1 µl 2.5 mM dNTPs (Fermentas, St. Leon-Rot, Germany), 0.2 µl forward primer and 0,2 µl reverse primer (50 pmol) (Eurofins MWG Operon, Ebersberg, Germany), one colony per tube and sterile water to 49 µl final reaction volume. PCR was carried out in a thermocycler (peqstar 96, Peqlab Biotechnologie, Erlangen, Germany) and started with an initial denaturation step at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 seconds (sec), annealing at 56.5 °C for 30 sec, and 72 °C for 1 minute (min) and the last elongation step was done at 72 °C for 10 min. An aliquot of the PCR reaction was analyzed on a 1 % agarose gel to verify the production of the expected fragment.

2.4.2.2 Cloning of genes

To obtain better quality of sequences, amplified 16S-rDNA gene fragments were further cloned with the StrataClone PCR Cloning Kit (Agilent Technologies, Böblingen, Germany). For the ligation reaction 3 µl StrataClone Cloning Buffer, 1 µl of StrataClone Vector Mix amp/kan and 20 ng of undiluted PCR product was used. The cloning procedure was done as noted in the manufacturers´ instructions. After cloning, the transformation mixture was spread on LB-ampicillin plates (plates contained 100 µg mL^{-1} ampicillin (Ampicillin sodium salt, Sigma-Aldrich, Steinheim, Germany)), which had been previously covered with 40 µl of 2 % X-Gal (Fermentas, St. Leon-Rot, Germany) according to the manufacturers´ protocol. On the next day, insert-positive clones (10 clones per sample) were selected by blue-white color screening and proliferated in 3 ml LB-media containing 50 µg mL^{-1} ampicillin.

Plasmid DNA was isolated from the E.coli host cells by using the NucleoSpin Plasmid Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Amplification of the insert plasmid was done by PCR using 0.2 µl primer M13f (10pM; 5' - TGT AAA ACG ACG GCC AG – 3'; Eurofins MWG Operon, Ebersberg, Germany), 1 µl BDT buffer (Applied Biosystems, Foster City, California, USA), 1 µl Big Dye Terminator (Applied Biosystems, Foster City, California, USA) and 100 ng Plasmid DNA. PCR was done in a thermocycler (peqstar 96, Peqlab Biotechnologie, Erlangen, Germany) and started with an initial denaturation step at 96 °C for 1 min, followed by 50 cycles at 96 °C for 10 sec, 50 °C for 5 sec (annealing), and 60 °C for 4 min. PCR products were purified by pipetting the whole volume of PCR product, each supplemented with 20 µl water, in filter wells (Multiscreen Column Loader 45µl, MilliPore, Billerica, MA, USA) containing Sephadex (Sephadex-G50 superfine, GE Healthcare, Uppsala, Sweden) according to manufacturers´ instructions after previous swelling (with 300 µl water) and washing of Sephadex with 150 µl water (water: LiChrosolv water for chromatography, Merck, Darmstadt, Germany; centrifuge: 4K15C, Sigma laboratory centrifuges, 910 g for 5 min). The presence of inserts with correct size was controlled on a 1 % agarose gel (horizontal gel electrophoresis apparatus: Peqlab Biotechnologie GmbH, Erlangen, Germany).

Subsequently, the inserts were given for sequencing (sequencer: 3730, Applied Biosystem, Foster City, California, USA).

From the obtained 40 sequences, the primer was removed and the sequences reduced to 500 bases each. Possible reading errors were checked within the chromatogram using software BioEdit (Ibis Biosciences, Carlsbad, CA, USA) and Finch TV 4.1 (Geospiza, USA). Chimera check of 16S-rRNA coding gene sequences was performed using the chimera test program Bellerophon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) (Huber et al., 2004) and chimeric

sequences excluded from further analysis. Subsequently, equal sequences were deleted and 3 of the 11 sequences differing in single bases were selected for further full sequencing.

2.4.2.3 Full-sequencing of 16S-rDNA coding genes

The full sequence of plasmid DNA inserts was done by the company Sequiserve (Vaterstetten, Germany) using the ABI 3730 DNA Analyzer (Applied Biosystems). For premium (primer T3) and full sequencing (primer T7), the below mentioned primers were used.

Table 4: Overview over primers used for sequencing of 16S-rRNA fragments.

Primer	Sequence
T3	5´- AAT TAA CCC TCA CTA AAG GG - 3´
T7	5´- TAA TAC GAC TCA CTA TAG GS - 3´

2.4.2.4 Analysis of sequencing data and reconstruction of a phylogenetic tree

All acquired 16S-rDNA sequences were fitted in an "alignment" of already identified sequences of the database SILVA (SSURef_108_SILVA_09_09_11_opt.arb, http://www.arb-silva.de; (Pruesse et al., 2007)). The tool "Aligner" served for this, which was implemented in the software package ARB (http://www.arb-home.de; Ludwig et al. (2004)). Not correctly aligned sequences were proofread manually. Here, it was paid attention that the homologous positions were placed at the same position after adjustment. In the variable areas of sequences, analysis of the secondary structure or smaller groups of preserved base orders served for orientation to determine homologous positions.

Phylogenetic trees were calculated by applying the methods "Maximum-Parsimony", "Neighbor-Joining" (Saitou and Nei, 1987) and "Maximum-Likelihood" (Felsenstein, 1981). All tools for tree calculation were included in the software package ARB (Ludwig et al., 2004). The matrix of paired distance values served as basis for reconstruction of phylogenetic trees according to the "Neighbor-joining" method (Saitou and Nei, 1987). The topology of phylogenetic trees was checked and, if necessary, corrected by means of "Maximum-Parsimony" (Saitou and Nei, 1987) and "Maximum Likelihood" (Felsenstein, 1981) analysis.

2.4.3 Diversity of IMMC

Accompanying to the isolation and identification of the IPU mineralizing strain, clone libraries were established in order to give information on the diversity of the IPU mineralizing microbial community.

To extract high molecular weight DNA from the microbial community, biomass established on Seramis® clay particles was removed from liquid culture (0.75 g) by a sterilized spoon and DNA

extracted by using the UltraClean® Microbial DNA Isolation Kit (MoBio, Carlsbad, California, USA). It was preceded according to the manufacturers´ protocol.

The 16S-rRNA genes of the extracted DNA were amplified by PCR using a thermocycler (PEQStar 96 Universal, PEQLAB Biotechnologie GmbH, Erlangen, Germany). For amplification, the primers 27f (5´-AGA GTT TGA TCC TGG CTC AG-3´) and 1492r (5´-GGT TAC CTT GTT ACG ACT T-3´) (Lane, 1991) (both primers Eurofins MWG Operon, Ebersberg, Germany) were applied. PCR mixture contained 0.15 µl Dream Taq™ DNA Polymerase (5u μl^{-1}; Fermentas, St. Leon-Rot, Germany), 1.5 µl forward primer and 0.1 µl reverse primer, 0.6 µl dNTPs (Fermentas, St. Leon-Rot, Germany), 1.5 µl BSA (Purified BSA, 100x, New England Biolabs, Beverly, MA, USA), 3 µl buffer containing $MgCl_2$ (10 x Dream Taq Buffer, Fermentas, St. Leon-Rot, Germany) and sterile water to 29 µl final reaction volume. 1 µl of DNA (50 ng μl^{-1}) was added per reaction. PCR was started by hot start with an initial denaturation at 95 °C for 4 min, followed by 50 cycles at 95 °C for 45 sec, 55 °C for 30 sec and 72 °C for 2 min and the last elongation step was done at 70 °C for 7 min. 5 µl aliquot of the PCR reaction was analyzed by horizontal gel electrophoresis to verify the success of PCR reaction.

PCR products were purified using the UltraClean® PCR Clean-up kit (MoBio, Carlsbad, California, USA) according to manufacturers´ instructions.

The gene fragments, amplified by PCR, were separated by cloning. For this, the DNA fragments were ligated in plasmids and transformed in competent *E.coli* cells. Cloning was performed with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) using chemically competent *E. coli* - cells (One Shot TOP 10F´chemically competent *E.coli*, Invitrogen, Carlsbad, CA, USA) according to manufacturers´ instructions. After the cloning reaction, the obtained cell suspension was spread on selective plates (LB plates containing kanamycin (50 µg mL^{-1}; Sigma-Aldrich, Steinkirchen, Germany) and X-Gal (40 mg mL^{-1}; Fermentas, St. Leon-Rot, Germany), prepared as noted in the cloning manual). On the next day, 25 insert-positive clones were selected by blue-white color screening and proliferated in 3 ml LB-media containing kanamycin (50 µg mL^{-1}).

Plasmid DNA was isolated by using the UltraClean® 6 Minute Mini Plasmid Prep Kit (MoBio, Carlsbad, California, USA).

16S-rDNA plasmid inserts were sequenced by the company Macrogen (Amsterdam, The Netherlands) using primer 27f (Lane, 1991).

2.5 Degradation capabilities of the IPU mineralizing microbial community in liquid culture

To investigate the degradation capabilities of IMMC, degradation respectively mineralization was examined in liquid cultures containing IPU, DD-IPU, MD-IPU or Diuron as the only source of C and N.

2.5.1 Degradation of IPU and its metabolites MD-IPU and DD-IPU by IMMC

2.5.1.1 Mineralization of ^{14}C-IPU and ^{14}C-MD-IPU

To measure the mineralization of ^{14}C-IPU and ^{14}C-MD-IPU, liquid cultures were prepared containing the respective compound as the only source of C and N.

Liquid culture studies were conducted in the test systems shown in Figure 3.

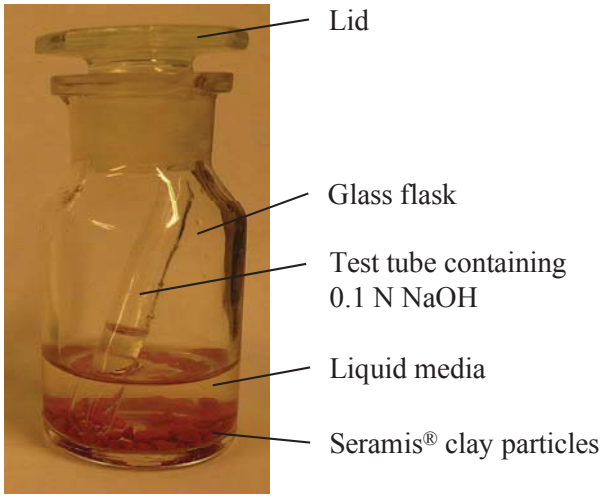


Figure 3: Liquid culture test system used for degradation tests with IPU, MD-IPU and DD-IPU.

Liquid cultures were prepared according to the following procedure:

(1) ^{14}C- and unlabeled standard, both dissolved in acetonitrile (ROTISOLV HPLC, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), were added separately to sterilized flasks of 100 mL volume. In case of replicates with IPU, 100 µl ^{14}C-IPU-standard I9 and 124 µl unlabeled IPU (10 mg mL^{-1}) (chapter 2.1.1) were added, resulting in a final IPU-concentration of 25 mg L^{-1} for the liquid culture. To replicates with MD-IPU, 250 µl ^{14}C-MD-IPU-standard 1 as well as 116.50 µl unlabeled MD-IPU (10 mg mL^{-1}) (chapter 2.1.1) were applied, resulting in a final concentration of 23.30 mg L^{-1}, which corresponds to the same molarity as 25 mg L^{-1} IPU (121.19 µM). Four replicates were done per variant.

(2) After evaporation of the solvent, 50 ml MS-medium (chapter 2.3.1) was added per flask. Subsequently, flasks were sealed with airtight stoppers and placed on an orbital shaker at 100 rpm (IKA Labortechnik KS 250 Basic Orbital Shaker, Staufen, Germany) in the dark at +20 ± 1 °C to assure the complete and homogeneous dilution of the added compounds in the liquid medium.

(3) On the next day, an aliquot of 100 µl was taken from each liquid culture and the exact amount of applied ^{14}C per liquid culture was determined by liquid scintillation counting (chapter 2.4.1.2).
(4) Sterile test tubes containing 2 mL of autoclaved 0.1 N NaOH (Merck, Darmstadt, Germany) were added to all replicates to trap evolved $^{14}CO_2$.
(5) Seramis® clay particles overgrown by IMMC (chapter 2.2.2) were removed by a sterile spoon from an ongoing liquid culture, containing ^{14}C-IPU as the only source of C and N and a mineralization rate just decreased below 2 % day^{-1}. To determine the number of living microbes attached to Seramis® clay particles, two particles were crushed and utilized for preparing dilution series as described in chapter 2.4.1. Four dilution series were done. 100 µl of dilutions were spread on LB plates (2.4.1.1) and subsequently stored at +20 ± 1 °C in the dark.
(6) Subsequently, more Seramis® clay particles were removed from the liquid culture that was used previously for determination of CFUs. Particles were placed on a sterile petri-dish (Greiner-Bio-One GmbH, Frickenhausen, Germany), divided into heaps of 40 particles each and immediately after partitioning added by the spoon to the test systems in order to avoid a drying-out of the particles and thus a damage of the microbial community.

The NaOH was sampled three times per week (Mondays, Wednesdays and Fridays) and measured for ^{14}C by liquid scintillation counting (chapter 2.4.1.2).

2.5.1.2 Cell dynamic of IMMC during mineralization of ^{14}C-IPU

To explore to what degree IPU mineralization was associated with a proliferation of IMMC, liquid cultures were prepared and the numbers of living cells of IMMC determined regularly.

Preparation of liquid cultures

Liquid cultures were prepared in the test systems shown in Figure 4 (objects 1-3), according to the following procedure:

(1) ^{14}C-IPU application standard 1 (specific radioactivity 5.06 kBq mg^{-1}) was applied to a sterile 100 mL-Erlenmeyer flasks (neoLab, Heidelberg, Germany) to a final concentration of 25 mg L^{-1}. Four replicates were done. After evaporation of the solvent, 25 mL of MS-medium (chapter 2.3.1) was added and the flasks placed on an orbital shaker (Model: 3005; GFL, Burgwedel, Germany) at +20 ± 1 °C to achieve a complete solution of the compound in the liquid.
(2) After shaking for 24 h, a 100 µl aliquot of the liquid was removed from each replicate in order to determine the amount of applied radioactivity by liquid scintillation counting (see below).

(3) Seramis® clay particles colonized with IMMC were removed from ongoing liquid cultures (chapter 2.2.2) and the number of living cells determined using LB plates as described in chapter 2.4.1.

(4) Again, Seramis® clay particles were withdrawn from the liquid culture and each newly prepared liquid culture was inoculated with 20 Seramis® clay particles according to chapter 2.5.1.1.

(5) After inoculation with IMMC, flasks were sealed with an upper part that was splitting in two tubes. To avoid the contamination of liquid cultures with external microorganisms, a filter unit (0.22µm pore size; MilliPore, Carrigtwohill, Ireland) was fixed to one opened side of the tube while on the other side a venting filter was connected (0.2µm pore diameter; Model: Midisart® 2000; Sartorious Stedim Biotech GmbH, Göttingen, Germany). The venting filter allows the attachment of the upper part to the laboratory system used for aeration of liquid cultures (see below). Further, to prevent the loss of air and thus $^{14}CO_2$ out of the flask, the two tubes were branched off by clamps between aeration periods.

(6) The number of living cells was determined regularly during IPU mineralization. At each sampling, two Seramis® clay particles were removed from liquid culture by a sterile spoon, dilution series were prepared and aliquots of dilutions spread on plate according to chapter 2.4.1. Dilutions were spread on LB plates. To avoid a loss of $^{14}CO_2$ by opening the test system for a removal of Seramis® clay particles, particles were in each case withdrawn immediately after sampling of $^{14}CO_2$. As for determination of living cells Seramis® clay particles were removed from liquid culture, the relation of biomass and culture media would have changed during the experiment and falsified mineralization measurements. To keep the relation of microbial cells and liquid constant during the experiment, each time when sampling Seramis® clay particles for cells counts also a percental equal aliquot of liquid was removed from the liquid culture. The measured $^{14}CO_2$ was then extrapolated to 25 mL liquid medium.

Sampling of $^{14}CO_2$

To measure the evolved $^{14}CO_2$, liquid cultures were attached to a laboratory system according to Lehr et al. (1996a) (Figure 4). Three times a week (Mondays, Wednesdays and Fridays), air was soaked through the trapping system to exchange the air within the flasks and measure mineralization of ^{14}C-IPU. Air was soaked through the incubation system for one hour with a flow rate of 1.0 L h^{-1}. This air flow assures a sufficient supply of the incubators with O_2. The air sucked off from the incubators was directed through one trap. The trap was filled with 10 ml 0.1 N NaOH (Merck, Darmstadt, Germany) in order to fix the released $^{14}CO_2$. After aeration, the 0.1 N NaOH from the trap was drained off, collected in a scintillation vial made auf polyethylene (PerkinElmer,

Shelton, USA) and the removed volume of liquid was determined gravimetrically. An aliquot of NaOH was measured for the amount of ^{14}C by liquid scintillation counting (below).

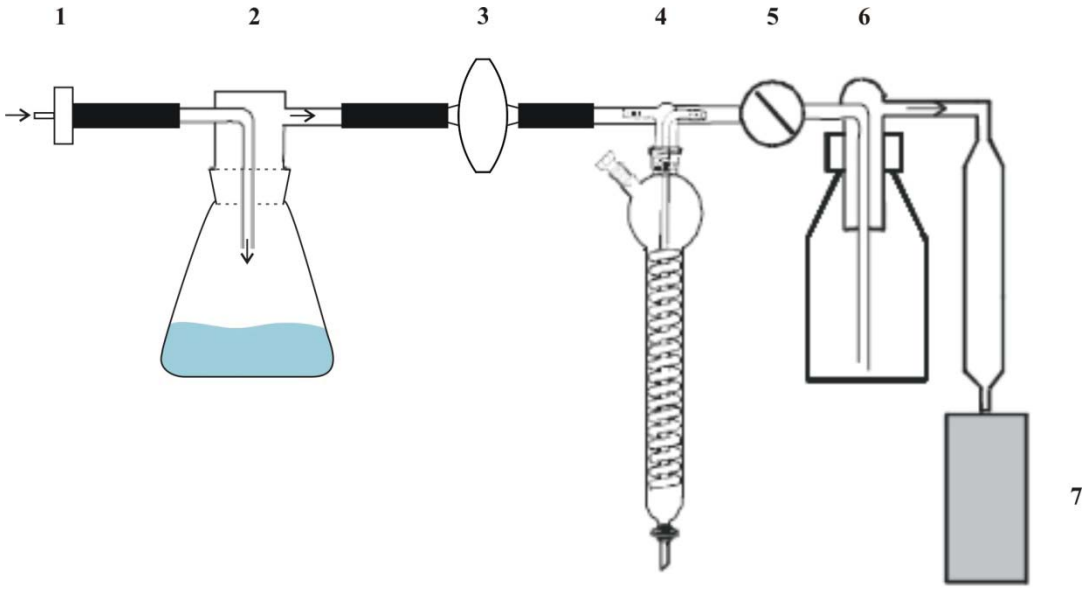


Figure 4: Laboratory system used for aeration of liquid cultures modified after Lehr et al. (1996a).
1: Filter unit, 2: Erlenmeyer flask containing liquid media, 3: Venting filter, 4: Trapping system for CO_2 , 5: Fine regulation valve, 6: Wulff bottle, 7: pump, → direction of air flow during aeration of samples.

The biodegradation systems were operated in a room with a constant temperature of +20 ± 1 °C. For storage between periods of aeration, the liquid cultures were placed also at +20 ± 1 °C on an orbital shaker (Model: 3005; GFL, Burgwedel, Germany) in order to improve the microbes´ supply with oxygen by agitation of liquid.

Quantification of mineralization by liquid scintillation scounting

Mineralization of ^{14}C-IPU was quantified by liquid scintillation counting. After aeration of liquid cultures, the NaOH containing ^{14}C was collected in scintillation vials made of polyethylene (PerkinElmer, Shelton, USA). The vials were weighed before and after sampling to get the exact volume of the liquid.

For measurement of evolved $^{14}CO_2$, an aliquot of 2 mL was mixed with 3 mL of scintillation cocktail Ultima Flo AF (PerkinElmer, Überlingen, Germany). The radioactivity in this aliquot was then referred to the total sample volume. For liquid scintillation counting, two liquid scintillation analyzers with identical characteristics were used: a Packard Tri-Carb 1900TR (Canberra-Packard GmbH, Dreieich, Germany) and a TriCarb 2800 TR (PerkinElmer, Überlingen, Germany). Customized quench curves were applied in order to correct results for quenching. Each sample was measured 3 times for 5 min. For data analysis, mean values were used.

To quantify the amount of ^{14}C actually added to a liquid culture, 100 µl liquid of each liquid culture was removed by a pipette prior to the application of microbial cells. Then, this aliquot was mixed

with 5 ml Ultima Gold XR (Packard, Dreieich, Germany) and measured for contained radioactivity by liquid scintillation counting. The ^{14}C was then extrapolated to the total volume of the liquid culture.

2.5.1.3 Degradation of IPU, MD-IPU and DD-IPU and the formation of metabolites

Setup of liquid cultures and sampling

To examine the formation and release of metabolites, analogue to ^{14}C-liquid cultures (chapter 2.5.1.1) liquid cultures containing just non-labeled IPU, MD-IPU and DD-IPU (chapter 2.1.1) were arranged in order to avoid a contamination of the measuring system with ^{14}C (see below). Either 25 mg L^{-1} IPU, 23.30 mg L^{-1} MD-IPU or 21.60 mg L^{-1} DD-IPU was added per flask, which corresponds to the same molarity as 25 mg L^{-1} IPU (121.19 µM). Four replicates were done per variant, each with 50 mL of MS-medium (chapter 2.3.1). To avoid a surplus of CO_2 in the air as well as its dissolution in the medium, NaOH in all replicates containing unlabeled compounds was replaced in the same mode as in replicates with labeled compounds. Two to three times per week aliquots of 1.5 mL were removed from the liquid media for measurements with **U**ltra **P**erformance **L**iquid **C**hromatography (UPLC). After sampling, the liquid was filtered (VectaSpin Micro, Anopore, 0.2 µm, Whatman, Maidstone, England) to remove microbial cells and fragments of Seramis® clay particles which were rubbed off during the experiment. Filtered samples were frozen at -20 °C until analysis.

All liquid cultures had been inoculated with 40 particles of Seramis® clay particles grown over by IMMC. Using aliquots of Seramis® clay particles, the number of applied living cells was determined immediately before inoculation, by preparing dilution series and spreading dilutions on LB plates (chapters 2.4.1 and 2.4.1.1 (plates)). Four dilution series were done. As for sampling for UPLC concentration measurements liquid was removed, the relation of microbial cells and culture media would have changed during the experiment and falsified UPLC concentration measurements if only liquid would have been removed. To keep the relation of microbial cells and liquid constant during the experiment, each time when sampling for UPLC also a percental equal aliquot of Seramis® clay particles and thus microbial cells was removed from the liquid culture. To keep the fraction of total sampled liquid respectively microbial cells for the experiment below 50 % of the initial amount, 50 mL MS-medium respectively 40 particles of overgrown clay particles were used.

To test if abiotic degradation of IPU, MD-IPU or DD-IPU might take place, control replicates were included in the experiment. Controls were prepared the same way as replicates inoculated with IMMC, but equipped with autoclaved clay particles instead of those overgrown with IMMC.

Concentration measurements

For identification and quantification of IPU and its metabolites, the aliquots taken from liquid cultures were measured by UPLC. The UPLC system consisted of the components shown in Table 5.

Table 5: Components of the UPLC system used for detection of IPU and its metabolites.

UV detector	PDA detector (Waters Corporation, Milford, MA, USA)
Pump	Binary Solvent Manager (Waters Corporation, Milford, MA, USA)
Autosampler	Sample Manager (Waters Corporation, Milford, MA, USA); samples were stored at 5 °C
Column	Acquity UPLC BEH C181,7 µm (2.1 x 100 mm); temperature column: 43 °C
Mobile phase	Acetonitrile (HPLC-grade, Merck, Darmstadt, Germany) and MilliQ water, both vented
Flow	0.4 mL min^{-1}

For quantification, calibrations for all compounds were done and peaks quantified by QuanLynx quantification software. Peaks were identified by retention time of the reference substance. Spectra were analyzed by software MassLynx (both software packages: Waters Corporation, Manchester, UK). The gradient program applied for analysis is noted in Table 6. Concentrations above 0.005 mg L^{-1} per compound could be detected and quantified.

Table 6: UPLC gradient program for detection of IPU and its metabolites.

	Time	Flow	% acetonitril	% water
1	0	0.4	40.0	60.0
2	2.50	0.4	45.0	55.0
3	2.60	0.4	40.0	60.0

2.5.2 Mineralization of ^{14}C-Diuron by IMMC

To investigate to what extent the IPU mineralizing microbial community was able to mineralize other phenylurea herbicides than IPU, a liquid culture experiment with ^{14}C-Diuron was done.

Diuron as a widely used herbicide is utilized for agricultural crops as well as for urban and industrial sites (Lapworth and Gooddy, 2006; Bazot et al., 2007). As it is well soluble in water and moderately adsorbed to soil particles, it was found to contaminate soils, sediments and water (Bazot et al., 2007; Sørensen et al., 2008). Metabolites have been reported to be more toxic to non target organisms than Diuron itself (Giacomazzi and Cochet, 2004). Therefore, this chemical was

included on the European Commission's list of priority substances for European freshwater resources (European Commission, 2008). As in case of IPU, microbial degradation was reported to be the main mechanism for Diuron degradation in soil and water (Giacomazzi and Cochet, 2004; Sørensen et al., 2008).

High Diuron concentrations were reported to have toxic effects on soil bacteria (Tixier et al., 2002; Sørensen et al., 2008) and fungi (Tixier et al., 2000), while there are also notes on soil bacteria capable to degrade high concentrations of Diuron: a soil slurry able to mineralize Diuron was shown to degrade Diuron concentrations of 20 mg L^{-1} (Cullington and Walker, 1999) while a bacterial strain capable to mineralize IPU was reported to degrade Diuron concentrations of 25 mg L^{-1} (Sørensen et al., 2001).

2.5.2.1 Pre-test: Estimate basic sensitivity of IMMC to high Diuron-concentrations

To examine the sensitivity of IMMC to a high Diuron concentration, as a first step a pretest with unlabeled Diuron was performed in liquid culture.

Liquid cultures were prepared in the test system shown in Figure 4 (objects 1 -3; chapter 2.5.1.2), according to the following procedure:

(1) Unlabeled Diuron (Dr. Ehrenstorfer Augsburg, Germany), dissolved in MeOH (LiChrosolv, gradient grade for liquid chromatography, Merck, Darmstadt, Germany), was applied to sterile Erlenmeyer flasks (neoLab, Heidelberg, Germany) to a final concentration of 25 mg L^{-1}. After evaporation of the solvent, 50 mL MS-medium (chapter 2.3.1) was applied and flasks were placed on an orbital shaker (Model: 3005; GFL, Burgwedel, Germany) at +20 ± 1 °C to achieve a complete solution of the compound in the liquid medium. After shaking for 24 h, a 500 µl aliquot of the liquid was removed from each replicate and frozen at – 20 °C until analysis with HPLC for determination of the exact Diuron concentration applied.

(2) Then, 40 particles overgrown by IMMC were applied to each liquid culture as described in chapter 2.5.1.1. The number of applied microbial cells was determined by preparing dilution series and spreading aliquots on LB plates (chapters 2.4.1 & 2.4.1.1).

Cell numbers were also examined 10, 20, 30 and 39 days after inoculation. Each time when sampling Seramis® clay particles for cells counts, a percental equal aliquot of liquid media was removed in order to maintain the relation of microbial cells and liquid constant during the experiment (chapter 2.5.1.2).

Two times a week (Mondays and Fridays), 500 µl liquid was removed from each replicate, filtered (VectaSpin Micro, Anopore, 0.2µm, Whatman, Maidstone, England) to remove microbial cells and parts of crushed clay particles and frozen at – 20 °C until analysis with HPLC. To keep the relation

of microbial cells and applied pesticide constant, at each sampling clay particles and thus microbial cells were removed from the replicates as described in chapter 2.5.1.3.

The components of the HPLC system are displayed in Table 7. Filtered samples were measured isocratically with 50 % water (Lichrosolv water for chromatography, Merck, Darmstadt, Germany) and 50 % acetonitrile (HPLC-grade, Merck Darmstadt, Germany). Absorption of Diuron was detected at 240 nm and at a retention time of 6.48 ± 0.1 min.

Peaks were identified by retention time of a reference standard. Quantification of Diuron was done by executing a calibration for the compound. The level of detection (LOD) and the level of quantification (LOQ) were determined according to Frehse and Thier (1991) to be at 0.52 (LOD) and 0.78 mg L^{-1} (LOQ). From each sample, 20 µl were injected to HPLC by autosampler. The acquisition and evaluation of the measuring signal was carried out with the software D-7000 HSM (Merck Hitachi, Darmstadt, Germany; version 4.1).

Table 7: Components of the HPLC system used for detection of Diuron in liquid culture.

UV detector	UV Detector D-7000 (Merck Hitachi, Darmstadt, Germany)
Radiodetector	HPLC Radioactovity Monitor LB 507 B (Berthold, Bad Wildbach, Germany)
Pump	Pump L-7100 (Merck Hitachi, Darmstadt, Germany)
Autosampler	Autosampler L-7200 (Merck Hitachi, Darmstadt, Germany)
Pre-column	LiChrospher 100 RP-18 (5 µm, 250 × 4 mm; Merck, Darmstadt, Germany)
Column	LiChrospher 100 RP-18 column (5 µm, 250 × 4 mm; Merck, Darmstadt, Germany)
Mobile phase	Acetonitrile (HPLC-grade, Merck, Darmstadt, Germany) and water (Lichrosolv water for chromatography, Merck, Darmstadt, Germany); both vented
Flow	1.0 mL min^{-1}

2.5.2.2 Mineralization of ^{14}C- Diuron by IMMC

As shown by the pretest, IMMC was not able to degrade Diuron at a high concentration level of 25 mg L^{-1} within 51 days (chapter 3.2.3.1). Thus, only 1 mg L^{-1} ^{14}C-Diuron was applied in the successive liquid culture experiment to avoid possible toxic effects on microbes. To investigate if IMMC was able to catabolize Diuron completely, i.e. including the cleavage of the ring structure, ^{14}C-Diuron was added ring- and side chain-labeled, respectively.

Liquid cultures were prepared in the test systems shown in Figure 3. Two variants of liquid cultures were prepared according to the procedure in chapter 2.5.1.1, containing (1) ring labeled ^{14}C-Diuron (1 mg L^{-1}; chapter 2.1.2) or (2) side chain labeled ^{14}C-Diuron (each 1 mg L^{-1}; chapter 2.1.2). Both variants were done in triplicates. Each replicate contained 25 mL MS-medium (chapter 2.3.1) and was inoculated with 20 Seramis® clay particles overgrown by IMMC. The number of applied

microbial cells was determined (chapter 2.4.1). NaOH was sampled three times per week (Mondays, Wednesdays and Fridays) and measured for trapped ^{14}C by liquid scintillation counting (chapter 2.4.1.2).

2.6 Degradation of IPU and its metabolites MD-IPU and DD-IPU by IMBS in liquid culture

2.6.1 Setup of liquid cultures

To examine the degradation capabilities of the IPU mineralizing strain for IPU, MD-IPU and DD-IPU as well as the formation of metabolites during degradation, a liquid culture experiment was run.

Liquid cultures were prepared in the test systems shown in Figure 4 (objects 1-3; chapter 2.5.1.2) according to the following procedure:

(1) Unlabeled IPU, MD-IPU or DD-IPU (chapter 2.1.1), all solved in methanol, was applied to sterile 100 mL-Erlenmeyer flasks (neoLab, Heidelberg, Germany). The compounds were added to a final concentration of 25, 23.3 and 21.6 mg L^{-1} for IPU, MD-IPU and DD-IPU, respectively, which corresponds to a concentration of 121.2 µM for each compound. These variants were set up for detection of metabolites that may be formed during degradation of the applied compound. Additionally, one variant received ^{14}C-IPU application standard 2 (specific radioactivity 10.04 MBq mg^{-1}; chapter 2.1.1) in order to estimate the extent of mineralization in the course of the experiment as those measurements were less time-consuming than concentration measurements. ^{14}C-IPU was applied to a final concentration of 25 mg L^{-1}. Eight replicates were done per variant.

(2) After evaporation of the solvent, 20 mL liquid media was added to each replicate: for each compound, four replicates were run with 20 mL MS-medium (chapter 2.3.1) while further four replicates were additionally supplemented with 0.1 g L^{-1} amino acid mix (chapter 2.3.1) as the presence of amino acids was indicated to play an important role during the isolation of *Sphingomonas* sp. AK1 (chapter 3.1.2.2). Then, flasks were placed on an orbital shaker for 24 h (Model: 3005; GFL, Burgwedel, Germany) at +20 ± 1 °C to achieve a complete solution of the compound in the liquid.

(3) Then, a 100 µl aliquot of the liquid was removed from the replicates containing ^{14}C-IPU in order to determine the amount of applied radioactivity (chapter 2.5.1.2). From replicates with unlabeled compounds, 300 µl of liquid were removed for concentration measurement and frozen at -20 ± 1 °C until analysis by HPLC (see below).

(4) For inoculation of flasks, several colonies of the IPU mineralizing strain *Sphingomonas* sp. AK1 were detached by a sterile inoculation loop from R2A plates (chapter 2.4.1.1) and the cells were mixed with 1x PBS (chapter 2.3.2) (= inoculation solution). A washing of cells

in 1xPBS in order to remove e.g. residues of agar prior to inoculation was not done as it was shown by a pre-test (for procedure see chapter 2.6.1.1) that centrifugation of a liquid that contains cells of *Sphingomonas* sp. AK1 results only in a fragmentary pelletizing of cells while the majority of cells get lost (for results see chapter A1.2, Appendix). 800 µl of the inoculation solution was applied per replicate. To determine the number of living cells applied per liquid culture, an aliquot of 100 µl was removed from the inoculation solution and diluted decimally with 1x PBS buffer (chapter 2.3.2), i.e. each time 100 µl of the concentrated sample was mixed homogeneously with 900 µl 1 x PBS buffer. Before each removal of a 100 µl aliquot, the content of tubes was mixed on a shaker (MS2 Minishaker, IKA, Staufen, Germany). Subsequently, 100 µl of several dilution grades was spread by a drygalski spatula on R2A agar plates. Several dilution series were done. For incubation, plates were stored at + 20 ± 1 °C in the dark and monitored regularly for growth of CFUs.

After inoculation with cells of *Sphingomonas* sp. AK1, flasks were sealed and placed on an orbital shaker.

Control

To assess if stimulation of microbial growth in replicates containing amino acids was induced by the amino acids themselves or by IPU or a metabolite, control replicates were introduced. Control replicates contained 20 mL of MS-medium supplemented with 0.1 g L^{-1} amino acid mix (chapter 2.3.1), but no IPU or one of its metabolites was provided as additional source of C and N. To monitor microbial growth, aliquots were taken for determination of CFUs one to two times a week.

2.6.1.1 Pre-test for the inoculation procedure: pelletizing of cells of *Sphingomonas* sp. AK1

To test if cells of *Sphingomonas* sp. AK1 may be washed with 1xPBS buffer prior to inoculation in order to remove e.g. residues of agar or liquid medium, a pre-test was performed. By means of this pre-test, it was measured to what extent the microbial cells floating in the medium were generating a pellet by centrifugation respectively the amount of cells lost by centrifugation and washing of cells with buffer.

It was preceded as follows:

(1) Partitioning of samples: 55 mL of distilled water was provided in an Erlenmeyer flask (neoLab, Heidelberg, Germany) and inoculated with microbial cells of *Sphingomonas* sp. AK1, grown on R2A plates (chapter 2.4.1.1) and detached with an inoculation loop. Subsequently, the mixed and slightly turbid solution (= solution A), showing no flocculation as liquid cultures inoculated with *Sphingomonas* sp. AK1 of Phase (A), was divided into 4 replicates of 12.5 mL each. Each replicate (= samples 1-4) was transferred to

a tube (Polypropylene conical tube, 50 mL, BD Biosciences, Erembodegem, Belgium). The residual volume of solution A was kept.

(2) Centrifugation: Samples 1-4 were centrifuged within the tubes for 4 min at 4000 rpm (centrifuge: Sorvall RC 6+, rotor: Sorvall 11788 PN; both ThermoScientific, Braunschweig, Germany) and the supernatant carefully soaked off with a pipette and discarded.

(3) Washing: The cells were washed with buffer in order to get rid of possible traces of ^{14}C-IPU of phase (A) in case of the main experiment. For that purpose, 3 mL of 1xPBS buffer (chapter 2.3.2) was added to each tube and the content of tubes mixed by a shaker (MS2 Minishaker, IKA, Staufen, Germany). Then, the mixture was again centrifuged as described in step (2) and the supernatant discarded. This washing procedure was performed two times. After both washing steps, 1 mL of 1xPBS was applied to all tubes of samples 1-4 and mixed by the shaker.

(4) Dilution series were conducted with dilutions -1 to -7 as described in chapter 2.6.1(4) for samples 1-4, giving the number of cells after the centrifugation and washing procedure, as well as for solution A, which represents the initial number of cells without any washing treatment.

(5) Preparations for cell counting: For solution A and samples 1-4, 10 µl of undiluted solution and 10 µl of all serial dilutions, respectively, were pipetted on wells of a glass slide (each well 6 mm in diameter, 8 wells per glass slide) (ThermoScientific, Braunschweig, Germany). Then, glass slides were transferred to a drying oven (+45 ± 1 °C) (Memmert, Schwabach, Germany). After evaporation of the liquid, cells in all wells were covered by Citifluor (Citifluor Ltd., London, UK) and the glass slides were supplied with a cover glass (ThermoScientific, Braunschweig, Germany).

(6) Enumeration of cells: The number of cells was determined using a Zeiss Axio-plan II epifluorescence microscope equipped with a 100x object lens (Zeiss, Oberkochen, Germany). The object lens was supported with a counting ocular, in which a grid was located (edge length 0.126 mm when using the 100x ocular). For counting of cells of solution A and samples 1-4, undiluted samples were selected as the grid then showed a countable number of cells; further dilutions partly did not show any cells. Per undiluted sample, the number of cells in 10 grids was counted. The total cell number per sample was then calculated as:

Total cell number = Mean of counts per grid * Microscoping factor * Dilution factor

Where the included Microscoping factor (M_F) is calculated as:

$$M_F = \frac{\text{well area}}{\text{grid area}}$$

Where M_F = 1780.95 when using wells of 6 mm in diameter and a grid with edge length of 0.126 mm.

The total cell counts were extrapolated to 1 mL liquid.

2.6.2 Quantification of mineralization by liquid scintillation counting

In the variant with ^{14}C-IPU, the production of $^{14}CO_2$ was measured using the laboratory system described in 2.5.1.2 (Figure 4).

2.6.3 Concentrations measurements and detection of metabolites

For concentration measurements and metabolite detection in replicates with unlabeled compounds, aliquots (300 µl each) were removed regularly from liquid cultures for analysis by HPLC. After sampling, aliquots were filtered (VectaSpin Micro, Anopore, 0.2 µm, Whatman, Maidstone, England) to eliminate biomass and subsequently stored at -20 ± 1 °C until analysis.

Concentration measurements were performed by a HPLC system. Its components are shown in Table 7 (chapter 2.5.2.1). Peaks were identified by retention time of reference substances. For identification of IPU, MD-IPU, DD-IPU and 4IA, the standard mix described in chapter 2.1.1 was used as a reference. The quantification of these substances was done by executing a calibration for each compound. 20 µl of each sample was injected to HPLC by autosampler. The acquisition and evaluation of the measuring signal was done out with the software D-7000 HSM (Merck Hitachi, Darmstadt, Germany; version 4.1). A gradient program as shown in Table 8 was run.

Table 8: HPLC gradient program for detection of IPU and its metabolites.

time (min)	acetonitrile (%)	water (%)
0.0	5	95
15.0	60	40
20.0	60	40
25.0	5	95
35.0	5	95

The level of detection respectively the level of quantification for compounds were determined according to Frehse and Thier (1991) and are shown in Table 9.

Table 9: LOD and LOQ for IPU and its metabolites at HPLC.

Compound	LOD (mg L^{-1})	LOQ (mg L^{-1})
IPU	2.22	3.62
MD-IPU	1.92	2.90
DD-IPU	0.75	1.12
4IA	1.64	2.47

2.6.4 Cell dynamic of IMBS during mineralization of ^{14}C-IPU

To determine to what extent the mineralization of IPU was linked with the reproduction of microbial cells by the IPU mineralizing strain, the number of living cells was determined. Three times a week, an aliquot of 100 µl was removed from each liquid culture and the number of living cells was determined as described in chapter 2.6.1(4).

2.7 Experiments with soils

2.7.1 Characteristics of soil materials

Four soils, representing typical but very different agricultural soils of Germany and Slovenia, were used for the experiments. The pedological characteristics, conditions governing location and the climatic data are given in Table 10.

Soil Feldkirchen derived from an agricultural farm in Feldkirchen which is located about 13 km from Munich centre. The soil was transferred in 1996 as soil monolith (4 replicates) to the lysimeter station of the Helmholtz Center Munich. In September 2009, soil material of the lysimeters was removed, handled and stored as described below for soil Kelheim. Several aspects of the IPU degradation history of this soil are essential for the conduction of soil experiments: Prior to summer 2003, soil material from the Ap-horizon was taken and ^{14}C-IPU applied once in the laboratory. According to the measured mineralization dynamics, this soil was described as mineralizing ^{14}C-IPU metabolically (Kühn, 2003). In September 2003, again soil material (top 0-5 cm) was taken from the same lysimeters and utilized for biodegradation experiments. This time, the specific soil function of IPU-mineralization was reduced drastically and the mineralization pattern showed a tendency for co-metabolic degradation after one application of ^{14}C-IPU. According to microcosm experiments, which simulated the harsh summer conditions of 2003, a change in the microbial community structure and therefore in specific soil functions of the community could be attributed to the extreme dry and hot weather conditions in summer 2003 (Levy et al., 2007). There are no records on the IPU application history before the soil was

transferred to the lysimeter station. While soil Feldkirchen was located at the lysimeter station, IPU was applied twice, in spring 1997 and spring 2001 (Grundmann et al., 2008).

Soil Scheyern was sampled in February 2010 on the agricultural research farm Scheyern (Field A02) which belongs to the Helmholtz Center Munich. Thus, it originates from an agricultural property of the Tertiary Hill Country about 50 km north of Munich. After sampling in the field, soil was air-dried until the water content was adequate for sieving. The sieved soil (≤ 2 mm) was homogenized and stored in sealed plastic bags at -20 °C until equilibration. No more IPU was applied on this field since 1991. For this soil, a co-metabolically degradation patter was observed after a single application of the pesticide (Folberth et al., 2009).

Soil Kelheim originated from an agricultural property about 100 km north from Munich located at the Donau river and was carried in 1996 as soil monolith (4 replicates) to the lysimeter station of the Helmholtz Center Munich. In September 2009, soil material from the top layer (5-10 cm) was removed, mixed and dried. The next day, soil material was sieved to ≤ 2 mm and stored for one week at +4 °C until it was frozen in sealed plastic bags at -20 ± 1 °C. Since its arrival at the lysimeter station, IPU was applied only once in 1997. Soil Kelheim was shown to degrade IPU co-metabolically after a single application of ^{14}C-IPU (Folberth et al., 2009).

Soil Konjišče originated from an agricultural farm of the Apace valley in the north-eastern part of Slovenia. It was sampled in 2007. The fresh soil material was sieved to ≤ 2 mm and stored in sealed plastic bags at +4 °C until it was frozen at -20 ± 1 °C. IPU was most likely spread on the field in 2003 and 2005. Before 2003, mainly maize was planted on this field. This soil was reported to mineralize IPU co-metabolically after one application of ^{14}C-IPU (Folberth et al., 2009).

The texture and composition of the soils from the lysimeter station as well as soil Scheyern were determined at the Bavarian main research station for agriculture (HVA) in Freising-Weihenstephan, Bavaria, Germany. Soil Konjišče was characterized at the institute Biotehniška at the University Ljubljana, Slovenia.

Table 10: Soil characteristics modified from Folberth (2008).

Natural site name	Feldkirchen	Kelheim	Scheyern (Field A02)	Konjišče
Soil type	Calcaric Regosol	Humic Cambisol	Mollic Gleysol	Fluvisol on Quaternary sediments
Soil characteristics				
Depth (cm)	0-10	0-10	0-15	0-20
Clay (%)	33	11	20	6
Silt (%)	34	19	40	60
Sand (%)	33	70	40	34
pH $CaCl_2$	7.2	6.9	7.8	6.9
TOC (%)	2.7	1.3	1.5	1.9
Total N (%)	0.3	0.1	0.2	0.2
$CaCO_3$ (%)	5.1	0.5	6.8	n.d.
Water content at -15 kPa (%)	32.80	17.34	29.90	38.90
Water content at filling level of 75 % of optimal water pool (%)	27.81	13.79	23.17	29.37
Geographical position				
Latitude (°)	48.150	48.917	48.493	46.710
Longitude (°)	11.733	11.867	11.432	15.821
Altitude (m)	521	348	497	230
Climate				
Annual precipitation (mm)	959	959	792	794
Temperature (average) (°C)	8	8	8.3	10.3

n.d. = not determined

2.7.2 Standard experimental conditions in soils

Soil experiments were conducted at a final water potential of -15 kPa and a soil density of 1.3 g cm^{-3}. These soil conditions were shown to be optimal for pesticide mineralization in soils at constant soil moisture conditions (Schroll et al., 2006) and thus regarded as comparable conditions for degradation experiments concerning soil water potential.

2.7.2.1 Determination of water content at a water potential of -15 kPa

The water content for soils corresponding to the water potential of -15 kPa was determined according to the following procedure: 13 g soil (dry weight) was stepwise pressed in small metal rings of 10 cm^{-3} volume to achieve a soil density of 1.3 g cm^{-3} (dry weight). Each sample was done in quadruplicates and placed on a cellulose filter with 0.45 µm pore size and 45 mm in diameter (Filter Paper Circles, Whatman, Dassel, Germany). Subsequently, samples on filters were saturated with double distilled water and transferred to a Sand/Kaolin Box (Model 08.02; Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands). The box itself was filled with very fine synthetic sand which was covered by a layer of kaolin clay. To avoid the pollution of the kaolin layer by the inserted samples, the surface of the kaolin was covered with one layer of filter cloth as recommended by the manufacturer. A pump created suction in a vacuum vessel and the under pressure was conveyed to the samples through the sand and kaolin layer. A lid prevented evaporation. Additionally, four filters were saturated and also introduced into the box in order to determine the mass of the filter separated from the soil sample. The pump was set to a pressure of -15 kPa. Samples were checked regularly gravimetrically for constant weight. When constant weight was reached, the mass of all filters and samples was determined by weighing and the water content of soil at -15 kPa was calculated. The setup of samples in the Sand/Kaolin Box is shown in Figure 5.

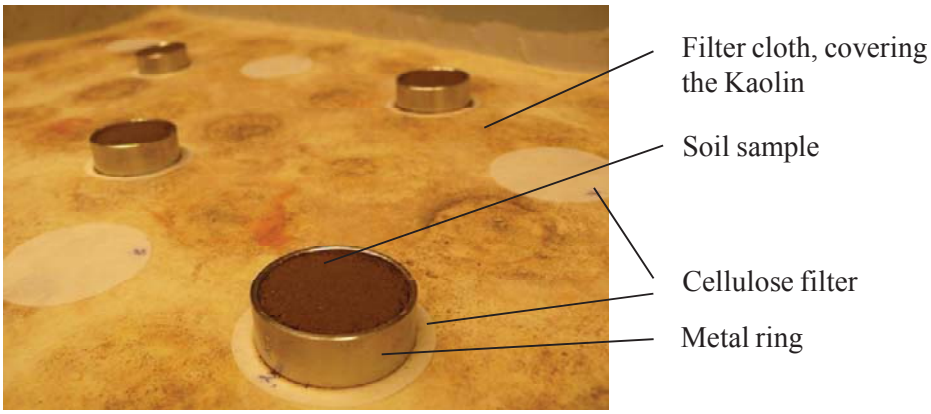


Figure 5: Compacted soil in metal rings in Sand/Kaolin Box.

2.7.2.2 Preparation of soils: Equilibration of soil

Soils for the respective experiments (≤ 2 mm) (chapter 2.7.; Table 10) were equilibrated prior to the start of the experiment in order to balance all biotic and abiotic processes and ensure comparable conditions with regard to soil water potential in the soil at the start of the experiment.

After sampling in the field, soil material was sieved and frozen at -20 ± 1 °C to avoid microbial activity and thus modifications in e.g. nutrient supply by time. If possible, soil was frozen in large amounts to ensure that always the identical soil material could be used for the different experiments.

For equilibration, the frozen soil was transferred to +4 ± 1 °C and stored for one week in a plastic bag, followed by another week of equilibration at room temperature (+20 ± 1 °C). When depositing the soil at +20 ± 1 °C, the soil was set to a water content corresponding to 75 % of the special water pool by adding distilled water. At this water content, the handling of the soil was better feasible than at a filling level of 100 % as no formation of clods was observed e.g. when mixing the dried aliquot for IPU application among the equilibrated soil. The filling degree of 75 % of the special water pool was calculated as follows: the water content corresponding to -20 MPa was subtracted from the water content corresponding to -15 kPa, the difference was then multiplied by 0.75 and the water content corresponding to -20 MPa was added. The water content for soils at -15 kPa and at a filling degree of 75 % of the optimal water pool (both at a soil density of 1.3 g cm^{-3}) are given in Table 10.

2.8 Sustainability of the enhanced IPU mineralization function induced by inoculation of IMMC into soil

Previous experiments within the working group have shown that mineralization of IPU in soils could be significantly enhanced by applying IMMC, established on Seramis® clay particles, as microbial hot spots into soil. This approach was shown to increase mineralization under laboratory and outdoor conditions (Grundmann et al., 2007). For these experiments, IPU mineralization was measured for a period of 46 days, starting with application of ^{14}C-IPU and IMMC to soil. To reduce effort and costs, it is desirable that inoculation is carried out as seldom as possible. The use of IPU is defined by the season of sowing (spring, autumn) and the growth of corn and pest plants. Hence, IPU is applied at most twice a year, in spring and autumn. Up to now, no data is available to what extent the increased metabolic IPU mineralization function, induced by inoculation of IMMC, will be preserved in soil when no more IPU was applied for several months, i.e. over periods realistic to a farmer's agricultural practice.

When specific microorganisms are introduced into soil for a certain purpose, a common problem is the fast decline of population size to a level no more sufficient to fulfil the desired goal (van Veen

et al., 1997). This observed decline in population size was mainly attributed to adverse abiotic and biotic environmental conditions (van Veen et al., 1997; Owsianiak et al., 2010).

Aim of the present experiment was to determine the sustainability of enhanced IPU mineralization, which was induced by inoculating IMMC attached on Seramis® clay particles, in soil.

For this study, soil Scheyern was selected as (1) the non-inoculated soil was reported to degrade IPU cometabolically after a single application of IPU (Grundmann et al., 2007; Folberth et al., 2009) and (2) an increase in IPU mineralization was shown when the soil was inoculated with IMMC attached to Seramis® clay particles (Grundmann et al., 2007). For soil characteristics see Table 10.

Variants A and B

Soil samples were set up (1) to identify and quantify ^{14}C-IPU residues extractable from soil by pore water and ASE (= variant A), allowing conclusions on the supply of microbes with IPU and its metabolites, and (2) to test how long the increased IPU mineralization function, which was induced by application of IMMC to soil, sustained in soil and could induce an increased mineralization of ^{14}C-IPU (= variant B). The set-up of variants A and B differed only by their specific radioactivity.

2.8.1 Overview on phases of the experiment

The experiment was split in two phases as described roughly in the following:

(1) In **phase 1**, ^{14}C-IPU and IMMC were applied to soil (variants A and B). Within the first 46 days, replicates of variant A were sampled regularly in order to determine the supply of microbes with IPU in soil.

(2) In **phase 2**, it was tested to which extent IPU mineralization was increased by applying ^{14}C-IPU again to replicates of phase 1, variant B. This application was executed after different periods of time: 1.5 (= reapplication 2a), 6 (= reapplication 2b) and 7.5 months (= reapplication 2c) after start of phase 1. Each time, ^{14}C-IPU was applied to four different replicates and each replicate received just one application in phase 2.

To investigate if inoculated microbes outlived more than one growing season and were still capable to mineralize ^{14}C-IPU, 7.5 months after application of ^{14}C-IPU a winter period was simulated in the laboratory by reducing temperature. ^{14}C-IPU was also applied a second time to those replicates (= reapplication 2d).

Additionally, soil of variant A was sampled at reapplications 2a-2d to analyze IPU residues at this particular time. Replicates of variant A received in total only one application of ^{14}C-IPU in phase 1 and no one in phase 2.

To examine indirectly if IMMC was able to survive on the carrier material itself for about one vegetation period, on day 322 of phase 1, Seramis® clay particles of four replicates

were separated from the surrounding soil and solely the carrier material was introduced to four replicates with newly equilibrated soil which was supplemented with ^{14}C-IPU.

In both phases, mineralization was measured and the amount of ^{14}C-IPU mineralized a well as the dynamics of mineralization used to evaluate if the enhanced mineralization, induced by inoculation of IMMC, was maintained for the respective period.

The detailed procedure for phases 1 and 2 is described in chapters 2.8.4 and 2.8.5, respectively.

2.8.2 The laboratory system

The laboratory systems used for incubation of soil is shown in Figure 6.

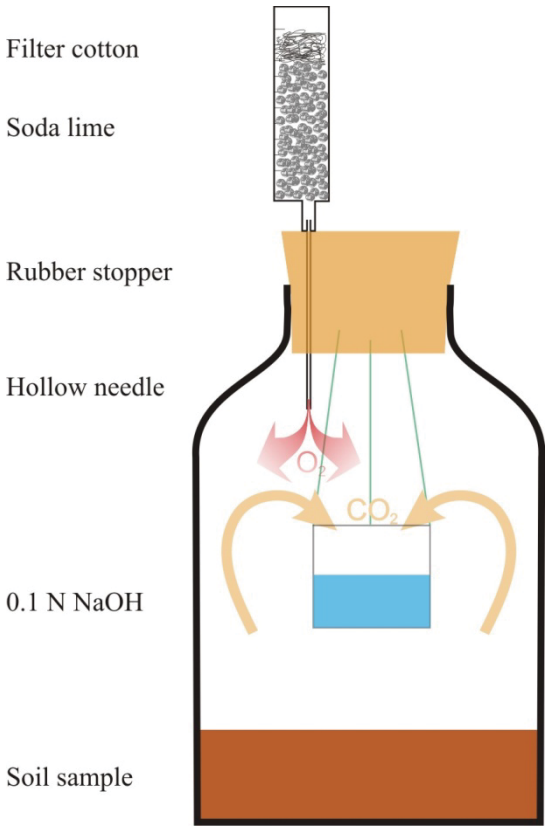


Figure 6: Laboratory system used for trapping of $^{14}CO_2$.

The laboratory system was build in approximation to the OECD guideline for testing of chemicals 304A (OECD, 1981): the opening of brown glass flasks (250 mL volume; neoLab, Heidelberg, Germany) at the top was closed by a rubber stopper (neoLab, Heidelberg, Germany) to which a small plastic beaker of 25 mL volume (VWR International, Darmstadt, Germany) was attached on the bottom side. The $^{14}CO_2$ resulting from mineralization of ^{14}C-IPU was trapped by 9 ml of 0.1 N NaOH (Merck, Darmstadt, Germany) which was filled into the beaker and replaced 3 times a week (Monday, Wednesday and Fridays). For exchange of the NaOH-solution, the upper part was removed and the solution sucked off with a pipette and collected in scintillation vials made of

polyethylene (PerkinElmer, Shelton, USA). Then, 9 mL of fresh NaOH solution was filled in the beaker. To allow a constant supply of soil microbes with O_2, a hollow needle (neoLab, Heidelberg, Germany) was conducted through the rubber stopper. To prevent saturation of the NaOH-solution with atmospheric CO_2 entering through the hollow needle and leading to an incomplete trapping of $^{14}CO_2$, a plastic syringe (neoLab, Heidelberg, Germany) was placed on the opening of the needle to the atmosphere. The syringe was filled with soda lime (Merck, Darmstadt, Germany) which was absorbing CO_2. To fix the soda lime particles within the syringe, filter cotton (Assistent, Sondheim, Germany) was inserted into the syringe.

2.8.3 Quantification of $^{14}CO_2$

After sampling of the NaOH-solution (chapter 2.8.2), its volume was determined gravimetrically. For quantification of the released $^{14}CO_2$, aliquots of 2 ml were mixed with 3 ml scintillation cocktail (Ultima Flo AF, Perkin Elmer, Inc., USA) and the amount of ^{14}C measured by liquid scintillation counting. For this, two liquid scintillation analyzers with identical characteristics were used (TRI-CARB 1900 TR, Canberra-Packard GmbH, Dreieich, Germany, and Tri-Carb 2800 TR, PerkinElmer, Überlingen, Germany, respectively). Results were corrected for quenching by using internally provided quench curves. All samples were measured 3 times for 5 minutes.

2.8.4 Phase 1: Application of ^{14}C-IPU and IMMC to soil

Soil Scheyern in the laboratory system was supplemented with ^{14}C-IPU and later inoculated with IMMC. Subsequently, mineralization in all replicates was measured until start of phase 2.

2.8.4.1 Procedure for application of ^{14}C-IPU to soil (variant B)

IPU was applied to soil Scheyern as presented in the following: per replicate, a total of 50 g soil (dry weight) was equilibrated before the start of the experiment according to chapter 2.7.2.2. Subsequently, an aliquot of 3.5 g (dry soil) out of it was dried at 105 ± 1 °C in a drier (Memmert, model: ULM 4000, Schwabach, Germany) and ground with mortar and ram. The ^{14}C-IPU pesticide application standard 5 (variant B: specific radioactivity of 84.48 kBq mg^{-1}) was then dripped on this aliquot by use of a Hamilton syringe (neoLab, Heidelberg, Germany) and intermixed for 1 min with a spatula to enable a homogeneous distribution of IPU within the soil material. Per 50 g of soil (dry weight), 250 µg IPU were applied, resulting in an herbicide concentration of 5 mg kg^{-1}. To avoid an impairment of native microorganisms by the MeOH in which the IPU was dissolved, the mixture of soil aliquot and application standard 5 was left for 30 min under the hood for evaporation of MeOH.

Subsequently, the dried soil aliquot (3.5 g) was mixed with the equilibrated soil material (46.5 g dry soil) for 2 minutes within the soil incubation flasks (Fig. 6 in chapter 2.8.2). Then, IMMC was introduced into soil as described in chapter 2.8.4.3, before the setup of incubators could

be completed. For this, soil material was compacted to a soil density of 1.3 g cm^{-3} with a spatula and distilled water was dropped carefully onto the compacted surface to achieve a final water potential of -15 kPa (chapter 2.7.2.1) in order to set optimum conditions for mineralization. Soil incubators were stored at 20 ± 1 °C in the dark.

In total, 24 soil incubators of variant B were prepared.

2.8.4.2 Modifications on ^{14}C-IPU-application for monitoring IPU residues in soil (variant A)

Parallel to the replicates of variant B which were dedicated to test the preservation of the IPU mineralization function by a second application of ^{14}C-IPU, additional 48 soil incubators of variant A were arranged to identify and quantify IPU residues in soil. Replicates of variants A and B differed solely in their specific radioactivity, the preparation and setting of incubators was apart from this identical. For replicates of variant A, the commercially available ^{14}C-IPU was no further diluted with unlabeled IPU. This application standard 6, having a higher specific radioactivity (10.04 MBq mg^{-1}) than the one applied to replicates of variant B, was added to better attain the detection limits of ^{14}C-IPU at the HPLC system even when a large portion of it was already mineralized. 250 µg of IPU were applied per 50 g of soil (dry weight), resulting in a final IPU concentration of 5 mg kg^{-1}.

According to the mineralization results by Kühn (2003) in soil Scheyern, which was at that time also inoculated with IMMC, mineralization of applied ^{14}C-IPU was mainly taking place within 46 days after application. To be able to cover this dynamics for the IPU residue situation in soil, during this period soil was sampled 1-2 times per week (8 samplings in total). Additionally, soil was sampled at days of reapplication 2a-2d. At each sampling point, 4 replicates were sampled. At sampling, soil in the respective incubators was mixed for 2 min by a spatula, the Seramis® clay particles were removed and the water content of soil determined gravimetrically in an soil aliquot. After determination of bioavailable IPU (chapter 2.8.6.1), the extracted soil as well as the residual non-extracted soil were stored at -20 ± 1 °C until further analysis for ^{14}C-IPU residues.

2.8.4.3 Inoculation of IMMC into soil

For inoculation of IMMC into soil, the Seramis® clay particles harbouring the IPU mineralizing microbial community were removed from liquid culture using a sterile spoon, transferred to a sterile petri-dish (Greiner-Bio-One GmbH, Frickenhausen, Germany) and divided into heaps of 20 middle-sized particles. To avoid a drying-out of the Seramis® clay particles and thus a possible impairment of attached microbial cells, the heaps were mixed for 2 minutes into the moist soil immediately after partitioning the Seramis® clay particles.

Parallel, the number of CFUs applied to soil was determined: Seramis® clay particles were removed from the same liquid culture from which the particles for inoculation into soil were

removed. Two particles were crushed and utilized to prepare one dilution series, then, 100 µl of each dilution were spread according to chapter 2.4.1 on LB plates (chapter 2.4.1.1). Four dilution series were done in total. Plates were stored at +20 ± 1 °C in the dark.

2.8.5 Phase 2: Reapplication of ^{14}C-IPU to test the sustainability of the enhanced IPU mineralization function

2.8.5.1 Reapplication periods 2a-2d

In phase 2, ^{14}C-IPU was applied a second time to 16 replicates of phase 1 (variant B) in order to control if microbes were still able to metabolize ^{14}C-IPU metabolically. IPU was applied to four replicates each time, after (a) 1.5 (= reapplication 2a), (b) 6 (= reapplication 2b) and (c) 7.5 months (= reapplication 2c). Each replicate received just one application of ^{14}C-IPU in phase 2.

To investigate if inoculated microbes outlived more than one growing season and were still capable to mineralize ^{14}C-IPU, (d) a winter period was simulated in the laboratory by reducing the temperature under which the laboratory systems were stored. For this, 7.5 months after the initial application of ^{14}C-IPU, the remaining four replicates were stored at +4 °C for one week to imitate decreasing temperatures of autumn and the onset of winter. Mineralization was still measured. Then, the incubators were transferred to -20 ± 1 °C. As microbial activity was reported to occur only as long as free water is available (Brooks et al., 1996), it was assumed that no microbial activity and thus no mineralization takes place at -20 °C. Therefore, the upper part of the laboratory system (Fig. 6 in chapter 2.8.2), which contained the NaOH-solution for trapping $^{14}CO_2$, was removed and the incubators were sealed with parafilm (Bemis, Neenah, WI, USA). To save time, the storage at -20 °C was limited to a period of two weeks. Thawing of soil in spring time was imitated by storing incubators for one week at +4 °C; to measure mineralization, the laboratory systems were again equipped with their upper parts. Subsequent to this, replicates were transferred to +20 ± 1 °C and ^{14}C-IPU was reapplied (= reapplication 2d).

Phases 2a, 2b, 2c and 2d lasted 24, 27, 27 and 21 days, respectively. During these periods, mineralization was measured.

2.8.5.2 Differentiation between $^{14}CO_2$ from applications 1 and 2

To be able to differentiate between the $^{14}CO_2$ originating from application 1 and the $^{14}CO_2$ deriving from application 2, four replicates of variant B did not receive an application of ^{14}C-IPU in phase 2. Nevertheless, mineralization of these replicates was further measured until replicates of reapplication 2d were stopped, and the amount subtracted from mineralization values in phase 2 in order to obtain mineralization values just from application 2.

2.8.5.3 Procedure for reapplication of ^{14}C-IPU to soil

^{14}C-IPU was reapplied to soil incubators according to the following procedure: the complete soil in the incubator was mixed by a spatula for 2 min. Subsequently, an aliquot corresponding to 3.5 g dry soil was withdrawn and dried overnight in a drier at +105 ± 1 °C (Memmert, model: ULM 4000, Schwabach, Germany). Parallel, the production of $^{14}CO_2$ was quantified by adding 9 ml of 0.1 N NaOH to the laboratory system (Fig. 6 in chapter 2.8.2). On the next day, the 0.1 N NaOH-solution was removed and the amount of ^{14}C determined by measuring an aliquot of 2 mL by liquid scintillation counting (chapter 2.8.3). Then, the dried soil aliquot was ground and mixed with ^{14}C-IPU-application standard 5 (specific radioactivity: 84.5 Bq μg^{-1}), which was already utilized for variant B of application 1, with an herbicide concentration of 5 mg kg^{-1} as described in detail in chapter 2.8.4.1.

2.8.5.4 Transfer of Seramis® clay particles to newly equilibrated soil

To investigate indirectly if inoculated microbes survived on the Seramis® clay particles for the duration of about one vegetation period and were still able to mineralize IPU efficiently, on day 322 of phase 1 Seramis® clay particles of four replicates (variant B) were separated from surrounding soil material and transferred to newly prepared soil incubators. This was done by the following procedure:

First, equilibrated soil (50 g dry soil) was supplemented with ^{14}C-IPU (specific radioactivity of 40.3 Bq μg^{-1}; 5 mg kg^{-1}) as described in chapter 2.8.4.1. Four replicates were prepared. Secondly, soil of four incubators (variant B, phase 1) was mixed homogeneously by a spatula and all Seramis® clay particles of a replicate removed carefully by a spatula and intermixed with one replicate of the newly prepared incubators. To avoid drying of the Seramis® clay particles and thus an impairment of the microbes attached, the carrier material was immediately homogeneously mixed with the soil of newly prepared replicates when removed from soil of variant B. Subsequently, soil of new incubators was compacted to a density of 1.3 g cm^{-3} and adjusted to a final water potential of -15 kPa (chapter 2.7). Soil incubators were stored at 20 ± 1 °C in the dark. $^{14}CO_2$ was collected by the laboratory system described in chapter 2.8.2 for a period of 51 days until mineralization decreased to a mineralization rate of < 1 $\mu g\ d^{-1}$.

2.8.6 Analysis of IPU in soil

The type and quantity of ^{14}C-IPU residues present in soil gives information about the current supply of soil microbes with IPU at time of sampling and also about the amount which might subsequently be delivered by desorption from the MeOH-extractable fraction to pore water. Residues of chemicals not extractable by organic solvents (in case of IPU this is methanol extraction) are called Non-Extractable Residues (Gevao et al., 2000). The bioavailability of these

NER is known to be very low and are also not expected to become bioavailable for microbes within a conceivable time span (Barraclough et al., 2005).

2.8.6.1 Determination of bioavailable IPU

A chemicals´ bioavailability in soil is one critical factor affecting its degradation by soil microorganisms. Folberth et al. (2009) tested different approaches for their suitability to depict the *in situ* bioavailability of IPU to soil microbes. Pore water (PW) extraction by centrifuging the soil was shown to be an adequate tool to assess the *in situ* bioavailability of IPU in soils.

Soil pore water was extracted from soil samples by using custom-built centrifugation containers, constructed as shown in Figure 7 (modified after Folberth (2008)).

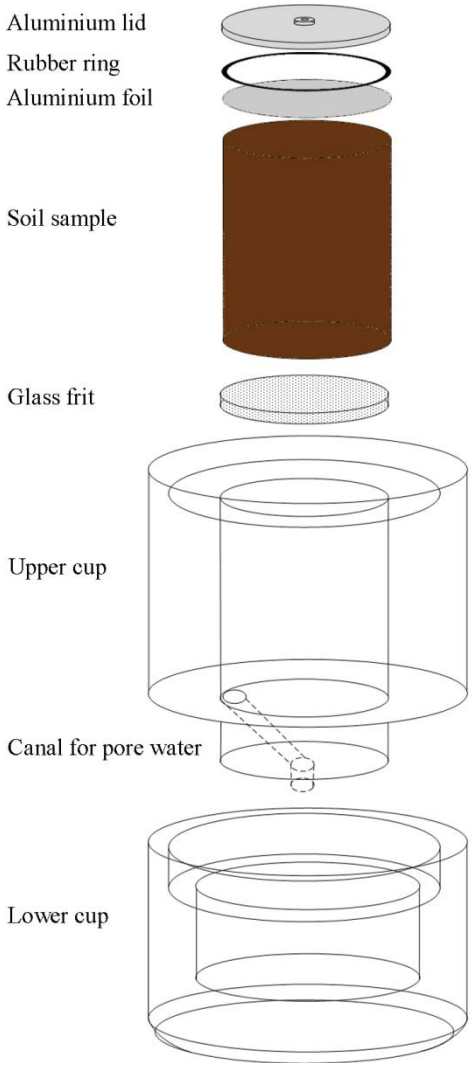


Figure 7: Centrifugation containers for extraction of pore water from soil.

Each container consisted of an upper and lower cup made of Teflon. The upper cup had a volume of 23.08 cm^3, which corresponds to a sample dry weight of 30 g at a soil density of 1.3 g cm^{-3}.

Seramis® clay particles were removed from the bulk soil before analysis. The soil to be extracted was filled gradually in the upper cup and compacted continuously to the density of 1.3 g cm^{-3} with a spatula. A canal of 2 mm diameter connected upper and lower cup and directed the extracted soil pore water into the basin of the lower cup. To prevent clogging of the canal by soil particles, a glass frit of 120 µm average pore size (VWR International, Darmstadt, Germany) was placed underneath the soil sample. To avoid drying of the soil after filling it into the upper cup, the soil surface was covered by a slice of aluminium foil, then a rubber ring was placed on top and the entire container was closed by screwing an aluminium lid onto the upper cup. The soil samples were centrifuged for 90 min at +20 °C (centrifuge: Sorvall 11788 PN, rotor: FIBERLite F21-8x50y; both ThermoScientific, Braunschweig, Germany).

After centrifugation, the volume of extracted soil pore water was determined by weighing the lower cup and the pore water was filtrated with a 0.4 µm pore polycarbonate filter (Nucleopore, Whatman, Maidstone, England) as occasionally a few soil particles passed the glass frit during centrifugation. From this filtrate, 100 µl were measured by liquid scintillation counting in order to quantify the dissolved ^{14}C-pesticide concentration in soil pore water. The total dissolved pesticide amount in soil was calculated by multiplying the pesticide concentration with the total water content of the soil sample. The pesticide amount adsorbed to soil was calculated by subtracting the dissolved amount from the total amount applied.

For HPLC analysis, filtered extracts were concentrated to a volume of about 1 mL by using rotary evaporators (Büchi Labortechnik, Konstanz, Germany). Then, extracts were transferred to 1.5 mL glass vials (neoLab, Heidelberg, Germany) and the sample herein was further reduced by a gentle N_2 stream to a volume of 50 µl. Samples were stored at -20 °C until metabolite analysis with HPLC (see chapter 2.8.6.5).

2.8.6.2 Determination of bioavailable IPU at application time of ^{14}C-IPU for soil Scheyern

To display the full dynamic of bioavailabe IPU in soil from time of IPU-application on, it was necessary to determine the amount of dissolved IPU at time of its application to soil.

When IPU is added to soil, it disperses and a portion remains dissolved, while another portion is adsorbed, mainly to SOM (Worrall et al., 1996; Ertli et al., 2004). To avoid the immediate beginning of degradation after application of IPU to soil, which may falsify sorption results, it was necessary to inhibit microbial activity, which was shown to play the major role in degradation of IPU (Hussain et al., 2009).

To determine the amount of bioavailable IPU at application time of ^{14}C-IPU (= T0), two successive experiments were conducted.

(1) In **experiment 1**, the aim was to determine the concentration of sodium azide (NaN_3) necessary to inhibit microbial activity to a sufficient extent.
(2) In **experiment 2**, soil samples were prepared with the NaN_3-concentration as defined adequate in experiment 1. By means of them, it was determined after which period of time the sorption equilibrium of IPU was achieved by measuring IPU concentration in pore water in time series parallel to mineralization.

Details for both phases are given below. By means of these results, the amount of dissolved IPU at T0 could be measured.

Experiment 1: Determination of optimum NaN_3-concentration for inhibition of microbial activity in soil Scheyern

The aim of this experiment was to determine the amount of NaN_3 necessary to suppress microbial activity in soil. As sorption of IPU to soil might be affected when NaN_3 is present in the soil at the same time, as little NaN_3 as possible should be applied. The aim was to reduce mineralization and thus microbial activity to a cumulative mineralization level < 0.5 % of applied ^{14}C-IPU within the course of the experiment.

For the inhibition of microbial activity, NaN_3 was applied to the soil in various concentrations. To evaluate the efficiency of NaN_3-incubation, three parameters were used:

(a) Mineralization of IPU
(b) The number of living cells as a measure for growth of microbes in soil
(c) The formation of metabolites as a measure for microbial activity and degradation in soil

Phase 1: Incubation of soil with NaN_3

At start of the experiment, before adding NaN_3, the equilibrated soil was homogenized and the number of CFUs was determined (see below). Then, aliquots of 3.5 g soil (dry mass) were dried at 105 °C for 24 hours. Later, four different concentrations of NaN_3 (1, 10, 100 and 1000 µg g^{-1} dry soil) in aqueous solution were applied to the oven-dry, ground soil and each of these soil aliquots was mixed with 32.5 g of equilibrated soil (dry weight). Each concentration variant of NaN_3 was done in quadruplicates. After transferring the soil to glass beakers, it was compacted to 1.3 g cm^{-3} and moistened with autoclaved distilled water to a water potential of -15 kPa. Subsequently, the beaker was covered with parafilm (Bemis, Neenah, WI, USA) and stored in a shaded exsiccator containing water at the bottom to avoid evaporation of water from the soil. The reliability of this procedure was confirmed by weighing the incubators before and after incubation with NaN_3.

Phase 2: Incubation of soil with ^{14}C-IPU and control for inhibition of microbial activity

After a pre-incubation period of three days with NaN_3, the soil was homogenized and the number of CFUs was determined (see below). Then, an aliquot of 3.5 g of the incubated soil (dry mass) was dried at 105 °C. Subsequently, ^{14}C-IPU application standard 7 (specific radioactivity 127.64 kBq mg^{-1}; 5 mg kg^{-1} soil per replicate of 33 g dry soil) was applied to the dry, ground soil aliquot of 3.5 g using a Hamilton syringe (neoLab, Heidelberg, Germany) and subsequently mixed with the moist soil. To be able to detect IPU mineralization, the soil was transferred to the soil incubators described in chapter 2.8.2. After transfer, the soil was compacted to a soil density of 1.3 g cm^{-3} and moistened with autoclaved distilled water to a water potential of -15 kPa (chapter 2.7). Soil incubators were stored at +20 ± 1 °C in the dark. Mineralization was measured 1, 2, 3, 4 and 7 days after application of ^{14}C-IPU.

Four and 7 days after application of ^{14}C-IPU, the number of CFUs was determined to ensure the suppression of microbial activity. On the latter, soil in all incubators was mixed and the water content gravimetrically determined. For later determination of extractable - and non-extractable-^{14}C-IPU-residues, soil was frozen at -20 ± 1 °C until analysis.

Determination of the number of bacteria and fungi in soil

The number of living bacteria and fungi in soil was determined to control to which degree microbial inhibition by application of NaN_3 to soil was working.

For extraction of cells from soil, 1 g of soil (fresh weight) was mixed with 9 ml of extraction solution (see Table 10) and suspended by a ball mill (5 min at speed 20; Retsch MM2, Retsch GmbH, Haan, Germany). Subsequently, the extract was transferred to sterile tubes (polystyrene conical tube, 15 mL, BD Biosciences, Erembodegem, Belgium) and soil particles were allowed to settle down for 10 min. 100 µl of the supernatant was removed to prepare dilution series according to chapter 2.4.1. 100 µl of each dilution were spread on MEA plates for cultivation of fungi and 100 µl on NB plates for cultivation of bacteria.

Table 10: Solution for extraction of microorganisms from soil.

Compound	Concentration (g L^{-1})
NaCl	0.1
CaCL2* 2 H2O	0.02
MgSO4 * 7 H2O	0.2
Tween 80	5.0
Aqua dest. until 1000 ml	

The **M**alt **E**xtract **A**gar (MEA) is a universal culture medium for fungi, its composition is given in Table 11. Bacterial growth is inhibited by the pH and the addition of the antibiotics Streptomycin and Chloramphenicol. Streptomycin and Chloramphenicol were purchased from Sigma-Aldrich, Steinheim, Germany while Agar agar was obtained by Bernd Euler Biotechnologie-Mikrobiologie, Frankfurt am Main, Germany.

Table 11: Composition of MEA plates for cultivation of fungi.

Compound	Concentration (g L^{-1})
Malt extract	20.0
Agar agar	15.0
Aqua dest. until 1000 mL; pH 6.0	
After autoclaving, sterile filtered Streptomycin and Chloramphenicol are added to a final concentration of 300 mg L^{-1} each to the hand warm media (about 45 °C).	

For growth of bacteria, Nutrient Solution Agar plates (NB plates) were used. The composition is given in Table 12. To inhibit growth of fungi, Cycloheximide (AppliChem, Darmstadt, Germany) was applied. Agar agar was obtained by Bernd Euler Biotechnologie-Mikrobiologie (Frankfurt am Main, Germany) while Meat extract was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Peptone from AppliChem GmbH (Darmstadt, Germany)

Table 12: Composition of NB plates for cultivation of bacteria.

Compound	Concentration (g L^{-1})
Peptone, pancreatic digested	5.0
Meat extract	3.0
Agar agar	12.0
Aqua dest. until 1000 ml	
After autoclaving, sterile filtered Cycloheximide was applied to a final concentration of 100 mg L^{-1} to the hand warm media (about 45 °C).	

Both types of plates were stored at +20 ± 1 °C in the dark until usage.

Experiment 2: Determination of the time period after which the sorption equilibrium for IPU was attained

The aim of this experiment 2 was to determine the period of time after which ^{14}C-IPU achieves sorption equilibrium between soil matrix and soil water. After application to soil, chemicals disperse by diffusion within the pore water and penetrate also into small soil pores. Diffusion in

soil is known to be a time–dependent process (Beulke et al., 2004). Therefore, in order to determine the maximum amount of IPU dissolved in pore water when the sorption equilibrium is attained, soil incubated with ^{14}C-IPU was sampled in time series and the IPU dissolved in pore water was extracted and analyzed for the amount of ^{14}C in pore water. To reduce a falsification of results by mineralization, soil samples were set up with a NaN_3-concentration of 1000 µg g^{-1} dry soil which was found adequate to inhibit microbial activity to a level < 0.5 % cumulative mineralization in experiment 1.

Phase 1: Incubation of soil with NaN_3

To enable the attainment of the sorption equilibrium, microbial activity in soil Scheyern was suppressed by pre-incubating the equilibrated soil with NaN_3 at a final concentration of 1000 µg g^{-1} dry soil. The detailed procedure is described in experiment 1.

Phase 2: Incubation of soil with ^{14}C-IPU and extraction of pore water

After 3 days of pre-incubation with NaN_3, ^{14}C-IPU application standard 8 (specific radioactivity 250.57 kBq mg^{-1}; 5 µg g^{-1} dry soil) was applied as noted in experiment 1.

One, 2, 3, 4 and 7 days after application of ^{14}C-IPU, in each case 4 replicates were centrifuged and the amount of radioactivity determined by measuring a mixture of filtered pore water (each 100 µl) with 5 mL of scintillation cocktail Ultima Gold XR (Packard, Groningen, Netherlands) by liquid scintillation counting (chapter 2.8.3). The total amount of dissolved IPU was obtained by referring the measured radioactivity to the total amount of pore water in soil.

Verification of results

To assess the results for plausibility, a ^{14}C-mass balance was set up: the centrifuged soil was combusted and, as pore water could not completely be extracted from soil, the measured ^{14}C was corrected by the radioactivity originating from pore water that was not extracted by centrifugation. The remaining ^{14}C thus derived from methanol-extractable and non-extractable ^{14}C-residues and was added up to the amount of ^{14}C in pore water.

2.8.6.3 Extraction of ^{14}C-IPU residues from soil

IPU and its metabolites were extracted from soil samples by **A**ccelerated **S**olvent **E**xtraction (= ASE) (ASE 200, Dionex, Germany). After sampling, soil samples were stored at -20 ± 1 °C. To enable a gentle thawing of the soil before extraction, frozen samples were placed at +4 °C in the fridge overnight. For ASE, samples were loaded in cartridges, which were then successively and automatically filled with methanol as extraction fluid. In three successive statically phases, the soil was extracted under elevated temperature (+90 °C) and pressure (10 MPa) and the extract was collected in glass vials. After this extraction step, a stream of compressed nitrogen was purging

solvent residues into the same vial. Each cartridge was extracted two times to guarantee an extensive extraction of IPU and its metabolites. It was shown by Schroll and Kühn (2004) that these conditions were suitable for a quantitative extraction of IPU from soils without producing any artefacts (e.g. synthesis of artificial ^{14}C-compounds) under these conditions.

If soil was too moist for filling it into a cartridge, diatomaceous earth (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added and mixed accurately with it in order to withdraw humidity and receive free flowing soil material. Results were corrected by the weight of the diatomaceous earth.

Each attained extract was measured for volume and the contained radioactivity, the latter by measuring a mixture of 100 µl extract with 5 ml scintillation cocktail (Ultima Goldt XR, Packard, Groningen, Netherlands) via liquid scintillation counting (chapter 2.5.1.2).

2.8.6.4 Clean-up of soil extracts

For analysis of ASE extracts by HPLC, a previous clean-up of extracts was needed in order to separate interfering components from the organic analyte. First, the sample extracts attained by ASE were concentrated to a volume of about 1 mL by using rotary evaporators (Büchi Labortechnik, Konstanz, Germany). For **S**olid **P**hase **E**xtraction (SPE), those samples were diluted with distilled water to a volume of 250 mL. The aqueous extracts were then passed over filters (20µm Frits, Varian, Middelburg, Netherlands) and SPE columns (Bond Elut-ENV, 200 mg, Varian, Darmstadt, Germany); the latter had been washed and conditioned before with methanol (Methanol gradient grade, Merck, Darmstadt) and distilled water. After drying in a soft nitrogen stream, the columns were eluted with 10 mL methanol and aliquots of the eluate were measured for radioactivity. After concentrating the eluate to a volume of about 1 mL by using a rotary evaporater, its volume was further reduced by a soft nitrogen stream to about 50 µl. Until HPLC-analysis, the samples were stored at +4 °C in the dark.

To detect possible losses during the clean-up process, aliquots of 100 µl were taken after the individual procedures, mixed with 5 ml scintillation cocktail (Ultima Goldt XR, Packard, Groningen, Netherlands) and measured for ^{14}C-activity by liquid scintillation counting (chapter 2.5.1.2).

2.8.6.5 HPLC-analysis of soil pore water and soil extracts

For identification and quantification of ^{14}C-IPU and its ^{14}C-metabolites, soil pore water as well as ASE soil extracts after clean-up were analysed by HPLC. The HPLC system was built up of the components presented in Table 13.

IPU and its metabolites were identified by comparing the ^{14}C-peaks (^{14}C-detector) with the retention time of unlabeled reference substances (UV/VIS detector) contained in a standard mix in

the following composition: IPU, MD-IPU, DD-IPU and 4IA (chapter 2.1.1). A linear gradient program as compiled in Table 14 was operated. From each sample, 20 µl were injected to HPLC manually by using a Hamilton syringe (neoLab, Heidelberg, Germany). Spectra were analysed by RadioStar software (version 4.6.0.0) (Berthold Technologies, Bad Wildbad, Germany). The level of detection and the level of quantification were determined according to Frehse and Thier (1991) and were identified to be at 350 dpm and 550 dpm, respectively.

Table 13: Components of the HPLC system used for measurement of ASE samples and extracted pore water.

UV/VIS detector	Merck-Hitachi L-4250 (240 nm, Merck KGaA, Darmstadt, Germany)
Radiodetector	Radioactivity monitor LB 506 C-1 (Berthold, Bad Wildbach, Germany)
Pump	L-6200 Intelligent Pump (Hitachi, Merck, Darmstadt, Germany)
Pre-column	LiChrospher 100 RP-18 (5 µm, 250 × 4 mm; Merck, Darmstadt, Germany)
Column	LiChrospher 100 RP-18 column (5 µm, 250 × 4 mm; Merck, Darmstadt, Germany)
Mobile phase	acetonitrile (= A) (HPLC-grade, Merck) and water (= B) (Lichrosolv water for chromatography, Merck, Germany); both vented
Flow	1.0 mL min^{-1}

Table 14: HPLC gradient program used for detection of IPU and its metabolites.

time (min)	acetonitrile (%)	water (%)
0.0	5	95
15.0	60	40
20.0	60	40
25.0	5	95
35.0	5	95

2.8.6.6 Adjustment of radioactivity extracted by ASE when using centrifuged soil

When extracting soil pore water by centrifugation, generally the contained pore water could not be extracted from soil completely. Thus, when extracting ^{14}C-IPU-residues by ASE from centrifuged soil, radioactivity of the residual pore water was also extracted by ASE, leading (1) to an overestimation of total ^{14}C extractable by ASE. Furthermore, the differing sample preparation for pore water samples (no SPE) and ASE extracts (with SPE) resulted in (2) modifications in relative distribution of peaks in the HPLC chromatograms and thus to a falsification in the amounts of IPU and its metabolites extractable just by ASE. Therefore, the raw ASE data was as follows:

(1) To calculate the total amount of radioactivity extractable just by ASE, it was proceeded as described in the following: Based on the soil water content before centrifugation and the volume of pore water extracted by centrifugation and the therein contained radioactivity, it was calculated how much radioactivity was still present in the residual pore water. This amount of ^{14}C was subtracted from the radioactivity of centrifuged soil samples treated with ASE, resulting in the total amount of ^{14}C just extractable by ASE.

(2) To calculate the correct amounts of the different metabolites extractable just by ASE, it was preceded as follows: preliminary tests with IPU and its metabolites showed that these compounds were not eliminated or reduced in their amount by performing ASE and the subsequent cleaning-up by SPE, also no artifacts could be detected by this procedure. At first, it was calculated as in (1) how much ^{14}C was still present in the residual pore water. Then, it was calculated by using the relative distribution of peaks in the HPLC chromatogram of the already extracted pore water, how much radioactivity accounted for the single peaks of the residual pore water. Parallel, it was calculated based on the relative peak distribution in the HPLC chromatogram of the ASE extract of the centrifuged soil, how much radioactivity was allocated for the different peaks. Then, the radioactivity of each peak contained in the residual water was subtracted from the radioactivity of the peak at same retention time in ASE extracts of centrifuged soil samples, giving the radioactivity for each peak extractable just by ASE.

2.8.6.7 Determination of non-extractable ^{14}C-residues

To measure the amount of NER in soil samples, soil aliquots were combusted following their extraction with ASE (chapter 2.8.6.3). For that purpose, three aliquots of about 300 mg were weighed into combustion cones (Packard, Zurich, Switzerland) and 3-4 drops of a saturated sugar solution were applied in order to optimize sample oxidation. Parallel to weighing in the aliquots for combustion, the water content of the soil sample was determined gravimetrically in order to refer the amount of ^{14}C-residues to soils´ dry weight. The prepared aliquots were oxidized in a sample oxidizer (Packard Model 307, Perkin Elmer, USA). The $^{14}CO_2$ was trapped automatically in absorber liquid (Carbo-Sorb; Packard, Groningen, Netherlands) and a scintillation cocktail (Permafluor E+; Packard, Groningen, Netherlands) was applied. ^{14}C was quantified by liquid scintillation counting (chapter 2.5.1.2).

2.9 Adaptation of microbes in soil to IPU mineralization by repeated IPU applications

In agricultural practice, IPU is applied at most twice a year. If crop rotation is conducted, it is used after much longer time intervals. In case of accelerated pesticide degradation, it is assumed that the chemical degrading population is maintained from one pesticide application to the next one (Arbeli

and Fuentes, 2007). Assuming that more chemical degrading microbes survive when the chemical itself is applied in shorter time intervals and that this promotes the development of accelerated degradation, then, this development might be speeded up by pesticide applications in short time intervals.

Aim of this experiment was to investigate if multiple applications of IPU, carried out in short intervals of about four weeks, may lead to accelerated degradation in non-inoculated soils. For this purpose, soil Feldkirchen was selected, which was reported to degrade IPU cometabolically after a single IPU application (Folberth et al., 2009).

2.9.1 Effect of repeated IPU-application on soil Feldkirchen

Four applications of ^{14}C-IPU to soil were conducted in intervals of about 4 weeks. To evaluate if an acceleration of IPU mineralization might have taken place by the repeated applications of ^{14}C-IPU, mineralization data was used. To determine to what extent a potential change from cometabolic to metabolic degradation pattern might be reflected in the type and quantity of IPU residues, additional soil samples were prepared for residue analysis.

During all four phases, mineralization was determined continuously by trapping $^{14}CO_2$ in NaOH (chapter 2.8.2) and measuring radioactivity in 2 mL aliquots of this solution by liquid scintillation counting (chapter 2.8.3).

2.9.1.1 First application of ^{14}C-IPU to soil Feldkirchen

IPU was applied to soil Feldkirchen as presented in the following: per replicate, a total of 50 g soil (dry weight) was equilibrated (chapter 2.7.2.2). An aliquot of 3.5 g (dry soil) of this equilibrated soil was then dried and ^{14}C-IPU pesticide application standard 3 (specific radioactivity of 0.64 MBq mg^{-1}) was dripped on the aliquot and intermixed with a spatula to enable a homogeneous distribution of IPU within the soil material. Then, the dried soil aliquot (3.5 g) was mixed with the equilibrated soil material (46.5 g dry soil) for 2 min within the soil incubation flasks described in chapter 2.8.2. Finally, the soil material was compacted to a soil density of 1.3 g cm^{-3} and a final water potential of -15 kPa was set by adding distilled water (chapter 2.7). For details on the application procedure for ^{14}C-IPU see chapter 2.8.4.1.

Sixteen replicates were prepared (= replicates F1-F16). The laboratory systems described in chapter 2.8.2 were used to trap the produced $^{14}CO_2$ for about 4 weeks after application 1 (= phase C1).

2.9.1.2 Second, third and fourth application of ^{14}C-IPU and sampling for residue analysis

Sampling at end of phase C1 and IPU application 2

Twenty-nine days after IPU application 1, four replicates (F13-F16) were stopped for IPU residue analysis. For this, the soil was mixed homogeneously for 2 min with a spatula and a 2 g aliquot (fresh weight) was taken to determine the water content gravimetrically. Then, the amount of bioavailable IPU was measured by centrifugation of 30 g soil (dry weight) (for analysis see chapter 2.8.6.1). To display the full dynamic of bioavailable IPU in soil Feldkirchen, the corresponding amount at time of ^{14}C-IPU application was adopted from Folberth et al. (2009), who conducted the necessary analysis in soil Feldkirchen. After centrifugation, the soil material was frozen at -20 ± 1 °C until further analysis with ASE (chapter 2.8.6.3). After extraction with ASE, the amount of non-extractable ^{14}C-residues was determined in the soil material by combustion (chapter 2.8.6.7).

Parallel, soil in laboratory systems F1-F12 was mixed for 2 min with a spatula and ^{14}C-IPU-standard 3 (specific radioactivity (0.64 MBq mg^{-1}) and concentration (5 mg kg^{-1}) as for application 1) was reapplied to the soil (= application 2 and start of phase C2). The procedure for reapplication of ^{14}C-IPU was carried out as described for application 1 (chapter 2.9.1.1).

Sampling at end of phase C2 and IPU application 3

Twenty-six days after application 2, another four replicates (replicates F9-F12) were stopped for residue analysis as done previously at the end of phase C1. Parallel, ^{14}C-IPU application standard 3 was applied to the remaining 8 replicates (replicates F1-F8) (= application 3 and start of phase C3; specific radioactivity 0.64 MBq mg^{-1} and concentration 5 mg kg^{-1}).

Sampling at end of phase C3 and IPU application 4

Thirty days after application 3, again four replicates were stopped for residue analyses (replicates F5-F8; analysis as described at the end of phase C1) and ^{14}C-IPU application standard 3 was applied to the remaining 4 replicates (= application 4 and start of phase C3; replicates F1-F4) (specific radioactivity 0.64 MBq mg^{-1} and concentration 5 mg kg^{-1}).

End of the study

Thirty-three days after application 4 (= 118 days after application 1), the final four replicates (F1-F4) were stopped and utilized for residue analysis as described previously for soil sampled at the end of phase C1.

2.9.2 Effect of repeated IPU-application on soils Scheyern, Kelheim and Konjišče

In the previous study it was shown that four applications of IPU to soil Feldkirchen in time intervals of about four weeks resulted in a change in mineralization pattern, from cometabolic to metabolic IPU mineralization. However, it was not possible to determine if the acceleration of IPU degradation (chapter 3.5.1.1) was caused by the adaptation of soil microbes during the experiment or if the specific soil history of soil Feldkirchen was responsible for this change in mineralization pattern (chapter 4.4.1.2).

To examine if a change from cometabolic to metabolic IPU degradation pattern may be generated by adaptation of soil microbes, three further agricultural soils were investigated (soils Scheyern, Kelheim and Konjišče) which were all shown to degrade IPU co-metabolically after a single application of the compound (Folberth et al., 2009) and which were not reported to degrade IPU metabolically previously. Application history regarding IPU was different for the three soils (chapter 2.7.1): in soil Scheyern, no more IPU was applied for 19 years before soil sampling, while soil Kelheim did not get any loading for 12 years. To soil Konjišče, IPU was applied two times within the last five years before sampling. Soon after sampling, all soils were frozen at -20 °C until equilibration, thus an adaptation of inherent microbes was not expected to develop during this period of time.

To evaluate if IPU mineralization may be accelerated by the repeated applications of ^{14}C-IPU, mineralization data from the three application phases were used. As a change of mineralization pattern might be detectable not only in the quantity of ^{14}C-IPU mineralized but also in the relation of mineralized, extractable and non-extractable ^{14}C, IPU residues were analyzed in several replicates per soil at the end of phases P1 and P3.

While IPU was applied to soil Feldkirchen four times (chapter 2.9.1), it was applied to soils Scheyern, Kelheim and Konjišče in total three times. Moreover, the duration of phases differed slightly between soil Feldkirchen and the other three soils. Therefore, phases for soils Scheyern, Kelheim and Konjišče (phases P1, P2 and P3) were named different from phases of soil Feldkirchen (phases C1, C2, C3, C4).

2.9.2.1 Setup of soil incubators and first application of ^{14}C-IPU

For the present study, the laboratory system described in chapter 2.8.2 was utilized. Replicates were prepared according to the procedure for soil Feldkirchen (chapter 2.9.1.1). Other than for soil Feldkirchen, ^{14}C-IPU application standard 4 (specific radioactivity: 1.32 MBq mg^{-1}) was applied in the present experiment (= application 1). This resulted in a final IPU concentration of 5 mg kg^{-1}, which corresponds to a supplement of 250 µg IPU per replicate. Per soil, eight replicates were arranged (soil Scheyern: replicates S1-S8, soil Kelheim: replicates KE1-KE8, soil Konjišče:

replicates K1-K8). The released $^{14}CO_2$ was trapped in 0.1 N NaOH and the radioactivity quantified by liquid scintillation counting (chapter 2.8.3).

2.9.2.2 Second and third application of ^{14}C-IPU as well as analysis of ^{14}C-IPU residues

Sampling at end of phase P1 and second application of IPU

After incubation for 27 days, four replicates per soil were stopped (replicates S5-8, KE5-8, K5-8). After mixing the soil for 2 min by a spatula, an aliquot of soil (about 2 g fresh weight) was used in order to gravimetrically determine the water content of the soil. The residual soil was frozen at -20 ± 1 °C until analysis with ASE (chapter 2.8.6.3). After ASE, the amount of NER was measured by combusting aliquots of the extracted soil material (chapter 2.8.6.7).

Parallel, ^{14}C-IPU was re-applied to replicates S1-S4, K1-K4 and KE1-KE4 (= application 2), by mixing the soil for 2 min, drying a soil aliquot of 3.5 g dry weight at 105 ± 1 °C and applying ^{14}C-IPU to this aliquot. As in application 1, ^{14}C-IPU application standard 4 was added to soil aliquots. To each replicate, 250 µg IPU were added. The soil aliquot was then homogeneously mixed with the moist soil in the incubator it was taken from. Subsequently, the soil was compacted again to a density of 1.3 g cm^{-3} and supplemented with distilled water to attain a water potential of -15 kPa. For details on the procedure see chapter 2.8.5.1.

Third application of IPU

Twenty-five days after IPU application 2 (= 52 days after application 1), ^{14}C-IPU was again reapplied to replicates S1-S4, K1-K4 and KE1-KE4 (= application 3) by the same procedure as for application 2. Mineralization was measured also after applications 2 and 3 as described for application 1 (chapter 2.9.2.1). ^{14}C-IPU residues were not determined at the end of phase P2.

End of the study

Twenty-five days after application 3 (= 76 days after application 1), all ongoing soil incubators were stopped. The soil material was mixed for 2 min, afterwards, the water content was determined gravimetrically by using an aliquot of 1-2 g soil (fresh weight). The residual soil was frozen at -20 ± 1 °C until analysis with ASE (chapter 2.8.6.3). After extraction with ASE, aliquots of the extracted soil material were combusted in order to quantify NER (chapter 2.8.6.7).

2.10 Data evaluation and statistical analysis

The analysis of sequencing data as well as the reconstruction of the phylogenetic tree were done with support from Dr. Michael Schmid, Department of Microbe-Plant-Interactions (AMP), Helmholtz Centre Munich, Germany.

For statistical analysis of variance, the t-test and, for more than two groups, the one-way ANOVA was calculated using IBM SPSS Statistics 14.0 (Inc., USA). A level of significance of 0.05 was used for evaluation of significance. Linear regression was done in Microsoft® Office Excel ® 2007 (Redmond, WA, USA).

For fitting of mineralization rates, the nonlinear Gompertz function was calculated using PROC NLIN of SAS 9.2 (SAS Institute Inc., Cary, NC, USA) by Dr. Hagen Scherb, Institute of Biomathematics and Biometry (IBB), Helmholtz Centre Munich, Germany.

Graphs were generated using Origin® 6.1 (OriginLab Corporation, Northampton, MA, USA).

3 Results

3.1 Isolation and identification of the IPU mineralizing bacterial strain from IMMC

3.1.1 Growth of IMMC cells on agar plates

After its extraction from soil, the microbial consortium of IMMC was established on Seramis® clay particles in a previous study (Kühn, 2003). To isolate the IPU mineralizing microbial species within IMMC and generate a pure culture of this consortium member, microbes of IMMC were detached from the Seramis® clay particles and then separated on LB, R2A and MS-IPU plates (chapter 2.4.1). Figure 8 summarizes the number of CFUs on all plate types counted 6, 17 and 48 days after plating. Microbes of IMMC could grow on all utilized plate types. The number of total colonies increased significantly on LB and R2A plates between days 6 and 17, as well as for MS-IPU and R2A plates between days 17 and 48. No new colonies established on LB plates after day 17.

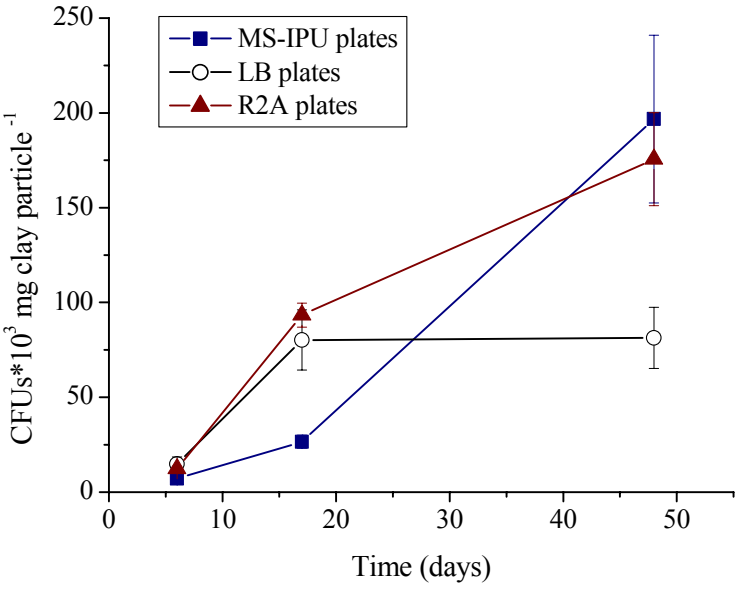


Figure 8: Development of total numbers of Colony Forming Units of IMMC grown on MS-IPU, LB and R2A- plates for a period of 48 days. Bars indicate standard deviation.

3.1.2 Mineralization of ^{14}C-IPU by isolated strains

Several colonies that grew on agar plates were detached from the plates and tested individually or in combinations for their ability to mineralize IPU. To do so, the respective colonies were applied

to liquid cultures that contained ^{14}C-IPU as the only source of carbon and nitrogen (chapter 2.4.1.2).

3.1.2.1 Colonies from LB and MS-IPU plates

Tested colonies grown on LB and MS-IPU plates for 8 days were not capable to mineralize IPU. Within 46 days, at most 0.9 % of applied ^{14}C-IPU was mineralized.

3.1.2.2 Colonies from R2A plates

Eight, 14 and 48 days after spreading (= samplings 1-3), colonies of R2A plates were detached from R2A agar and their capability to mineralize ^{14}C-IPU was tested in liquid culture systems.

IPU mineralization by colonies of samplings 1 and 2

At sampling 1 (= 8 days after spreading of IMMC on plate), two colonies were tested for IPU mineralization, but mineralized at most only 6.6 % of applied ^{14}C-IPU within 32 days. At sampling 2, 10 colonies were detached, which subsequently mineralized at most 3.6 % of applied ^{14}C-IPU within 40 days.

IPU mineralization by colonies of sampling 3

Sørensen et al. (2001) isolated an IPU mineralizing strain and reported that IPU mineralization was substantially increased when amino acids were added to the liquid medium.

In the present thesis, the impact of amino acids on IPU mineralization was tested for the 16 colonies (= colonies C1-C16) that were detached at sampling 3 (= 48 days after spreading of IMMC on plate). After removal from plate, colonies were divided in halves and each half used to inoculate one liquid culture system: one with MS-medium and one with MS-medium supplemented with an amino acid mix (0.1 g L^{-1}) (chapter 2.4.1.2). Mineralization by colonies C3-C16 was low in both types of liquid medium: within a period of 28 days, they mineralized at most 1.1 % of applied ^{14}C-IPU in MS-medium and 2.2 % in MS-medium which contained amino acids. In contrast, inoculation with colonies C1 and C2 resulted in good mineralization of ^{14}C-IPU in both types of medium (Fig. 9a). The isolate that was inoculated with colony C1 was selected for further purification (chapter 2.4.1.2) which should verify that a pure culture containing solely microbes of the IPU mineralizing strain were present in the liquid culture. The obtained purified strain which originated from colony C1 was designated strain AK1 and was subsequently identified (chapter 3.1.3). While the unpurified colony C1 showed good mineralization in both types of media (Fig. 9a), this was not the case for the purified strain AK1 (Figure 9b): mineralization of IPU in the liquid culture supplemented with the amino acid mix (47.5 % of applied ^{14}C-IPU) was significantly higher than in the one without (1.1 % of applied ^{14}C-IPU).

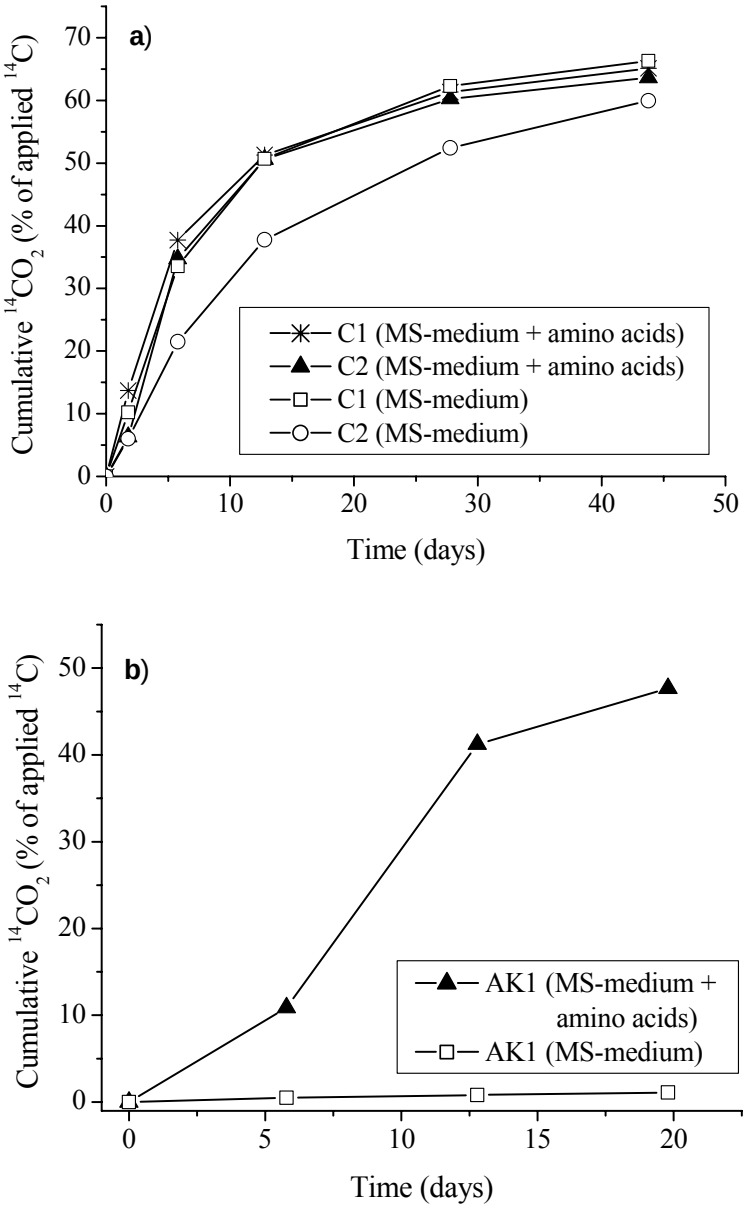


Figure 9: Development of ^{14}C-IPU mineralization in liquid culture systems containing either MS-medium or MS-Medium plus 0.1 g L^{-1} amino acid mix: (a) colonies C1 and C2 (unpurified), (b) purified strain AK1.

To test the capability of strain AK1 to establish not only on R2A but also on MS-IPU and LB plates, dilution series were prepared and several dilutions plated on the three plate types (chapter 2.4.1.1). No growth of colonies was detected for a period of three months on LB and MS-IPU plates, indicating that strain AK1 could not grow at all on these plate types. Colonies on R2A plates were visible about 4-5 weeks after spreading on plates.

3.1.3 Phylogenetic identification of the isolated IPU mineralizing strain AK1

A phylogenetic dendrogramm illustrating the results of comparative analysis of 16S-rDNA sequence data is summarized in Figure 10. The three 16S-rDNA almost full gene sequences (1449,

1390 respectively 1390 bases), which were obtained from strain AK1 (chapter 2.3.2.3), were compared with sequences from the GenBank database NCBI (http://www.ncbi.nlm.nih.gov/). The highest similarity (98.4, 98.3 and 98.6 % for clones 1, 2 and 3, respectively) was obtained with the 16S-rDNA gene sequence of an uncultered bacterium (GenBank Ac. No. EF516566). The highest similarity with a described bacterial species (95.3, 95.3 respectively 95.5 % for clones 1, 2 and 3) was attained with the gene sequence of *Sphingomonas fennica.* Given the phylogenetic relationship of the isolated strain AK1 with several *Sphingomonas* spp., the now isolated strain was designated *Sphingomonas* sp. AK1.

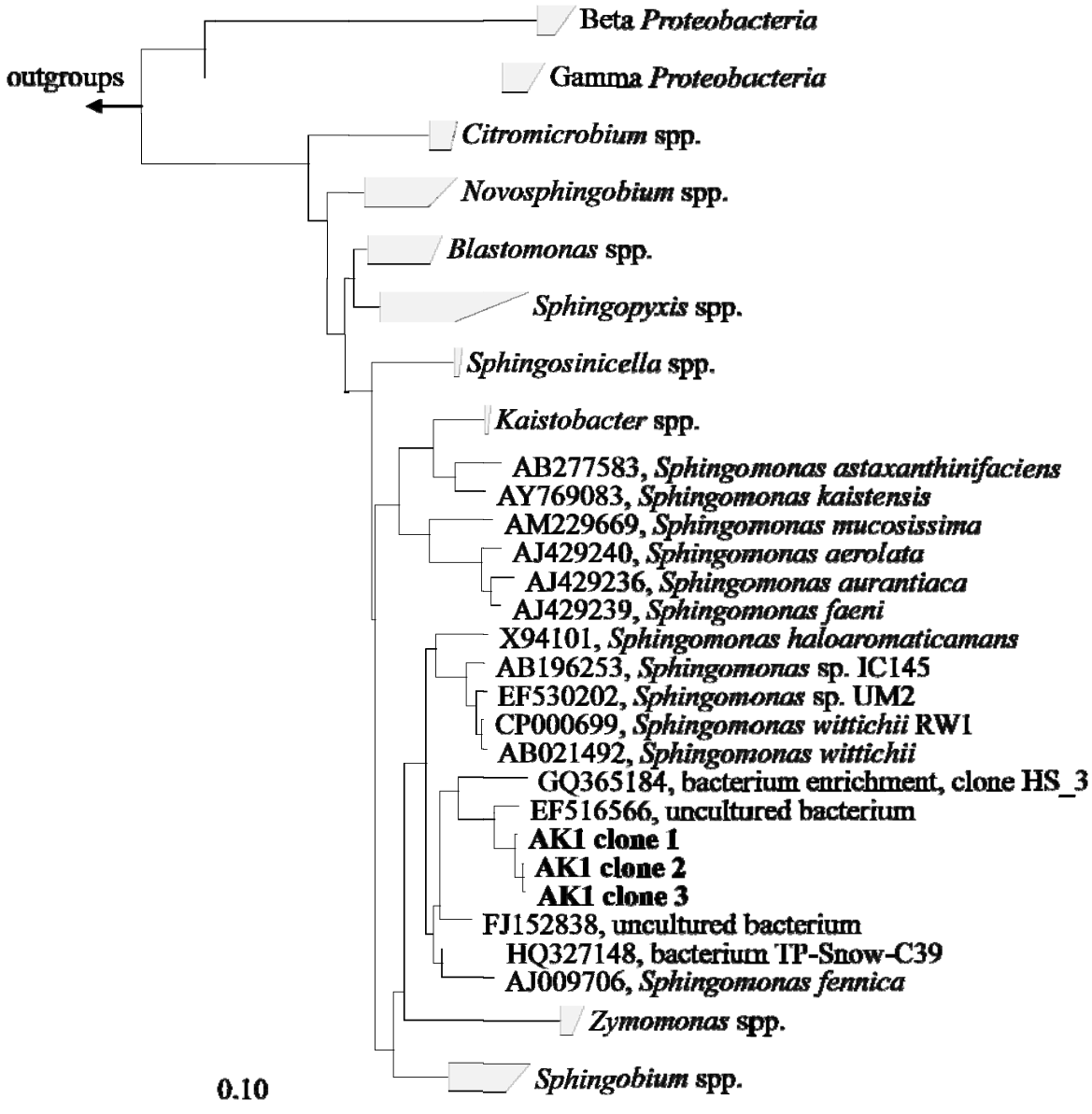


Figure 10: Phylogenetic tree (calculated by Neighbor-Joining) including the identified clones 1-3 from the IPU mineralizing strain AK1 that was isolated from IMMC. The clones identified in this examination are highlighted with bold letters. The bar indicates a divergence of sequences of 10 %. The GenBank accession numbers are given in prefixed capital letters.

3.1.4 Diversity analysis of IMMC

Cells of IMMC established on Seramis® clay particles served as raw material for the identification of microorganisms participating in the mineralization of ^{14}C-IPU. Microbial cells were harvested and their DNA extracted, subsequently, 16S-rRNA genes were amplified by PCR and the obtained gene fragments separated by cloning. Finally, the 16S-rDNA plasmid inserts were sequenced (chapter 2.4.2). Twenty five clones of IMMC were examined. Upon comparison of the received 16S-rDNA gene sequences (489-1007 bases) with sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/), the highest similarity was obtained with the gene sequences of microbes compiled in Table A1, Appendix. In case the next relative was an uncultured bacterium, then the next hit of a characterized species or genus is given. Due to the brevity of sequences a more detailed classification as well as a reliable phylogenetic dendrogramm could not be developed.

The obtained sequences were assigned to different bacterial classes illustrating the diversity of IMMC. All in all and within the phylum of Proteobacteria, the class of γ-Proteobacteria was represented in highest abundance (8 clones), while α- and β-Protecobacteria were also detected in high numbers (6 respectively 7 clones). Three clones were affiliated to the phylum of Acidobacteria, while one belonged to the phylum Bacteroidetes. Within the phylum of Proteobacteria, the genera Pseudomonas (8 clones) and Azohydromonas (4 clones) were detected most frequently.

3.2 Degradation of IPU, MD-IPU, DD-IPU and Diuron by IMMC in liquid culture

Several liquid culture studies were conducted to elucidate the capability of IMMC to degrade IPU, its metabolites MD-IPU and DD-IPU, as well as the phenylurea herbicide Diuron (chapter 2.5).

3.2.1 Mineralization of ^{14}C-IPU and ^{14}C-MD-IPU

Figure 11a shows the mineralization of IPU and its metabolite MD-IPU in liquid medium by IMMC. To each liquid culture (50 mL MS-medium), 1.2 ± 0.1 *10^7 CFUs were applied. Replicates had a final pesticide concentration of 25 mg L^{-1} (IPU) and 23.30 mg L^{-1} (MD-IPU), respectively, which corresponds both to 121.19 µM. For experimental setup see chapter 2.5.1.1.

Although the total quantities mineralized within 24 days did not significantly differ between IPU and MD-IPU (50.5 ± 9.1 and 58.0 ± 8.0 % of applied ^{14}C, respectively; Fig. 11a), the mineralization dynamics varied clearly (Fig. 11b): the maximum rate for MD-IPU (8.2 ± 3.0 % d^{-1}) was significantly higher than the one for IPU (4.2 ± 0.6 % d^{-1}) and was achieved much earlier (day 7) than the maximum rate for IPU (day 14).

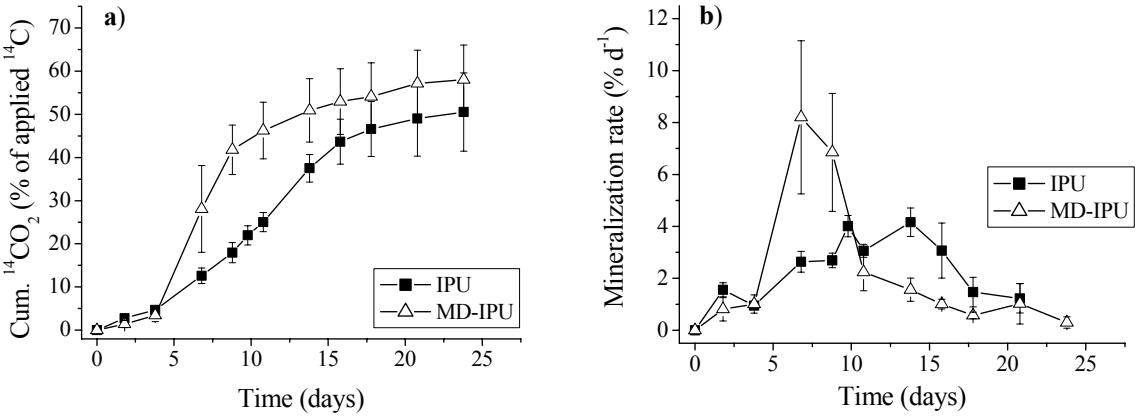


Figure 11: Development of mineralization of IPU and MD-IPU in liquid cultures inoculated with IMMC: (a) cumulative mineralization, (b) mineralization rates. Bars indicate standard deviation.

Cell dynamic of IMMC during IPU mineralization

To examine to what extent IPU degradation was linked to a proliferation of IMMC, liquid cultures were set up and IPU mineralization and cell numbers were determined regularly (for experimental setup see chapter 2.5.1.2). For this, liquid cultures were prepared with 25 mL MS-medium and 25 mg L^{-1} ^{14}C-IPU, subsequently a total of 0.6 ± 0.0 *10^7 CFUs of IMMC were applied to each replicate at the start of the experiment.

Figure 12 shows the mineralization rate for ^{14}C-IPU as well as the number of living microbial cells in liquid culture in the course of the experiment.

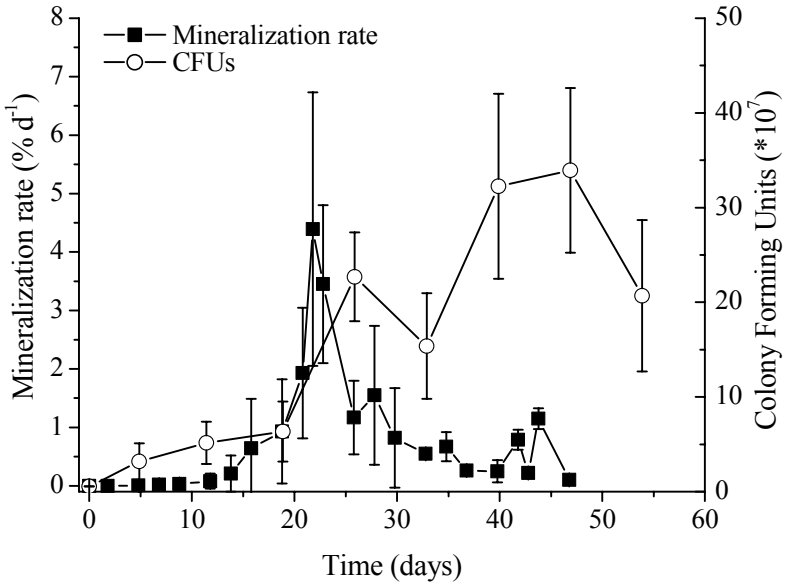


Figure 12: Development of IPU mineralization rates (closed squares) and CFUs (open circles) in liquid cultures inoculated with IMMC. To each replicate (25 mL MS-medium) 25 mg L^{-1} ^{14}C-IPU was applied. Bars indicate standard deviation.

Mineralization rates were below a level of 1 % d^{-1} for a period of 19 days, then, mineralization rates increased until a clear maximum at day 22 (4.4 ± 2.3 % of applied ^{14}C d^{-1}) and decreased subsequently. At the end of the experiment, mineralization rates below 0.3 % of applied IPU d^{-1} were measured.

The number of living cells increased considerably in the course of the experiment. Maximum numbers of living cells were measured at day 47 (33.9 ± 8.7*10^7 CFUs = 56.5 times more CFUs than at day 0).

3.2.2 Degradation of IPU, MD-IPU and DD-IPU and the formation of metabolites

Figures 13a,c,e give the concentration of IPU or its metabolites in liquid cultures that were inoculated with IMMC and supplemented with IPU (Fig. 13a), MD-IPU (Fig. 13c) or DD-IPU (Fig. 13e). Per liquid culture, 1.2 ± 0.1 *10^7 CFUs were applied. Replicates were supplemented with 25 mg L^{-1} IPU, 23.3 mg L^{-1} MD-IPU or 21.6 mg L^{-1} DD-IPU, respectively, which all correspond to 121.19 µM. For experimental setup see chapter 2.5.1.3.

Abiotic controls that obtained IPU, MD-IPU and DD-IPU, respectively, are given in Figures 13b,d and f.

In all replicates that were inoculated with IMMC (Figure 13a,c,e), a considerable disappearance of the applied compound was measured. Additionally, a formation of metabolites was detected: In replicates supplemented with IPU, just traces of MD-IPU (maximum 0.03 mg L^{-1}) and 4IA (maximum 0.03 mg L^{-1}) were measured. When MD-IPU was applied, no formation of DD-IPU was detected whereas 4IA was formed in considerable quantity (at most 2.04 mg L^{-1} on day 7) and degraded subsequently. In replicates provided with DD-IPU, traces of 4IA (at most 0.19 mg L^{-1}) were detected.

The applied compounds in replicates with IMMC disappeared in different speed. In case of DD-IPU, the target concentration of 21.6 mg L^{-1} was not met well in all replicates, resulting in a high standard deviation (Figure 13e). Nevertheless, to enable a comparison of degradation speeds for applied IPU, MD-IPU and DD-IPU, the decrease in applied amounts was calculated in percent of the applied amount (Figure 14). On days 4, 7 and 9, the concentration of MD-IPU was significantly lower than for DD-IPU and IPU. From day 14 on, MD-IPU and DD-IPU were below the quantification limit of UPLC. From day 7 on, IPU was present in liquid culture in significant higher quantity than MD-IPU and DD-IPU. IPU was detected in liquid culture until the end of experiment at day 21.

In abiotic control replicates (Fig. 13b,d,f), no decrease in concentration or formation of metabolites was detected.

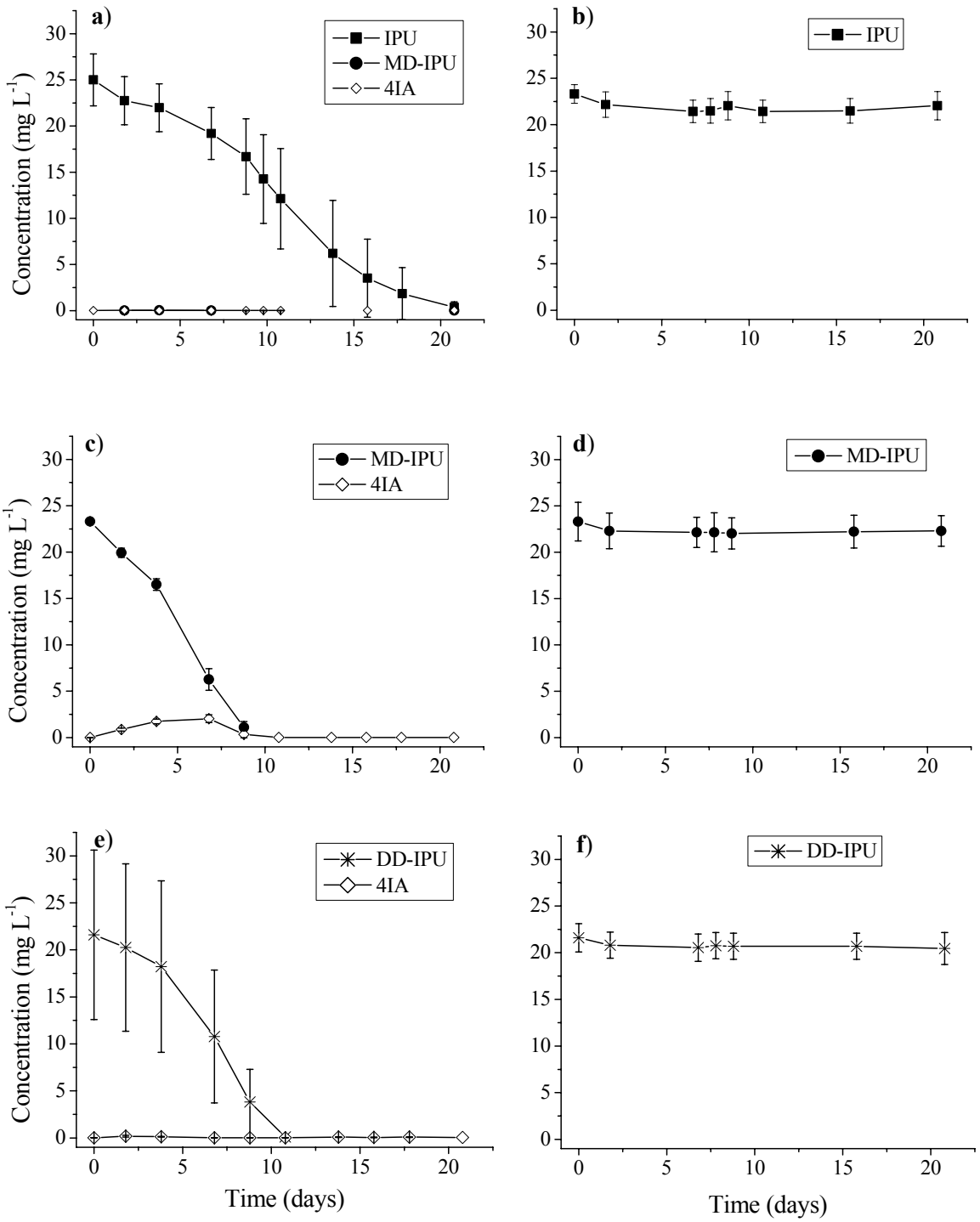


Figure 13: Development of concentration of applied IPU (a, b), MD-IPU (c, d) and DD-IPU (e, f) in MS-medium: (a), (c), (e): inoculation with IMMC, (b), (d), (f): abiotic control replicates. Bars indicate standard deviation.

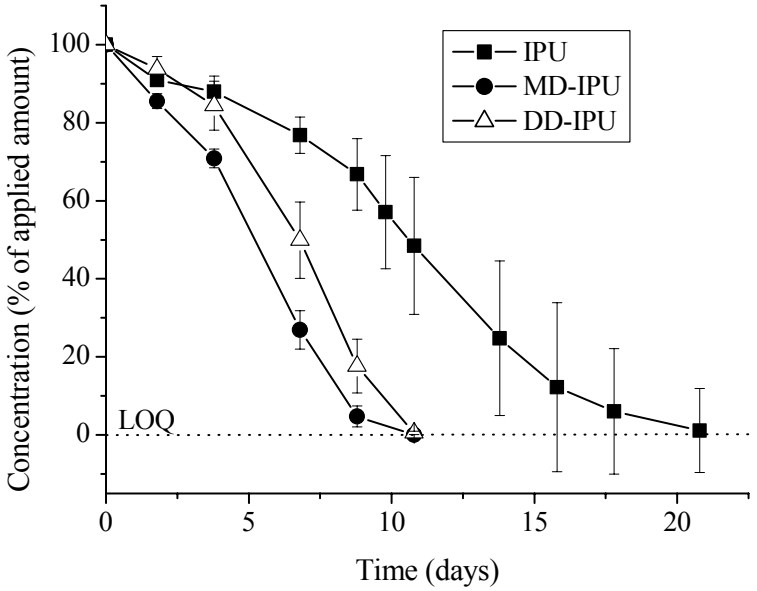


Figure 14: Development of the degradation of IPU, MD-IPU and DD-IPU in MS-medium inoculated with IMMC. Bars indicate standard deviation.

3.2.3 Degradation of Diuron

Liquid culture experiments were performed in order to elucidate if the degradation enzymes of IMMC that were responsible for the mineralization of IPU were also able to degrade other phenylurea herbicides. Diuron belongs to the group of N,N-dimethyl phenylureas. Two successive experiments were conducted: in a pre-test, the sensitivity of IMMC towards a high Diuron concentration (25 mg L^{-1}) was assessed (for experimental setup see chapter 2.5.2.1). In a second liquid culture study, a much lower Diuron concentration (1 mg L^{-1}) was utilized (for experimental setup see chapter 2.5.2.2).

3.2.3.1 Pre-test: Estimate the basic sensitivity of IMMC to high Diuron-concentrations

Disappearance of Diuron

The concentration of Diuron measured in liquid cultures inoculated with IMMC is shown in Figure 15. No degradation was discovered for a period of 51 days when 25 mg L^{-1} of Diuron was applied.

At start of the experiment, 5.6 ± 0.3 *10^6 CFUs of IMMC were applied per replicate (chapter 2.5.2.1). Unfortunately, the number of CFUs from Diuron replicates could not be utilized at days 10, 20 and 30 due to a contaminated buffer that had been used. Nevertheless, at day 39 just 0.2 ± 0.0 *10^6 CFUs were detected per replicate, which corresponds to 3.1 % of applied cells.

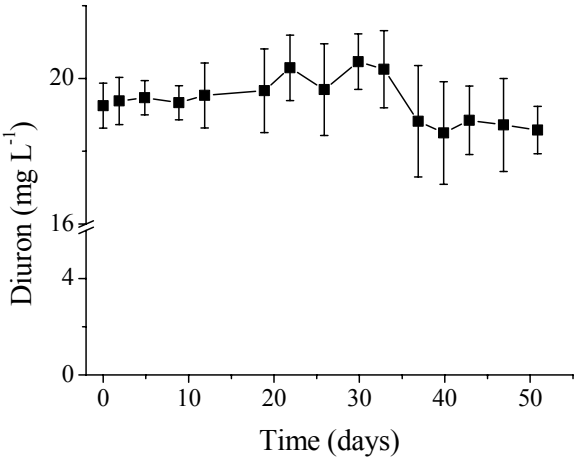


Figure 15: Development of Diuron concentration in 25 mL MS-medium, to which 25 mg L^{-1} Diuron and IMMC were applied. Bars indicate standard deviation.

3.2.3.2 Sensitivity of IMMC to low concentrations of Diuron

The pre-test showed that IMMC was not capable to degrade Diuron at high concentrations such as 25 mg L^{-1} (chapter 3.2.3.1). To avoid possible toxic effects of high Diuron concentrations on microbes, a further liquid culture experiment with IMMC was conducted with a clearly reduced concentration of 1 mg L^{-1} ^{14}C-Diuron (chapter 2.5.2.2). Figure 16 gives the cumulative mineralization curves for ring ^{14}C-labeled and side chain ^{14}C-labeled Diuron.

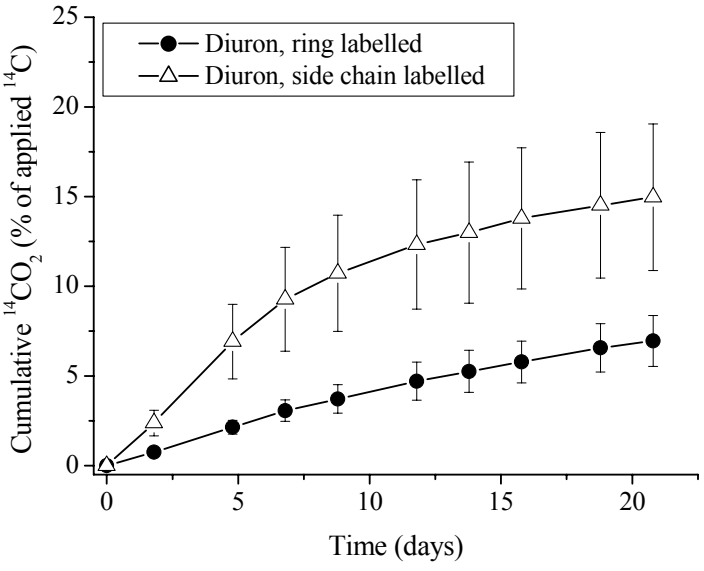


Figure 16: Development of mineralization of ring ^{14}C-labeled and side chain ^{14}C-labeled Diuron (each 1 mg L^{-1}) in MS-medium inoculated with IMMC. Bars indicate standard deviation.

Although the Diuron concentration was lowered to 1 mg L^{-1}, side chain labelled as well as ring labeled Diuron were mineralized by IMMC to a manageable extent, with a cumulative mineralization of 15.0 ± 4.1 and 7.0 ± 1.4 % of applied radioactivity, respectively.

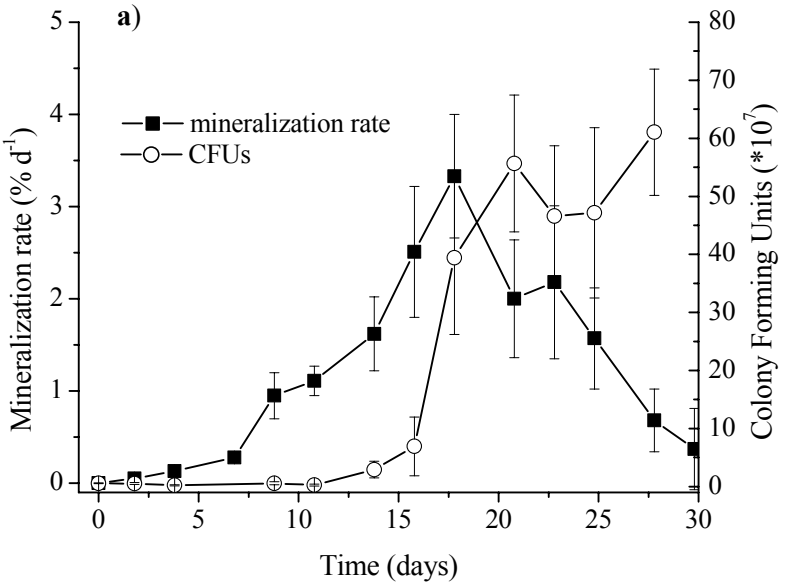
3.3 Degradation of IPU and its metabolites MD-IPU and DD-IPU by *Sphingomonas* sp. AK1 in liquid culture

Several liquid culture studies were performed to elucidate the capability of the isolated strain *Sphingomonas* sp. AK1 to degrade IPU and its metabolites MD-IPU and DD-IPU.

3.3.1 Mineralization of ^{14}C-IPU and cell dynamic of *Sphingomonas* sp. AK1

Results obtained during the isolation of *Sphingomonas* sp. AK1 indicated an influence of amino acids on its degradation performance (chapter 3.1.2.2). To further investigate the importance of amino acids for the degradation performance of *Sphingomonas* sp. AK1, a liquid culture study was conducted with MS-medium or MS-medium + amino acid mix (0.1 g L^{-1}). For experimental setup see chapter 2.6.1. At start of the experiment, 25 mg L^{-1} ^{14}C-IPU as well as a total of 0.6 ± 0.1*10^7 living cells were applied to each liquid culture (20 mL medium).

Figure 17a-b presents mineralization rates for IPU as well as the number of living cells for *Sphingomonas* sp. AK1 in both types of liquid medium. The mineralization pattern as well as the proliferation of cells was completely different depending on whether amino acids (Fig. 17a) or no amino acids (Fig. 17b) were applied to the MS-medium. In replicates with amino acids, the mineralization rate increased to a clear peak maximum (3.3 ± 0.7 % of applied ^{14}C d^{-1}) at day 18. The number of living cells proliferated considerably between day 16 and 18, up to a maximum at day 28 (61.1 ± 10.9 *10^7 CFUs $replicate^{-1}$ = 101.8 times more CFUs than applied at day 0).



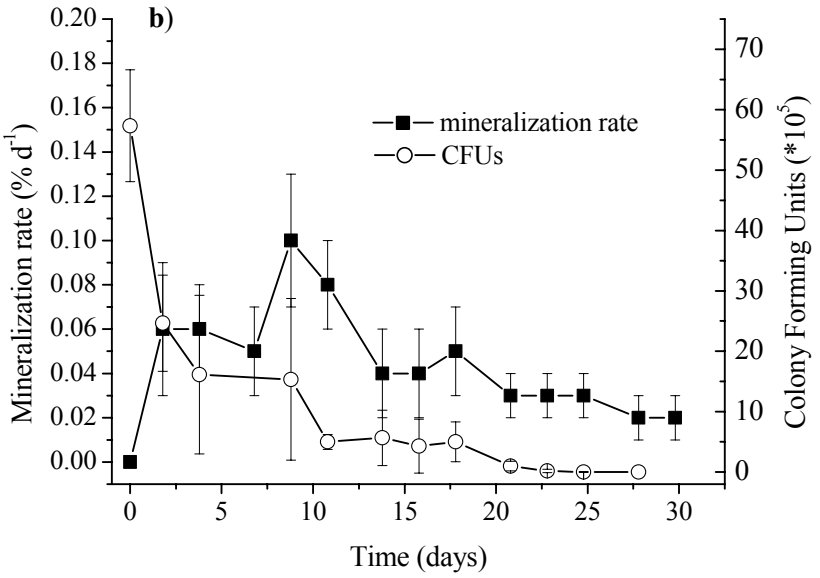


Figure 17: Development of IPU mineralization rates (closed squares) and CFUs formed by Sphingomonas sp. AK1 (open circles)in 20 mL MS-medium with 25 mg L^{-1} IPU: (a) MS-medium supplemented with 0.1 g L^{-1} amino acid mix, (b) MS-medium without amino acids. Bars indicate standard deviation.

When no amino acids were applied to the liquid medium (Fig. 17b), mineralization rates remained on a very low level for the entire experiment and cell numbers decreased from the start of experiment on. The maximum mineralization rate was measured at day 9 (0.1 ± 0.0 % d^{-1}).

In replicates that contained amino acids but not IPU or one of its metabolite, basically stable cell numbers were detected (Figure 18).

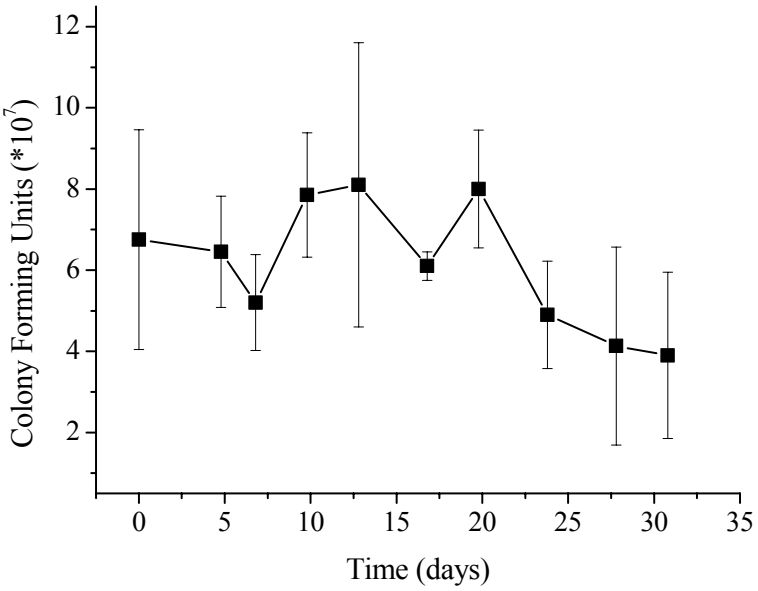


Figure 18: Development of CFUs formed by Sphingomonas sp. AK1 in liquid culture (MS-medium + amino acid mix (0.1 g L^{-1})). Neither IPU nor one of its metabolites was applied. Bars indicate standard deviations.

3.3.2 Degradation of IPU, MD-IPU and DD-IPU and formation of metabolites

Figures 19a-f show the pesticide concentrations in liquid cultures (20 mL medium) to which IPU (Fig. 19a, b), MD-IPU (c, d) or DD-IPU (e, f) was applied (25, 23.3 and 12.6 mg L^{-1} (= 121.2 µM), respectively). All liquid cultures were inoculated with *Sphingomona*s sp. AK1 (5.7 ± 0.9 *10^6 CFUs per replicate). As for the study with ^{14}C-IPU, liquid cultures contained either MS-medium (Fig. 19b, d, f) or MS-medium + amino acid mix (0.1 g L^{-1}) (a, c, e). For experimental setup see chapter 2.6.

Clear differences in degradation pattern were observed between variants with pure MS-medium (b, d, f) and those additionally supplemented with amino acid mix (a, c, e).

In all replicates supplied with the amino acid mix, a clear decrease in the concentration of the applied compound was detected. Applied IPU (Figure 19a) was no more detected by HPLC after day 11, while added MD-IPU (Figure 19c) was detectable until day 18 and applied DD-IPU until the end of experiment at day 36 (Figure 19e). At this point of time, 31.5 ± 3.0 % of applied DD-IPU was recovered in the medium. During disappearance of the applied IPU, MD-IPU or DD-IPU, respectively, just one metabolite was formed per variant. In replicates supplemented with IPU, MD-IPU was formed while 4IA was the only metabolite formed when MD-IPU or DD-IPU were applied. The disappearance of applied compounds varied clearly in speed. DD-IPU was degraded slower than MD-IPU and IPU.

Replicates that were not provided with amino acids showed a low decrease in concentration of the applied compound (Fig. 19b,d,f). At day 30, 88.2 %, 54.1 % and 78.6 % of the applied concentration was recovered in liquid cultures with IPU (Figure 19b), MD-IPU (Figure 19d) and DD-IPU (Figure 19f), respectively. No metabolites were formed in replicates amended with IPU and DD-IPU, respectively. During degradation of MD-IPU, 4IA was detected once.

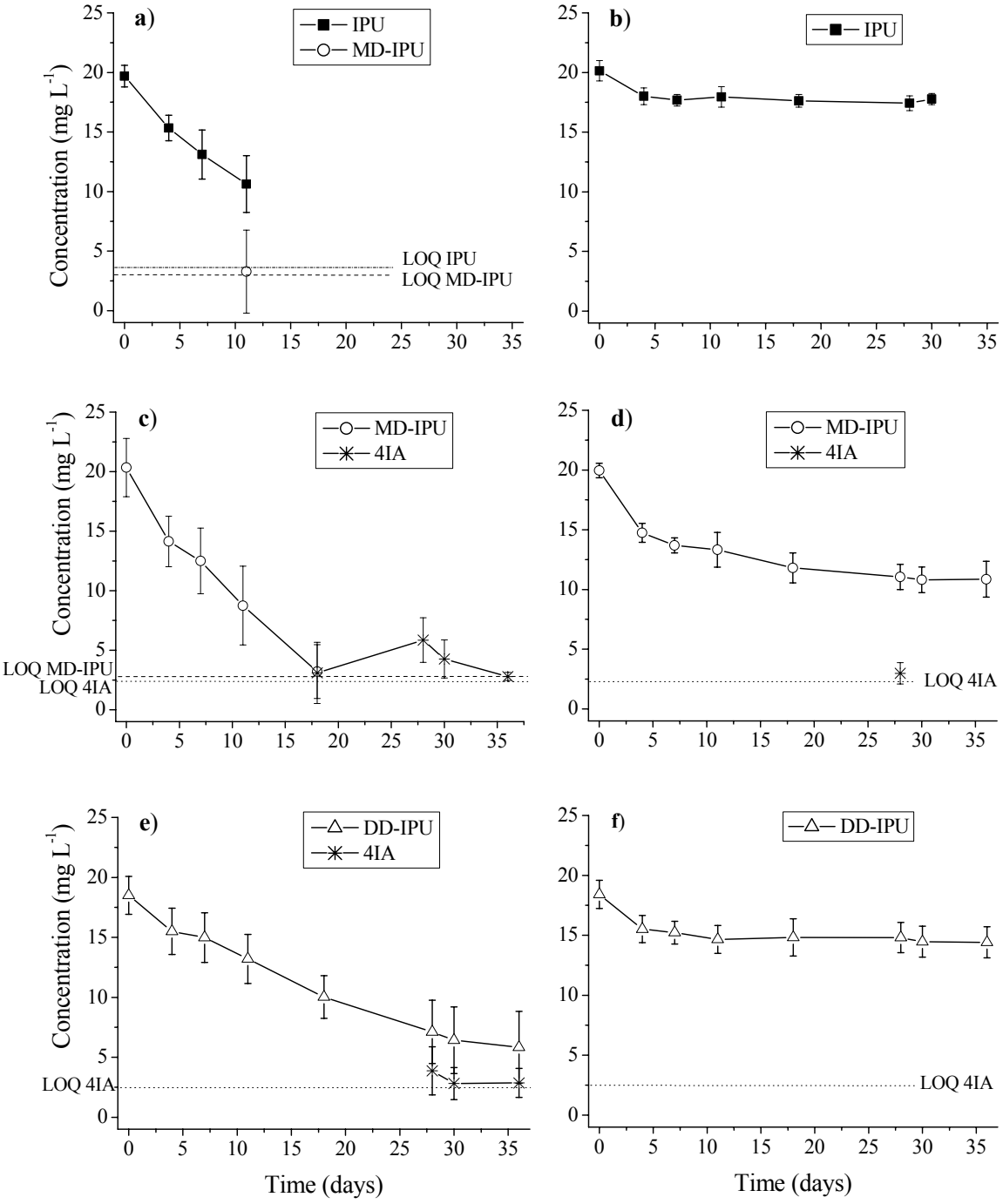


Figure 19: Development of the concentration of applied IPU (a,b), MD-IPU (c,d) and DD-IPU (e,f) and formation of metabolites by Sphingomonas sp. AK1 in liquid cultures containing MS-medium (b, d, f) or MS-medium + amino acid mix (0.1 g L^{-1}) (a, c, e). Bars indicate standard deviation.

3.4 Sustainability of the enhanced IPU mineralization function induced by inoculation of IMMC into soil

Previous inoculation experiments with IMMC in soil were restricted to a period of only 46 days (Kühn, 2003; Grundmann et al., 2007). The aim of the present experiment was to investigate how long and to what extent the increased mineralization function, which is induced by inoculation of IMMC, is preserved in soil when no more IPU is applied for several months. The experiment in this thesis was done in two phases: in phase 1, IMMC was inoculated to 50 g dry soil that contained 250 µg ^{14}C-IPU (= 5 mg kg^{-1} dry soil), while after different time intervals 250 µg ^{14}C-IPU were reapplied to the soil (phase 2) in order to test if IPU mineralization was still enhanced. For details on the experimental setup see chapter 2.8.

3.4.1 Mineralization of IPU in soil

3.4.1.1 Mineralization after inoculation with IMMC and a single application of IPU to soil (= phase 1)

Figure 20 shows the cumulative mineralization of IPU in soil Scheyern over a time period of 322 days after the first IPU-application (250 µg = 5 mg kg^{-1} dry soil) and the addition of IMMC to soil (5.0 ± 1.9 *10^5 CFUs). Along this mineralization curve the different times are marked with arrows when the second IPU applications (each 250 µg) were conducted in parallel replicates. After these time intervals, it was tested if IPU mineralization in soil was still increased by reapplying IPU to soil (chapter 2.8.1). The reapplications of IPU were designated applications 2a-2d. As the first application in the field in the course of the growing season is usually conducted in spring, application 1 might be regarded as spring application and reapplication 2b (after 6 months) can be designated as autumn application. To investigate if inoculated microbes outlived more than one growing season, after 7.5 months a winter period was simulated in the laboratory by storing soil samples in the fridge (+ 4 °C) and freezer (- 20 °C) (chapter 2.8.5.1). IPU reapplication 2d, which was done after this winter simulation period, might thus be designated as spring application of the next growing season.

When ^{14}C-IPU was added a second time to four replicates after a period of 1.5 months (= reapplication 2a) 134.7 µg of the applied 250 µg IPU in phase 1 were already mineralized to $^{14}CO_2$ whereas 160.9 µg of this amount were mineralized after a period of 6 months (= reapplication 2b, simulated autumn) and 162.1 µg after 7.5 months (= reapplication 2c). After simulating the winter period, a total of 164.4 µg IPU were mineralized (= reapplication 2d). At the end of phase 1 after 322 days, 174.7 µg IPU were mineralized.

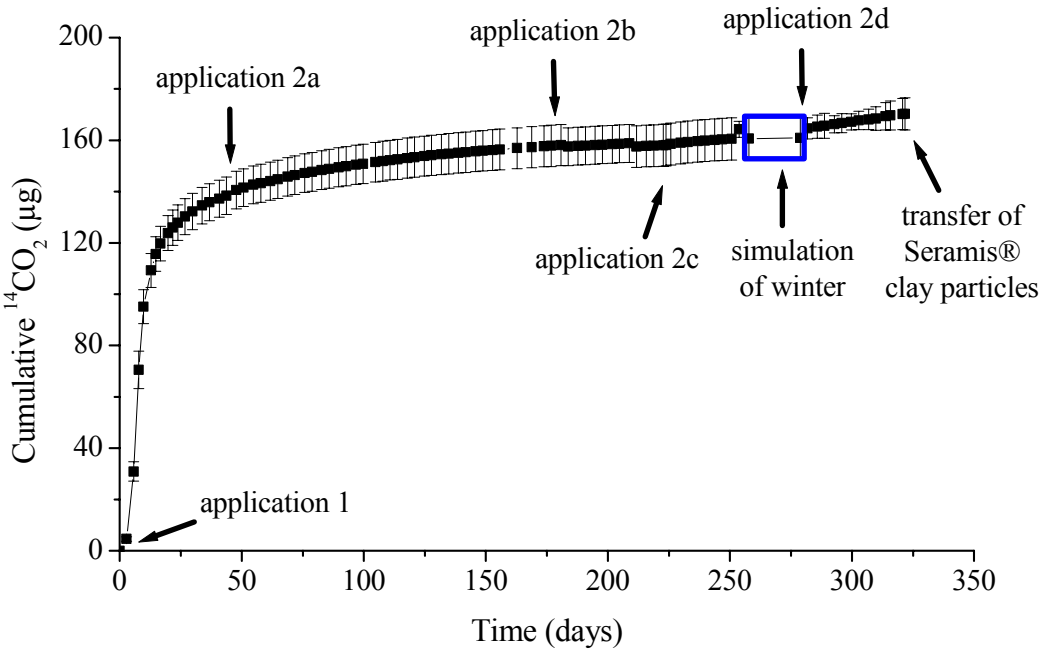


Figure 20: Development of cumulative mineralization of IPU after IPU application 1 to soil Scheyern inoculated with IMMC. Arrows are highlighting times of application 1 (= day 0) and applications 2a to 2d (1.5, 6 and 7.5 months after application 1 as well as after simulation of a winter period) as well as the removal of clay particles for testing the mineralization capability of attached microbes at day 322. Bars indicate standard deviation.

The mineralization rate for phase 1 is shown in Figure 21a. After application of ^{14}C-IPU to soil, the mineralization rate increased rapidly and reached a maximum daily rate of 19.8 µg d^{-1} at day eight. Afterwards, rates declined slowly and fell below a level of 1µg d^{-1} after 24 days. Rates stayed below this level until the end of the experiment, with a mineralization rate < 0.2 µg d^{-1} at day 322.

3.4.1.2 Mineralization of IPU after reapplication of IPU to soil (= phase 2)

To monitor to what extent the increased IPU mineralization function was preserved in soil, 250 µg ^{14}C-IPU were added a second time to 4 replicates 1.5, 6 and 7.5 months after application 1 as well as after a simulated winter period (= reapplications 2a to 2d) (chapter 2.8.5.1). Mineralization in phase 2 was measured for 24, 27, 27 and 21 days for reapplications 2a to 2d.

As the ^{14}C-IPU applied in phase 1 was not mineralized completely until ^{14}C-IPU was reapplied at beginning of phase 2, the $^{14}CO_2$ originating from phase 1 was subtracted from the measured mineralization in phase 2 in order to get the amount of ^{14}C-IPU, solely originating from application 2 (chapter 2.8.5.2). To determine the mineralization derived from phase 1 (background mineralization), the respective mean mineralization rate was calculated for each reapplication 2a-d in those replicates not receiving application 2 at this point of time.

The ^{14}C-IPU applied in phase 2 was mineralized quickly and in similar quantities as in phase 1. At the end of phase 2a, 167.3 µg were mineralized to $^{14}CO_2$ and thus a higher amount than in phase 1

(127.9 µg). However, mineralization at the end of phases 2b-d (144.1 µg, 145.1 and 141.6 µg, respectively) was lower than at the end of phase 2a, but still higher than at the respective time points of phase 1 (130.3, 130.3 respectively 124.9 µg).

Figures 21b-e show the daily mineralization rates for all reapplications as well as for application 1 (Fig. 21a). After reapplication of 250 µg ^{14}C-IPU, the mineralization rate increased for all reapplications until a maximum daily rate was detected, then, rates declined slowly until the end of phase 2. For phase 2a, the maximum daily rate amounted to 31.1 µg d^{-1}, while for phases 2b to 2d maximum rates of 27.3, 22.3 and 23.8 µg d^{-1} were detected.

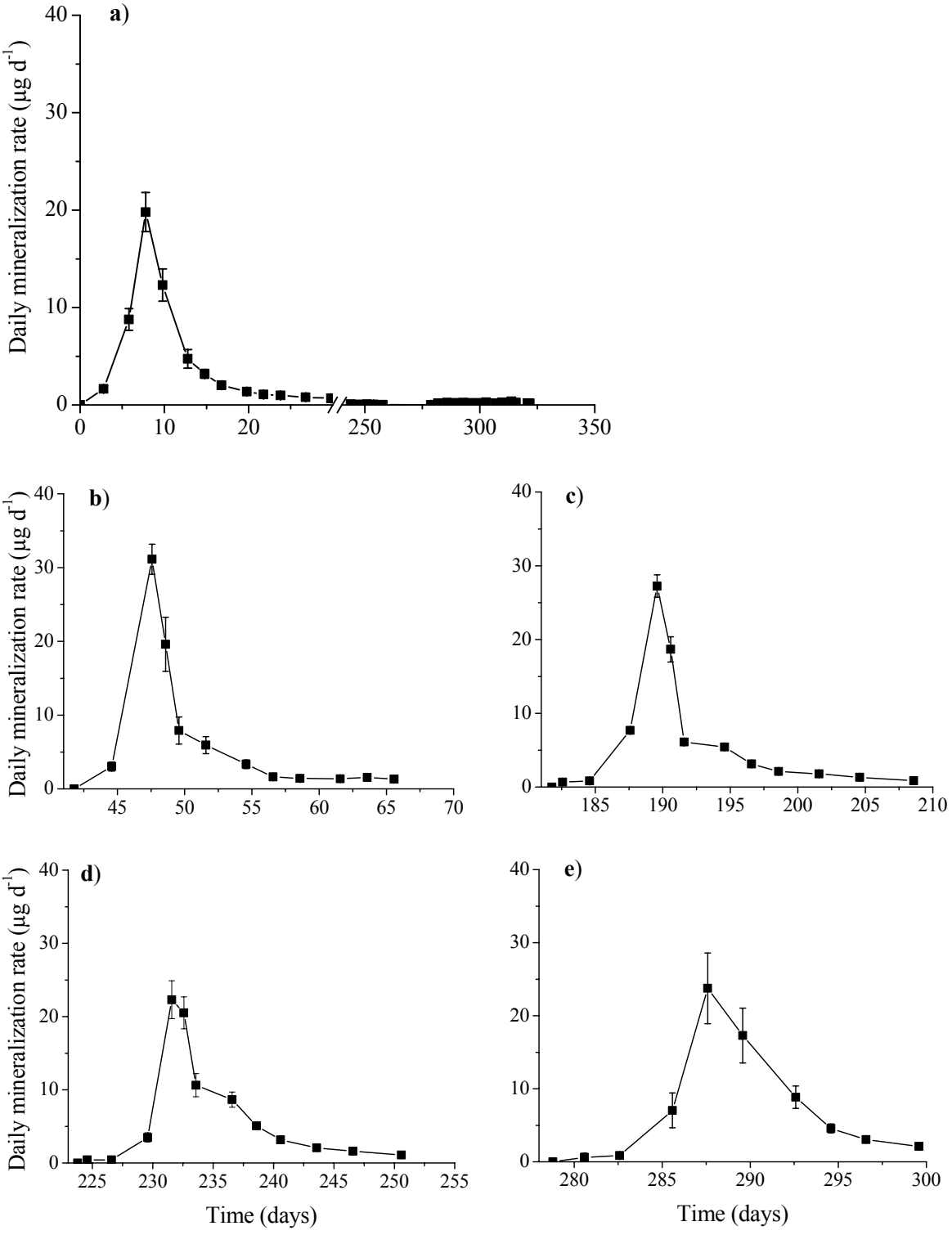


Figure 21: Development of daily IPU mineralization rates for IPU applications 1 and 2 in soil Scheyern inoculated with IMMC: (a) application 1, (b-e) applications 2a to d (1.5, 6 and 7.5 months after application 1 as well as after simulation of winter). Bars indicate standard deviation.

Figure 22 enables a direct comparison of peak positions for IPU applications 1 and 2a to 2d, by defining the respective last application of IPU as day 0. The maximum daily mineralization rate

after application 1 was measured at day 8, while the maximum rates for reapplications 2a to 2d were detected 5.8 ± 0.0, 7.8 ± 0.0, 8.3 ± 0.6 and 9.3 ± 1.0 days after reapplication of ^{14}C-IPU to soil.

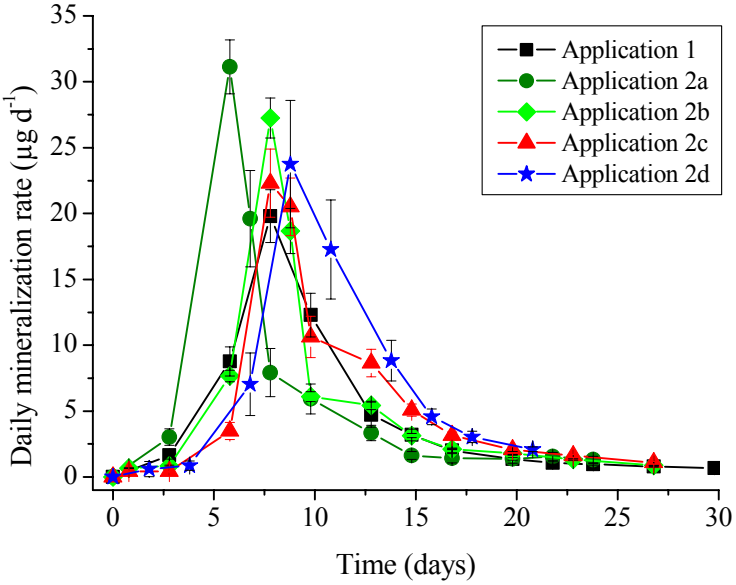


Figure 22: Development of daily IPU mineralization rates for IPU applications 1 and 2 with the respective last application of IPU defined as day 0, allowing a direct comparison of peak positions. Applications 2a to 2d were carried out 1.5, 6 and 7.5 months after application 1 as well as after simulation of winter. Bars indicate standard deviation.

3.4.1.3 Statistical analysis of mineralization dynamics in phases 1 and 2

Investigated Parameters

To evaluate if mineralization dynamics in phases 2a-d were statistically different from the one in phase 1, two parameters were used:

(1) maximum mineralization rates

(2) the period of time until the maximum mineralization rate was attained

Quality of fitting

Mineralization rates were fitted by the nonlinear Gompertz function

$$y = a*\exp(-0.5*(\log(x/x0)/b)^2)$$

where *a* is the maximum mineralization rate, *x0* the day at which the maximum rate is attained and *b* a shape parameter.

Fitted curves for mineralization rates in phases 1 and 2 are shown in chapter A1.3.1, Appendix.

In linear regression models, the coefficient of determination, known as R^2, is used to express the quality of fit of a model. As in nonlinear regression the quality of a fit is not readily defined, a measure relatively closely corresponding to R^2 in the nonlinear case is used, the "Pseudo-R^2". It was calculated according to the following formula

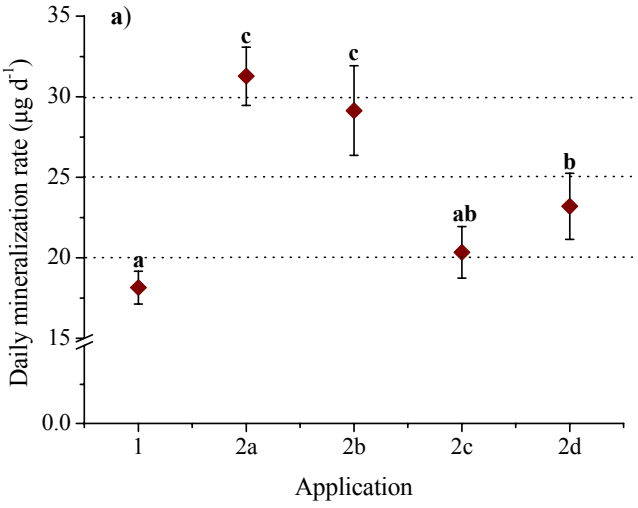
$$\text{Pseudo - } R^2 = \frac{\text{Sum of squares model}}{\text{Sum of squares total}}$$

The calculated values for Pseudo-R^2 are given within chapter A1.3.1, Appendix. The Gompertz function resulted in a good fitment to measured data with exact modeling for height and position of peaks.

Statistical significance

In Figure 23, estimates and confidence intervals (95 %) are given for the maximum mineralization rates (Fig. 23a) and the number of days when the maximum rate was reached (Fig. 23b). Values are given for applications 1 and 2. In case of the maximum mineralization rate, the estimate quotes the value that the determined curve indicates at the respective day *x0*. For the number of days to maximum mineralization, the estimate gives the value that the fitted curve indicates at the respective maximum mineralization rate *a*.

As presented in Figure 23a, the estimated daily maximum mineralization rate for application 1 (18.2 µg d^{-1}) was lower than the maximum rates for applications 2 a-d (31.4, 29.1, 20.3 and 23.2 µg d^{-1}, respectively). However, according to the position of confidence intervals (95 %), the estimated daily maximum mineralization rate of application 1 (confidence interval (CI) 17.1 – 19.2 µg d^{-1}) was not significantly different from the one of application 2c (CI 18.7 – 21.9 µg d^{-1}), while it differed significantly from the estimated maximum rates of applications 2a (CI 29.5 – 33.1 µg d^{-1}), b (26.4 – 31.92 µg d^{-1}) and 2d (CI 21.1 – 25.3 µg d^{-1}).



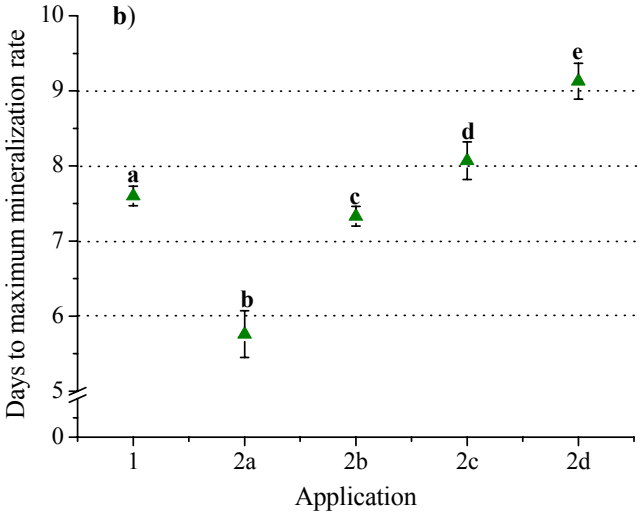


Figure 23: Estimates and confidence intervals (95 %) for maximum mineralization rates of IPU and time periods to maximum rates for applications 1 and 2: (a) maximum mineralization rates, (b) days passing by until the maximum mineralization rate was measured. Values are given for phase 1 and phases2a-d (1.5, 6 and 7.5 months after application 1 as well as after simulation of winter). Values with the same letter assigned are not significantly different (α = 0.05) according to One-way ANOVA and Tukey HSD test.

According to Figure 23b, estimated 7.6 days passed until the maximum mineralization rate for application 1 was reached. The period estimated for application 1 was significantly longer than the ones estimated for reapplications 2a and 2b (5.7 respectively 7.3 days), while it was significantly shorter than the estimates for reapplications 2c and 2d (8.1 respectively 9.1 days). The confidence intervals encompassed generally a very narrow time range (application 1: 7.5 – 7.7 d; applications 2 a–d: 5.5 – 6.1, 7.2 – 7.5, 7.8 – 8.3, 8.9 – 9.4 d), which might be attributed to the very small standard errors (application 1: ± 0.1; applications 2 a–d: ± 0.2, 0.1, 0.1 and 0.1).

3.4.2 Identification and quantification of extractable ^{14}C-IPU residues in soil

3.4.2.1 Extractable IPU residues after a single application of IPU to soil (phase 1)

To determine the supply of soil microbes with IPU during phase 1, extractable ^{14}C-IPU residues were identified and quantified at different time intervals. The residues dissolved in the pore water indicate the fraction that is bioavailable to the microbes at the time of sampling, while the MeOH-extractable fraction might be subsequently delivered by desorption.

Amount of IPU residues dissolved in pore water and MeOH extract

As shown in Figure 24, the quantity of ^{14}C-IPU residues dissolved in pore water decreased rapidly with time after IPU application 1. While at time of application 1 (= day 0), 67.3 µg ^{14}C (= 26.9 ± 0.8 % of applied ^{14}C) were dissolved in pore water and were thus bioavailable (for

determination see chapter 2.8.6.1, for results see chapter A1.4, Appendix), at day 6 just 48.3 ± 1.8 µg ^{14}C (= 19.3 %) were present in soil solution. After 4 weeks (day 30), only marginal amounts were detected in pore water (1.8 ± 0.1 µg ^{14}C (= 0.7 % of applied ^{14}C)). Further on, the amount of ^{14}C in soil pore water was at a very low level until the end of the experiment, with 0.6 ± 0.0 µg ^{14}C (= 0.2 % of applied ^{14}C) measured at day 322.

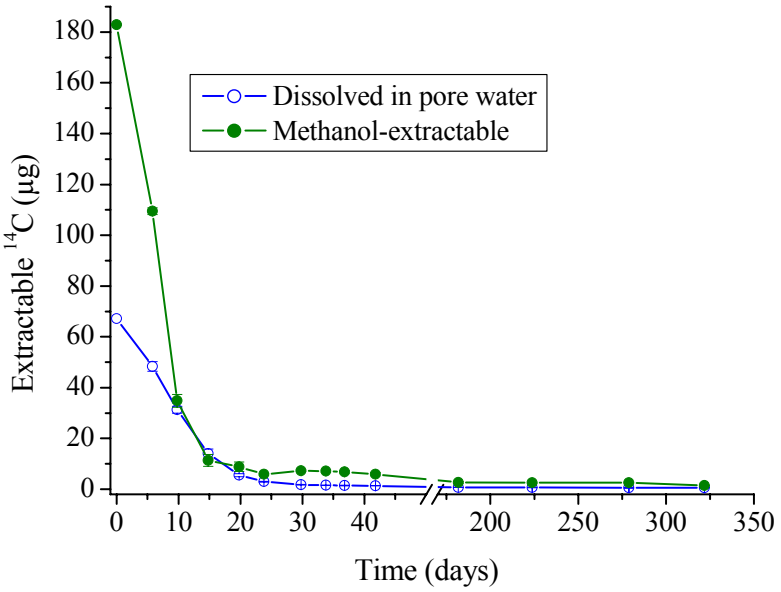


Figure 24: Development of the quantity of ^{14}C-IPU residues dissolved in pore water and extractable by MeOH, respectively, after IPU application 1. Quantities are given in µg IPU equivalents. Bars indicate standard deviation.

The amount of MeOH-extractable IPU residues is also shown in Figure 24. Parallel to ^{14}C in pore water, the quantity of MeOH-extractable ^{14}C decreased fast within 4 weeks after application. At day 0, 182.8 µg ^{14}C (= 73.1 ± 0.8 % of applied ^{14}C) were detected, while after 30 days just 7.3 ± 0.3 µg ^{14}C (= 3.0 % of applied ^{14}C) were measured.

Identification of IPU residues in pore water

In Figure 25, the metabolites detected in pore water are given. The analysis by HPLC allowed to distinguish IPU and 8 different metabolites in pore water. Four of these compounds could be identified due to their retention time by means of reference substances (chapter 2.1.1). IPU and its metabolites could be detected only for a maximum period of 24 days after application 1, afterwards their concentrations fell below the LOD. IPU was detected in pore water in highest quantity compared to other metabolites just for day 0 (66.4 µg = 26.6 % of applied ^{14}C; for determination see chapter 1.4, Appendix) and day 6 (31.7 ± 1.4 µg), at day 15 its amount fell already below 1 % (0.8 µg) and was no more detected in later samplings. The metabolites 2-OH-MD-IPU, MD-IPU and Unknown 3 were formed in a maximum quantity of 7.2 (day 15), 3.9 (day 6) and 14.3 µg (day

10) respectively and were subsequently degraded. The metabolites Unknown 1, Unknown 2, Unknown 5 and DD-IPU were detected during the experiment just in marginal quantities (at most 0.2, 0.2, 0.3 and 0.1 µg, respectively).

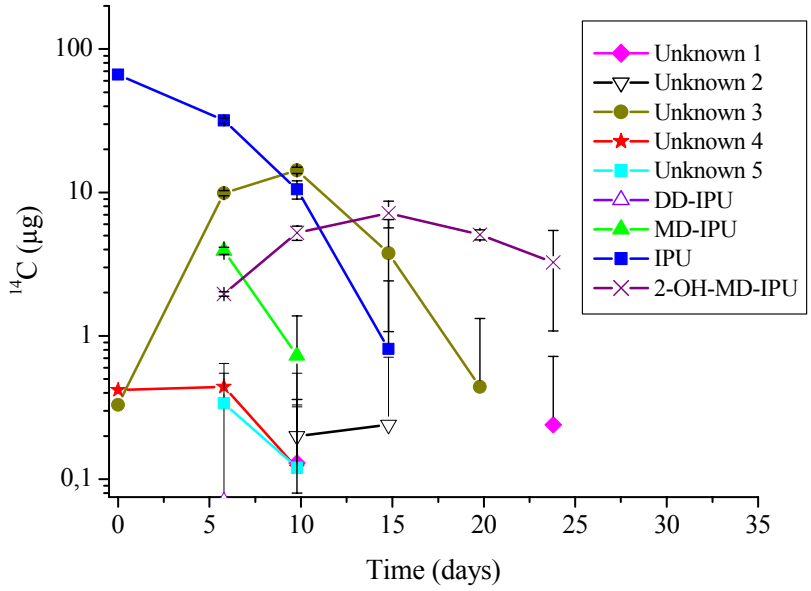


Figure 25: Composition of ^{14}C-IPU residues dissolved in pore water after application 1. Metabolites Unknown 1-5 could not be identified as standards were not available. After day 24, no more metabolites were detected by HPLC. Bars indicate standard deviation.

Identification of Methanol-extractable IPU residues

Methanol-extractable metabolites after IPU application 1 are shown in Table 16. IPU and 15 metabolites were distinguished by HPLC, 5 of them were identified due to their retention time by means of reference substances (chapter 2.1.1). At all samplings, IPU was the compound in highest abundance. MD-IPU was the only metabolite that was formed in high quantity (maximum of 19.9 µg at day 6). The metabolites DD-IPU and Unknown 4, Unknown 5 and Unknown 6 were at most measured in quantities of 1.1, 1.6, 1.7 and 4.5 µg, while all other metabolites were detected in marginal quantities (< 1 µg). Metabolites were detected until the end of phase 1 (day 322).

Table 16: Composition of Methanol-extractable IPU residues for a period of 322 days after IPU application 1 (see also next page). Metabolites Unknown 1-11 could not be identified by HPLC as standards were not available. See next page for days 37-322.

Peak	Retention time (min)	Methanol-extractable ^{14}C (µg)							
		Days after application 1							
		0	6	10	15	20	24	30	34
Unknown 1	10.52				0.03 ± 0.05				
2-OH-MD-IPU	11,82 ± 0.16		0.57 ± 0.20				0.15 ± 0.34	0.13 ± 0.04	0.09 ± 0.09
Unknown 2	12.32 ± 0.02					0.04 ± 0.07			
Unknown 3	12.82 ± 0.11	0.90	4.52 ± 0.27		1.69 ± 2.08	1.36 ± 0.55	1.46 ± 0.45	0.57 ± 0.20	0.58 ± 0.23
Unknown 4	13.75 ± 0.11		1.56 ± 0.44	1.70 ± 0.36	1.46 ± 0.24	0.76 ± 0.12	0.59 ± 0.12	0.29 ± 0.12	0.33 ± 0.16
Unknown 5	14.04 ± 0.05	1.15			0.13 ± 0.22	0.05 ± 0.08			
Unknown 6	15.09 ± 0.09			0.24 ± 0.03	0.24 ± 0.03	0.15 ± 0.14			
Unknown 7	16.10 ± 0.12		0.64 ± 0.05	0.44 ± 0.17	0.21 ± 0.02	0.06 ± 0.10			
DD-IPU	17.41 ± 0.07		1.07 ± 0.00	0.45 ± 0.06	0.24 ± 0.08	0.16 ± 0.03	0.09 ± 0.10	0.11 ± 0.14	0.16 ± 0.06
MD-IPU	18.41 ± 0.07		19.89 ± 0.85	4.70 ± 0.75	1.82 ± 0.90	1.12 ± 0.09	1.34 ± 0.54	1.08 ± 0.48	0.98 ± 0.27
Unknown 8	18.90								
IPU	19.57 ± 0.07	180.78	76.68 ± 1.44	19.42 ± 0.96	4.01 ± 0.22	3.21 ± 0.17	3.25 ± 1.40	2.68 ± 1.24	2.17 ± 0.72
4IA	20.63 ± 0.45				0.07 ± 0.07	0.05 ± 0.08			
Unknown 9	21.49 ± 0.10		0.43 ± 0.01	0.27 ± 0.06	0.15 ± 0.15	0.22 ± 0.08	0.05 ± 0.00		
Unknown 10	22.17 ± 0.05		0.23 ± 0.07						
Unknown 11	23.70 ± 0.09		0.16 ± 0.02		0.05 ± 0.10				
Sum		182.83	105.75	27.25	10.10	7.17	6.91	4.86	4.32

Continuation of Table 16: Composition of Methanol-extractable IPU residues for a period of 322 days after IPU application 1 (see also page before). Metabolites Unknown 1-11 could not be identified by HPLC as standards were not available.

Peak	Retention time (min)	Methanol-extractable ^{14}C (µg)					
		Days after application 1					
		37	42	182	224	279	322
Unknown 1	10.52						
2-OH-MD-IPU	11,82 ± 0.16	0.06 ± 0.07	0.05 ± 0.07				
Unknown 2	12.32 ± 0.02			0.14 ± 0.12	0.02 ± 0.04	0.06 ± 0.07	0.12 ± 0.02
Unknown 3	12.82 ± 0.11	0.31 ± 0.25	0.34 ± 0.16	0.11 ± 0.11	0.22 ± 0.08	0.10 ± 0.08	0.08 ± 0.01
Unknown 4	13.75 ± 0.11	0.17 ± 0.11	0.20 ± 0.09	0.10 ± 0.03	0.09 ± 0.06	0.02 ± 0.03	
Unknown 5	14.04 ± 0.05						
Unknown 6	15.09 ± 0.09	0.05 ± 0.09		0.09 ± 0.08			
Unknown 7	16.10 ± 0.12			0.04 ± 0.07			
DD-IPU	17.41 ± 0.07	0.10 ± 0.12	0.14 ± 0.05	0.03 ± 0.06			
MD-IPU	18.41 ± 0.07	0.95 ± 0.34	0.85 ± 0.33	0.43 ± 0.07	0.37 ± 0.06	0.40 ± 0.09	0.32 ± 0.08
Unknown 8	18.90			0.02 ± 0.03			
IPU	19.57 ± 0.07	2.17 ± 0.45	2.14 ± 0.81	0.95 ± 0.84	0.93 ± 0.15	0.91 ± 0.24	0.49 ± 0.08
4IA	20.63 ± 0.45						
Unknown 9	21.49 ± 0.10	0.04 ± 0.08		0.05 ± 0.09		0.03 ± 0.06	
Unknown 10	22.17 ± 0.05						
Unknown 11	23.70 ± 0.09						
Sum		3.84	3.71	1.95	1.63	1.52	1.01

3.4.2.2 Extractable IPU residues after reapplication of IPU to soil (phase 2)

The amount of extractable ^{14}C-IPU residues in phase 2 was only determined at the end of phase 2 when soil incubators were stopped. Although 250 µg IPU had been applied to soil at applications 2a to 2d, only marginal extractable amounts were detected at the end of the individual phases. At the end of phase 2a, a total of 2.2 ± 0.9 µg ^{14}C was dissolved in pore water and MeOH extracts. Similar amounts were measured at the end of phases 2b (4.1 ± 2.5 µg), 2c (3.5 ± 2.2 µg) and 2d (5.7 ± 4.4 µg). Phases 2a, b, c and d lasted 24, 27, 27 and 21 days, respectively.

Due to the low radioactivity in soil samples of phase 2 (chapters 2.8.4.1 & 2.8.4.2), the LOQ of the HPLC system was not achieved. Thus, extractable IPU residues at the end of phase 2 could not be identified.

3.4.3 Distribution of ^{14}C during phase 1 and at the end of phases 2a to 2d

3.4.3.1 Distribution of ^{14}C during phase 1

96.5 –110.8 % of the applied ^{14}C was recovered in the investigated four fractions (mineralization, residues dissolved in pore water, Methanol-extractable IPU residues (MER) and non-extractable IPU residues). Figure 26 shows the fractions for all samplings during phase 1.

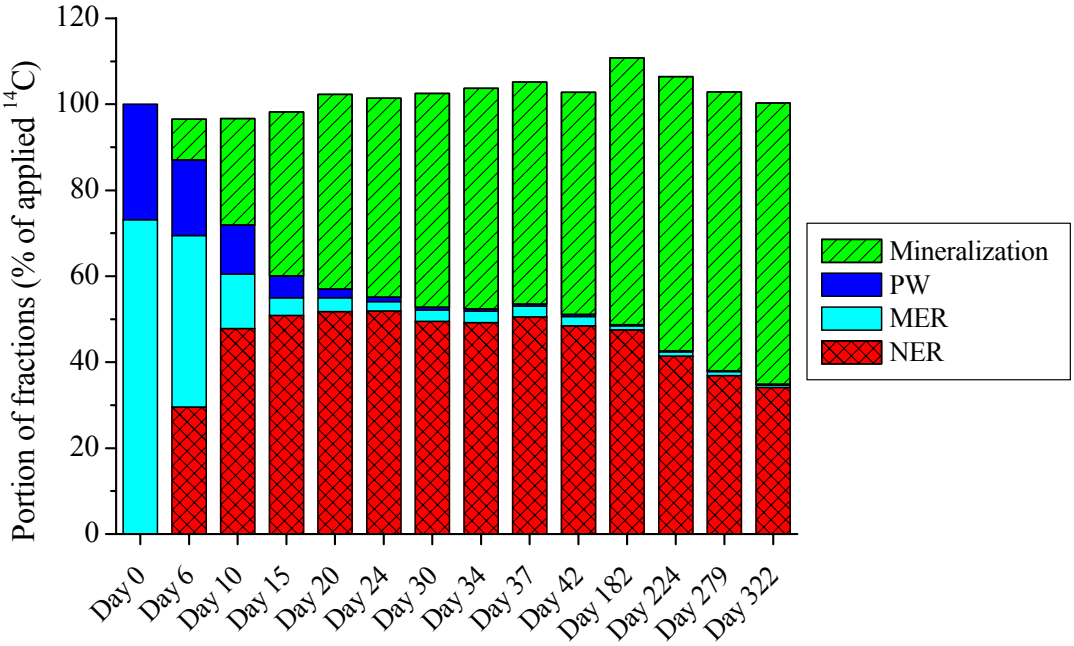


Figure 26: Development of the distribution of applied ^{14}C in soil Scheyern inoculated with IMMC after IPU application 1 (= day 0). Applications 2a to 2d were done 42, 182, 224 and 279 days, respectively, after application 1. Complexes of IMMC and Seramis® clay particles were transferred 322 days after application 1.

While at start of the experiment (day 0), the applied ^{14}C was existent either dissolved in pore water or Methanol-extractable (for determination see chapter A1.4, Appendix), these two fractions

decreased quickly in the course of experiment. At day 15, these fractions already accounted together for < 10 % of applied ^{14}C. Parallel to the decrease of these fractions, similar portions for mineralization and NER were measured. The ratio of all investigated pathways stayed at a similar level from days 20 to 41, with similar quantities of ^{14}C mineralized and detected as NER. From day 182 (= application 2b) on, the amount of NER decreased while mineralization increased. However, the increase in mineralization was lower than the decrease of NER amounts.

3.4.3.2 Distribution of ^{14}C at the end of phases 2a to 2d

At the end of phases 2a to 2d, 91.3 to 96.7 % of ^{14}C applied in phases 1+2 were recovered. Table 17 presents the values measured at the end of phases 2a to 2d as well the share of fractions in % of the ^{14}C totally applied in phases 1+2.

A different distribution of ^{14}C portions in soil was detected after two applications of IPU (Table 17) than it has been determined after one application (Figure 26): at the end of applications 2a to 2d, approximately 2/3 of the ^{14}C that was applied in phases 1+2 was mineralized (column "Cum. mineralization", "Phase 1+2", "%"), while about 1/3 was detected as NER (column "NER", "%"). Only marginal amounts of extractable IPU residues were detected at the end of phases 2a to 2d (column "MER + PW").

Table 17: Distribution of applied ^{14}C in soil Scheyern inoculated with IMMC on Seramis® clay particles at the end of phases 2a to 2d. Sampling was done 24, 27, 27 respectively 21 days after IPU applications 2a to 2d. The percentages given refer to the total amount of ^{14}C applied in IPU applications 1 respectively 2a to 2d (250 µg + 250 µg = 500 µg).

	Recovery of applied ^{14}C									
	Cum. mineralization				MER + PW		NER		Sum	
	Phase 1	Phase 2	Phase 1+2							
Application	µg	µg	µg	%	µg	%	µg	%	µg	%
2a	142.0 ± 8.4	167.8 ± 16.3	309.7 ± 16.3	61.9 ± 3.3	2.2 ± 0.9	0.4 ± 0.2	166.6 ± 6.7	33.3 ± 1.3	478.5 ± 11.3	95.7 ± 2.3
2b	170.4 ± 7.2	144.3 ± 6.6	314.7 ± 6.6	62.9 ± 1.3	4.1 ± 2.5	0.8 ± 0.5	143.2 ± 10.1	28.6 ± 2.0	462.0 ± 16.0	92.4 ± 3.2
2c	175.2 ± 5.8	145.4 ± 6.2	320.6 ± 6.2	64.1 ± 1.2	3.5 ± 2.2	0.7 ± 0.4	144.2 ± 8.1	28.8 ± 1.6	468.3 ± 13.9	93.7 ± 2.8
2d	178.0 ± 5.3	141.2 ± 19.8	311.5 ± 19.8	62.3 ± 4.0	5.7 ± 4.4	1.1 ± 0.9	131.7 ± 23.2	26.3 ± 4.6	456.6 ± 26.3	91.3 ± 5.3

3.4.4 Survival of IMMC on Seramis® clay particles

To examine indirectly if microbes of IMMC may have survived on Seramis® clay particles until reapplication of IPU and may thus be responsible for IPU mineralization in phase 2, Seramis® clay particles were removed from surrounding soil material 322 days after IPU application 1 and were immediately transferred to new soil incubators (chapter 2.8.5.4). The new soil incubators contained 50 g (dry weight) of equilibrated soil Scheyern and 250 µg ^{14}C-IPU (= 5 mg kg^{-1} dry soil).

The cumulative mineralization as well as the mineralization rate in this phase 2 is shown in Figures 27a,b. The mineralization dynamics exhibited a clear metabolic mineralization pattern for all replicates. Until day 51, when the mineralization rate decreased to a rate of < 1 µg d^{-1}, 137.9 µg of the applied 250 µg were mineralized (= 55.1 % of applied ^{14}C-IPU). In phase 1, a similar amount was mineralized (141.2 µg after 51 days = 56.5 % of applied ^{14}C-IPU). Nevertheless, compared to the dynamics of phase 1, IPU was mineralized slower, with 15 days until the maximum rate was achieved (phase 1: 8 days) and a maximum rate of 5.7 µg d^{-1} (phase 1: 19.8 µg d^{-1}).

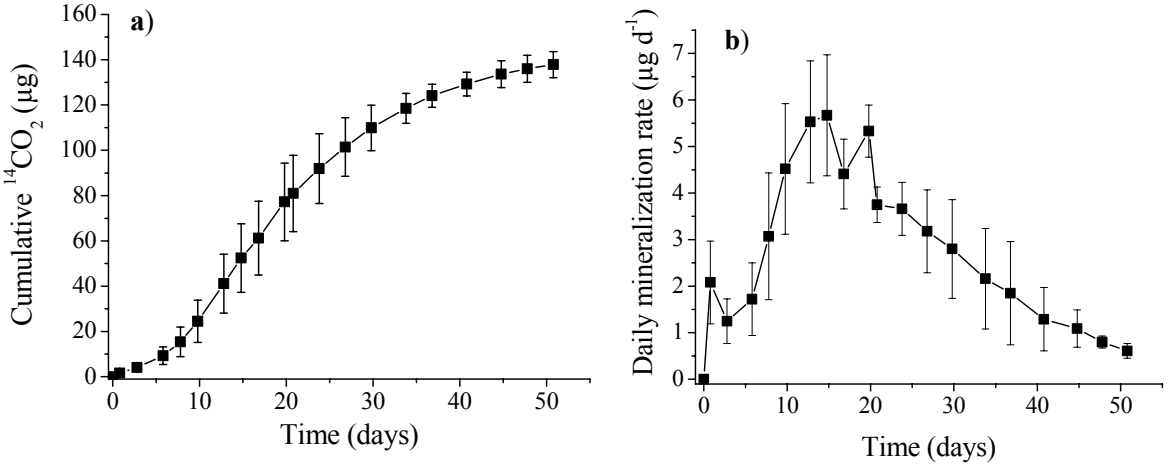


Figure 27: Development of ^{14}C-IPU mineralization in soil Scheyern inoculated with Seramis® clay particles that were removed from phase 1 322 days after IPU application 1: (a) cumulative mineralization, (b) mineralization rates. IMMC had been established on the Seramis® clay particles before IPU application 1. Bars indicate standard deviation.

3.5 Adaptation of microbes in native soils to IPU mineralization by repeated IPU applications

Aim of this experiment was to investigate if a multiple application of IPU, carried out in short time intervals of about four weeks, may lead to an accelerated degradation of IPU in soils.

3.5.1 Effect of repeated IPU application on IPU degradation in soil Feldkirchen

For this experiment, soil Feldkirchen was selected as is was demonstrated to mineralize IPU cometabolically after a single application of IPU to soil (Folberth et al., 2009). For experimental setup see chapter 2.9.1.

3.5.1.1 Effect on IPU mineralization

Mineralization rates for applications 1-4 are given in Figure 28. At start of the experiment, 16 replicates (= F1-F16) were set up (chapter 2.9.1.1). For residue analysis, four replicates were stopped at the end of phases C1, C2 and C3, respectively. To display the mineralization dynamics during all four phases C1-C4, mineralization rates are shown for the six replicates (= F1-F6) that passed through all four phases and were stopped at the end of phase 4 (= 118 days after application 1) and utilized subsequently for analysis of ^{14}C-IPU residues.

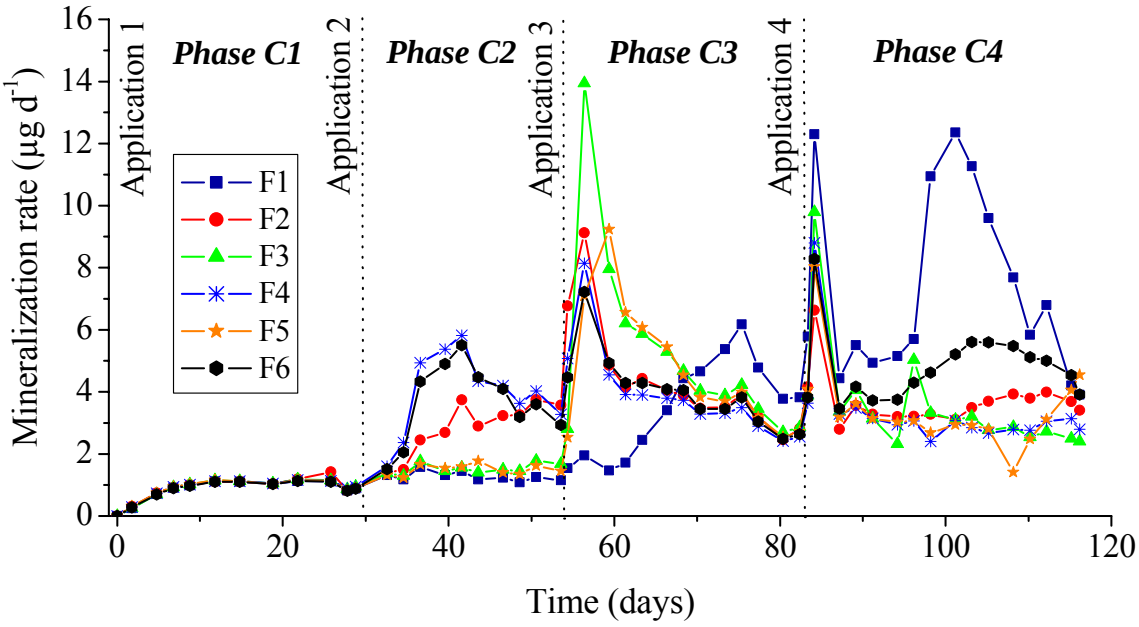


Figure 28: Development of IPU mineralization rates in soil Feldkirchen after 1, 2, 3 and 4 applications of ^{14}C-IPU. ^{14}C-IPU was applied at days 0, 29, 55 and 85.

Mineralization measurements revealed constant rates at low level (mean 0.9 ± 0.0 µg d^{-1}) after application 1 (= phase C1). In phase C2, mineralization rates were elevated in three replicates (F2, F4, F6), causing a maximum standard deviation of 2.0 µg d^{-1} (phase C1: 0.1 µg d^{-1}) at day 42. In phases C3 and C4, the maximum rate was elevated compared to phase C2 (phase C2: 3.3 ± 2.0 µg d^{-1}, phase C3: 9.1 ± 2.8 µg d^{-1} (except replicate F1), phase C4: 8.3 ± 1.2 µg d^{-1} (except replicate F1)).

While in phase C1 constant rates were observed, the period to the maximum mineralization rate reduced in the course of the experiment: the maximum rate in phase C2 was measured 12.2 ± 2.6 days after IPU application 2, in phase C3 it was detected 3.4 ± 1.3 days after application 3 (except replicate F1) and in phase C4, the maximum mineralization rate was observed 0.8 ± 0.0 days after application 4 (except replicate F1).

Replicate F1 showed a distinct delayed mineralization pattern: in phase C3, the maximum rate was measured after 21.8 days while in phase C4, even two peaks with high rates were detected (after 0.8 and 7.8 days).

Statistical analysis of mineralization dynamics in phases C1, 2, 3 and 4

Changes in mineralization dynamics for phases C1-4 were evaluated by

(1) maximum mineralization rates

(2) the period of time until the maximum mineralization rate was detected

As conducted for the inoculated soil Scheyern, mineralization rates were fitted by a nonlinear Gompertz function and the goodness of fits was calculated as Pseudo-R^2 (chapter 3.4.1.3). In chapter A1.3.2, Appendix, the fitted curves for mineralization rates as well as corresponding Pseudo-R^2 are displayed. Values for Pseudo-R^2 show a good adjustment of fitted curves to measured data of phases C1-C3. In phase C4, a reasonable fit of the Gompertz function (0.95) could only be achieved when excluding replicate F1 as this replicate showed a considerably different mineralization dynamics than replicates F2-F6.

Estimates and confidence intervals (95 %) for the maximum mineralization rates and the number of days to the maximum rate are shown in Figure 29a,b.

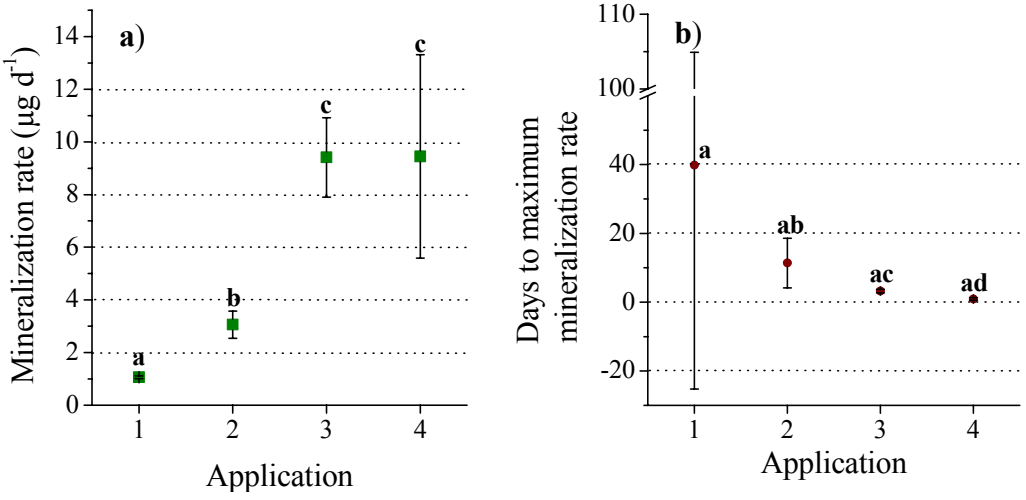


Figure 29: Estimates and confidence intervals (95 %) in soil Feldkirchen after applications 1-4 of ^{14}C-IPU for (a) maximum mineralization rates of IPU and (b) time periods to maximum mineralization rates. Values with the same letter assigned are not significantly different (α = 0.05) according to One-way ANOVA and Tukey HSD test.

The maximum mineralization rate increased significantly from phase C1 to C2 as well as from C2 to C3 (Figure 29a). No statistical difference was detected between phase C3 and C4. The standard error was highest in phase C4.

According to Figure 29b, the period to the maximum mineralization rate decreased significantly from phase C2 to C3 and from phase C3 to C4. Standard errors were very small in these phases. In contrast, the standard error after application 1 was huge, ranging from -25.3 to 104.9 days, reflecting that for cometabolic degradation normally no clear peak is detectable.

3.5.1.2 Distribution of ^{14}C at the end of phases C1 to C4

The distribution of ^{14}C at the end of phases C1-C4 is given in Table 18. The fate of ^{14}C-IPU was investigated by measuring radioactivity in the following fractions: mineralization, extractable and non-extractable IPU residues.

The portion of ^{14}C in the various fractions changed clearly in the course of the experiment: the portion of mineralized ^{14}C increased significantly from phase C1 (10.9 ± 0.3 % of applied ^{14}C) to C4 (33.9 ± 3.5 %), while the amount of extractable radioactivity (column "PW + MER") decreased significantly (phase C1: 42.7 %; phase C4: 13.4 % of total applied ^{14}C).Thus, by repeated application of IPU, an acceleration of mineralization as well as a change in distribution of radioactivity in the investigated fractions was discovered. From phase C1 to C3, the amount of ^{14}C mineralized increased by 255.1 % while the amount of extractable IPU residues decreased by 48.4 %.

Table 18: Development of the distribution of applied ^{14}C in soil Feldkirchen after 1, 2, 3 and 4 applications of ^{14}C-IPU. At the end of all phases, the amount of NER, MER and IPU residues dissolved in pore water (PW) was determined. [1]

		Recovery of applied ^{14}C in %					
Application	Replicate	MIN	PW	MER	PW + MER	NER	Sum
1	F1	10.6	6.6 ± 0.4	36.1 ± 3.0	42.7 ± 3.4	53.7 ± 1.7	101.9 - 112.3
	F2	11.3					102.6 - 113.0
	F3	10.9					102.2 - 112.6
	F4	10.9					102.2 - 112.6
	F5	10.9					102.2 - 112.6
	F6	10.6					101.9 - 112.3
2	F1	11.4	6.3 ± 0.6	20.9 ± 10.6	27.2 ± 11.2	57.7 ± 1.6	83.9 - 109.5
	F2	18.9					91.7 - 117.3
	F3	12.7					85.3 - 110.9
	F4	23.8					96.7 - 122.3
	F5	12.5					85.1 - 110.7
	F6	22.2					95.1 - 120.7
3	F1	21.6	4.6 ± 0.2	17.4 ± 1.0	22.0 ± 1.2	59.3 ± 2.6	99.1 - 106.7
	F2	28.6					106.1 - 113.7
	F3	28.6					106.1 - 113.7
	F4	30.5					108.0 - 115.6
	F5	27.0					104.5 - 112.1
	F6	30.0					107.5 - 115.1
4	F1	39.0			7.2	62.3	108.6
	F2	32.6	3.0	12.1	15.1	56.2	103.9
	F3	31.7	3.5	13.9	17.4	50.6	99.7
	F4	32.8	3.5	10.2	13.7	49.1	95.5
	F5	30.1	n.d.	n.d.	n.d.	n.d.	n.d.
	F6	37.4	2.6	10.9	13.5	55.4	106.2

n.d. = not determined

[1] From the 16 replicates (= F1-F16), which were set up at the start of the experiment, four were stopped for IPU residue analysis at the end of phases C1, C2 and C3, respectively. Replicates F1-F6 passed through all four phases and were stopped at the end of phase C4 and subsequently analyzed for IPU residues. Thus, just mean values could be shown in phases C1-C3 for these parameters as well as a range for the sum of percentages. At the end of phase C4, ^{14}C in pore water and MeOH-extractable ^{14}C for replicate F1 was not analyzed separately. To compare this replicate with others, the column PW + MER was introduced.

3.5.1.3 Identification of extractable IPU residues

Extractable IPU residues in pore water

Analyzed IPU residues in pore water are shown in Figure 30. During the course of the experiment, IPU and three metabolites were found in pore water. Two of the metabolites could be identified by HPLC (MD-IPU, 2-OH-MD-IPU). At the end of phase C1 and C2, IPU and the three metabolites were detected, while in phases C3 and C4 just IPU and the unidentified metabolite were discovered.

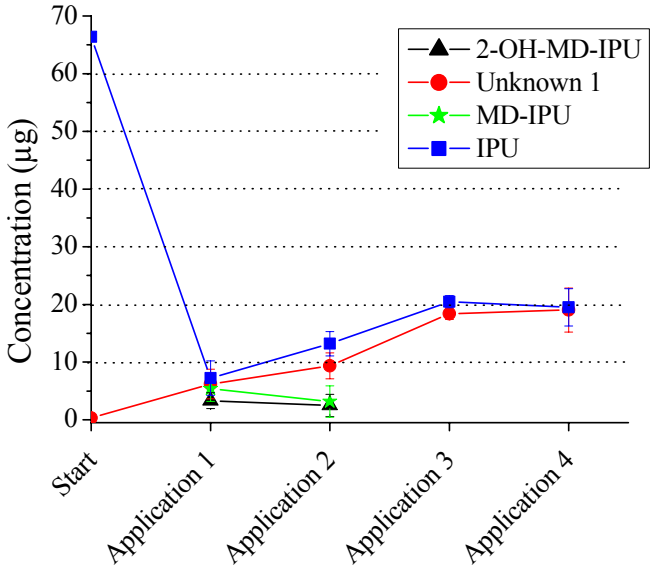


Figure 30: Composition of IPU residues dissolved in pore water for soil Feldkirchen after 1, 2, 3 and 4 applications of ^{14}C-IPU. Quantities are given in µg IPU equivalents for not identified metabolites. Metabolite Unknown 1 was not identified by HPLC. Samples were collected at the end of phases C1 to C4. Bars indicate standard deviation.

In all phases, IPU was the compound in highest concentration, with a considerable decrease during phase C1 (from 66.4 µg at start to 7.2 ± 3.0 µg at end of phase C1). Although IPU was applied three more times, concentration in pore water was only 19.5 ± 3.2 µg at the end of phase C4, which is less than 2 % of the ^{14}C-IPU applied in total. The metabolite Unknown 1 was formed during phases C1-C4.

Methanol-extractable IPU residues

Figure 31 gives the MeOH-extractable metabolites that were identified by HPLC. IPU and total of 7 metabolites were detected. IPU and 3 of the metabolites were identified.

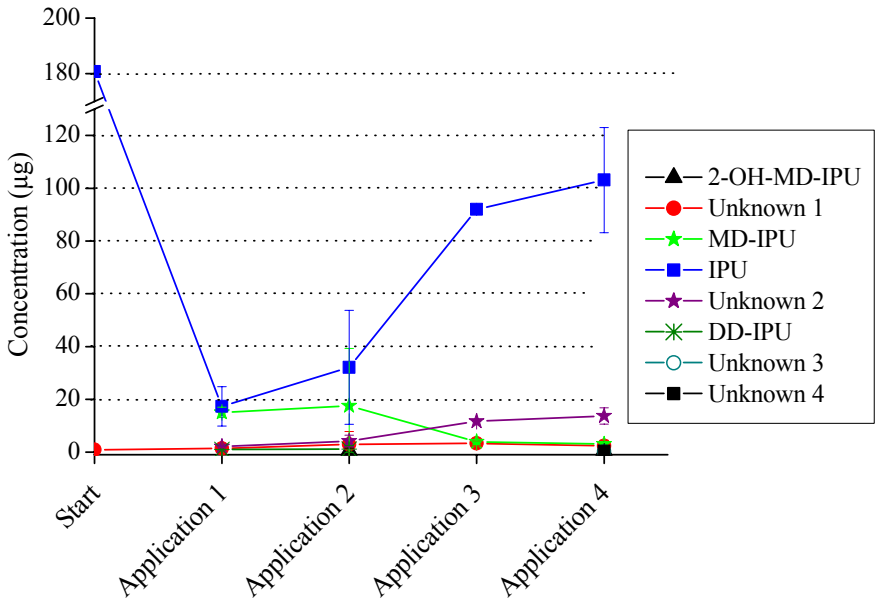


Figure 31: Composition of Methanol-extractable IPU residues for soil Feldkirchen after 1, 2, 3 and 4 applications of ^{14}C-IPU. Quantities are given in µg IPU equivalents for not identified metabolites. Metabolites Unknown 1-4 were not identified by HPLC. Bars indicate standard deviation.

As in pore water, IPU was the compound in highest concentration. During the course of the experiment, just IPU and the metabolite Unknown 2 increased in concentration. At the end of phase C4, still 103.1 ± 19.9 µg IPU were extractable by MeOH (= 10.3 % of total IPU applied during phases C1-C4).

3.5.2 Effect of repeated IPU applications on IPU degradation in soils Scheyern, Kelheim and Konjišče

In the previous experiment it was shown that four applications of IPU to soil Feldkirchen in time intervals of about four weeks resulted in a change in IPU mineralization pattern, from cometabolic to metabolic IPU mineralization (chapter 3.5.1.1). However, as soil Feldkirchen was able to mineralize IPU metabolically already before its IPU degradation function was impaired during summer 2003 (Levy et al., 2007), it was not possible in the previous experiment to determine if the acceleration of IPU degradation (chapter 3.5.1.1) was generated by the adaptation of soil microbes during the experiment or if the soil history and especially the metabolic capabilities before summer 2003 were responsible for this change in mineralization pattern (chapter 4.4.1.2).

To test if a change from a cometabolic to a metabolic IPU degradation pattern might be caused by adaptation of soil microbes, three further soils were investigated (soils Scheyern, Kelheim and Konjišče) which were not able to mineralize IPU metabolically before. For experimental setup see chapter 2.9.2.

While IPU was applied to soil Feldkirchen four times, it was added to soils Scheyern, Kelheim and Konjišče three times. Furthermore, the length of phases differed slightly between soil Feldkirchen and the other three soils. Thus, phases for soils Scheyern, Kelheim and Konjišče (phases P1, P2 and P3) were designated different from phases of soil Feldkirchen (phases C1, C2, C3, C4).

3.5.2.1 Effect on IPU mineralization

To estimate if an adaptation of soils towards an accelerated mineralization of IPU occurred, mineralization data from the three consecutive applications was used.

Mineralization rates for the three soils are shown in Figure 32. As the mineralization pattern of replicates within a soil varied sometimes clearly, single replicates instead of mean values are given. The response of soils to repeated application of ^{14}C-IPU was similar in the three soils. In phase P1, low mineralization with constant rates predominated in all three soils (1.2 ± 0.5, 1.0 ± 0.2 and 0.9 ± 0.6 µg d^{-1} for soils Scheyern, Kelheim and Konjišče, respectively; calculated without values of day 0.8). In soil Scheyern, 34.4 µg of applied ^{14}C were mineralized in this phase. In phase P2, elevated mineralization rates were observed, especially in soil Scheyern (15.2 ± 8.9, 8.0 ± 2.5 and 8.2 ± 6.5 µg d^{-1} for soils Scheyern, Kelheim and Konjišče, respectively). Replicates of soil Konjišče and particularly the ones of soil Scheyern varied in maximum mineralization rates and the period until the maximum rate was achieved. In phase P3, replicates of all soils (except replicate KE1) were characterised by increased mineralization rates (21.6 ± 3.3, 28.7 ± 7.5 and 24.7 ± 8.9 µg d^{-1} for soils Scheyern, Kelheim (without replicate KE1) and Konjišče, respectively). Maximum mineralization rates were mostly higher than in phase 2 and the time span, until the maximum rate was detected, was shorter. Replicates varied less than in phase 2 regarding the maximum mineralization rate and the point in time, when it was measured.

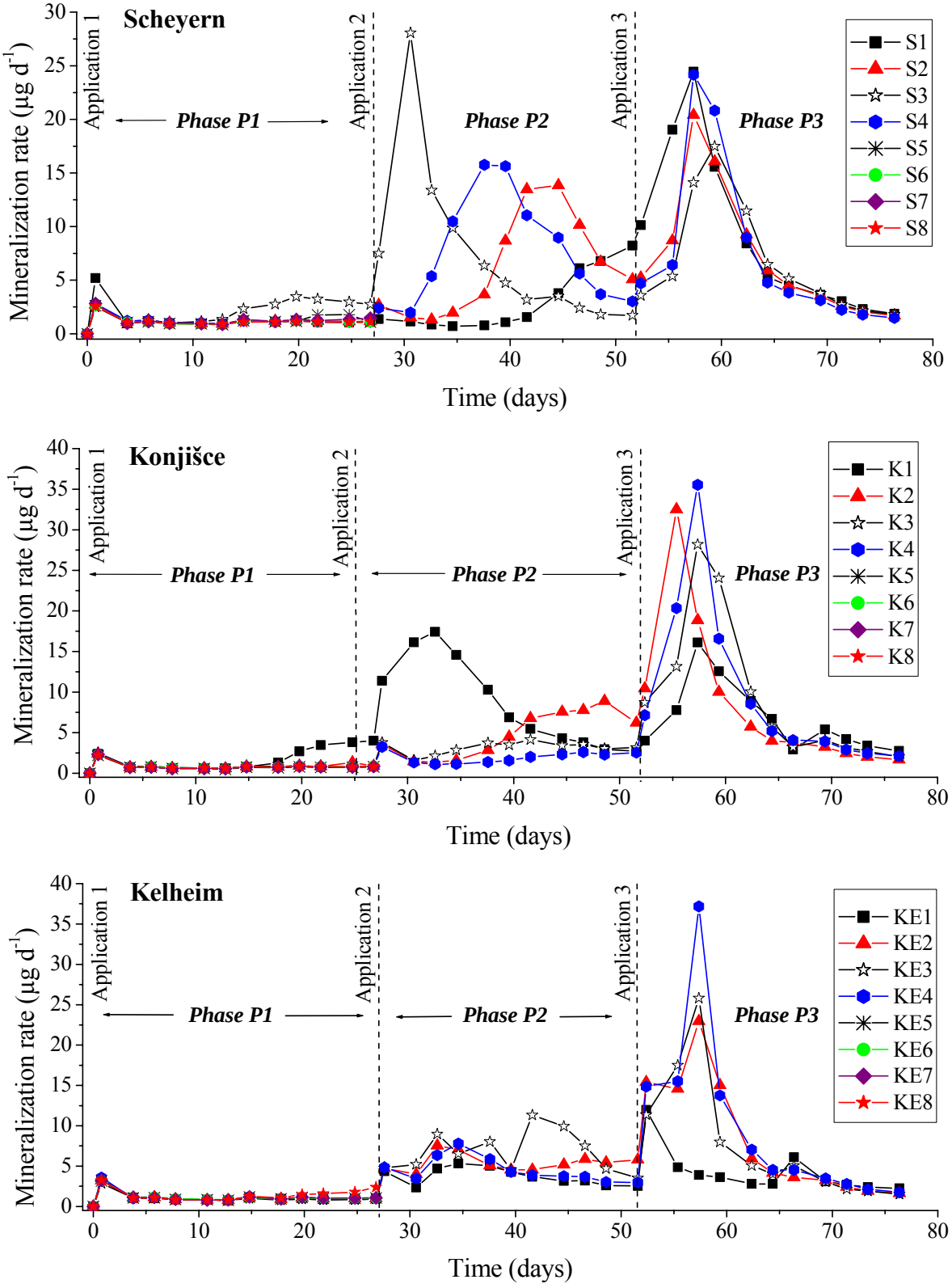


Figure 32: Development of IPU mineralization rates for soils Scheyern, Konjišče and Kelheim after 1, 2 and 3 applications of ^{14}C-IPU for individual replicates (S1-8, K1-8 and KE1-8, respectively). At the end of phase P1, replicates 5-8 of all soils were stopped for analysis of IPU residues. ^{14}C-IPU was applied at days 0, 27 and 52.

Statistical analysis of mineralization dynamics in phases P1, P2 and P3

To evaluate if the mineralization dynamics altered by repeated application of IPU, the following parameters were applied:

(1) maximum mineralization rates

(2) the period of time until the maximum mineralization rate was attained

Mineralization rates were fitted by a nonlinear Gompertz function and the goodness of fits was calculated as Pseudo-R^2 as it was previously also done for soil Scheyern that was inoculated with IMMC (chapter 3.4.1.3). In chapter A1.3.3, Appendix, fitted curves for mineralization rates as well as corresponding Pseudo-R^2 are given. Values for Pseudo-R^2 show a good alignment of fitted curves to recorded data in all soils and phases except phase 2 for soil Scheyern and Konjišče, reflecting higher standard deviations due to a change of mineralization dynamics.

Figure 33 shows estimates and confidence intervals (95 %) for the maximum mineralization rates and Figure 34 the ones for the number of days to the maximum rate. Values are given for applications 1-3 for soils Scheyern, Kelheim and Konjišče.

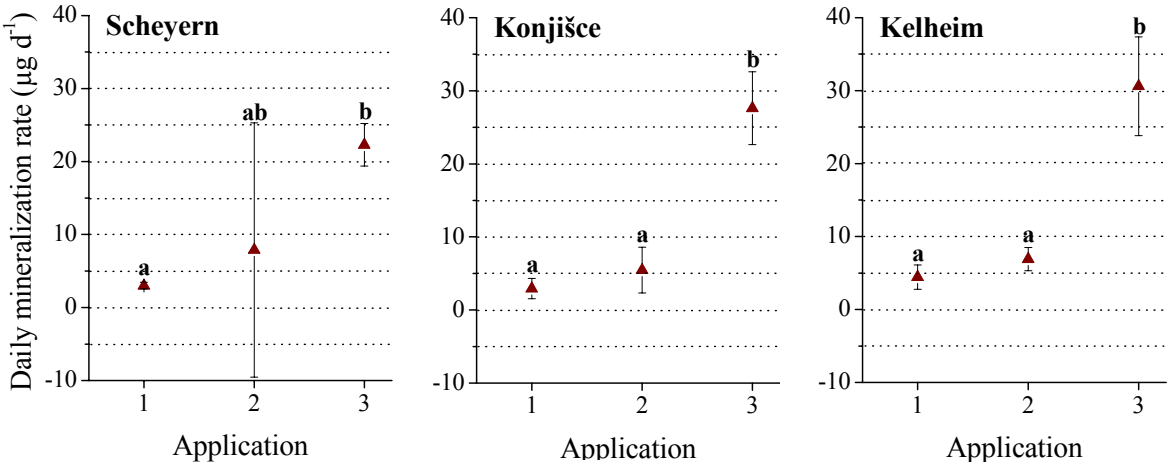


Figure 33: Estimates and confidence intervals (95 %) for maximum mineralization rates of IPU in soils Scheyern, Konjišče and Kelheim for applications 1-3 of ^{14}C-IPU. Values with the same letter assigned are not significantly different (α = 0.05) according to One-way ANOVA and Tukey HSD test.

In all soils, the maximum mineralization rate in phase P3 was significantly increased compared to phase P1 (Figure 33). In soil Scheyern, the rate in phase P2 had a huge standard error. In soils Kelheim and Konjišče rates of phase P2 were not significantly different from phase P1. In these two soils, the highest standard error and a clear rise in mineralization rate was detected for phase P3.

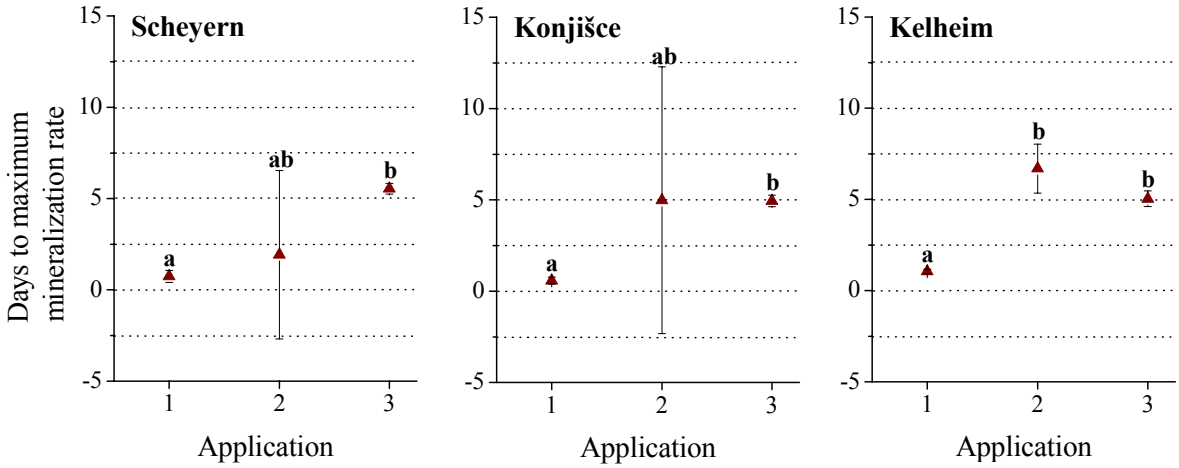


Figure 34: Estimates and confidence intervals (95 %) for time periods to maximum mineralization rates of IPU in soils Scheyern, Konjišče and Kelheim for applications 1-3 of ^{14}C-IPU. Values with the same letter assigned are not significantly different (α = 0.05) according to One-way ANOVA and Tukey HSD test.

According to Figure 34a-c, the period until the maximum rate was attained, increased significantly from phase P1 to P3.

However, after IPU application 1 an immediate and short-term increase in mineralization rates was observed in all three soils, which also constituted the highest peak of phase P1 (Fig. 32). This short-term increase in mineralization rates was also detected at the beginning of phases P2 and P3 (Fig. 32).

3.5.2.2 Distribution of ^{14}C at the end of phases P1, P2 and P3

Figure 35 shows the ^{14}C fractions at the end of phases P1, P2 and P3 as a possible change from cometabolic to metabolic mineralization pattern might also be reflected in the type and quantity of IPU residues. Since at the end of phase 2 no IPU residue analysis was carried out, only mineralization data are available for this phase.

The magnitude of the individual fractions altered clearly from phase P1 to P3 in all soils. At the end of phase P1, the applied ^{14}C was detected in large shares in the extractable (MER + PW) and non-extractable fraction, while only a small portion of ^{14}C-IPU was mineralized.

Until the end of phase P3, the distribution of ^{14}C changed clearly: the amount of extractable ^{14}C decreased significantly from phase 1 to 3, while the amount of mineralized ^{14}C increased considerably from phase P1 to P3 in all soils. In detail, 50.6, 47.7 and 48.6 % of the total applied 750 µg were mineralized until the end of phase P3 in soils Scheyern, Konjišče and Kelheim (replicates KE2-4), respectively. This corresponds to an increase by 367.1 %, 377.2 % and 484.3 %, respectively, from phase P1 to P3. Parallel, the amount of extractable ^{14}C decreased significantly from phase P1 to P3, with a decrease by 97.0 %, 81.9 % and 76.9 % in soils Scheyern,

Konjišče and Kelheim, respectively. It was not analyzed which IPU metabolites were in these extracts. The percentage of NER present in phase P1 was on a similar level as in phase P3.

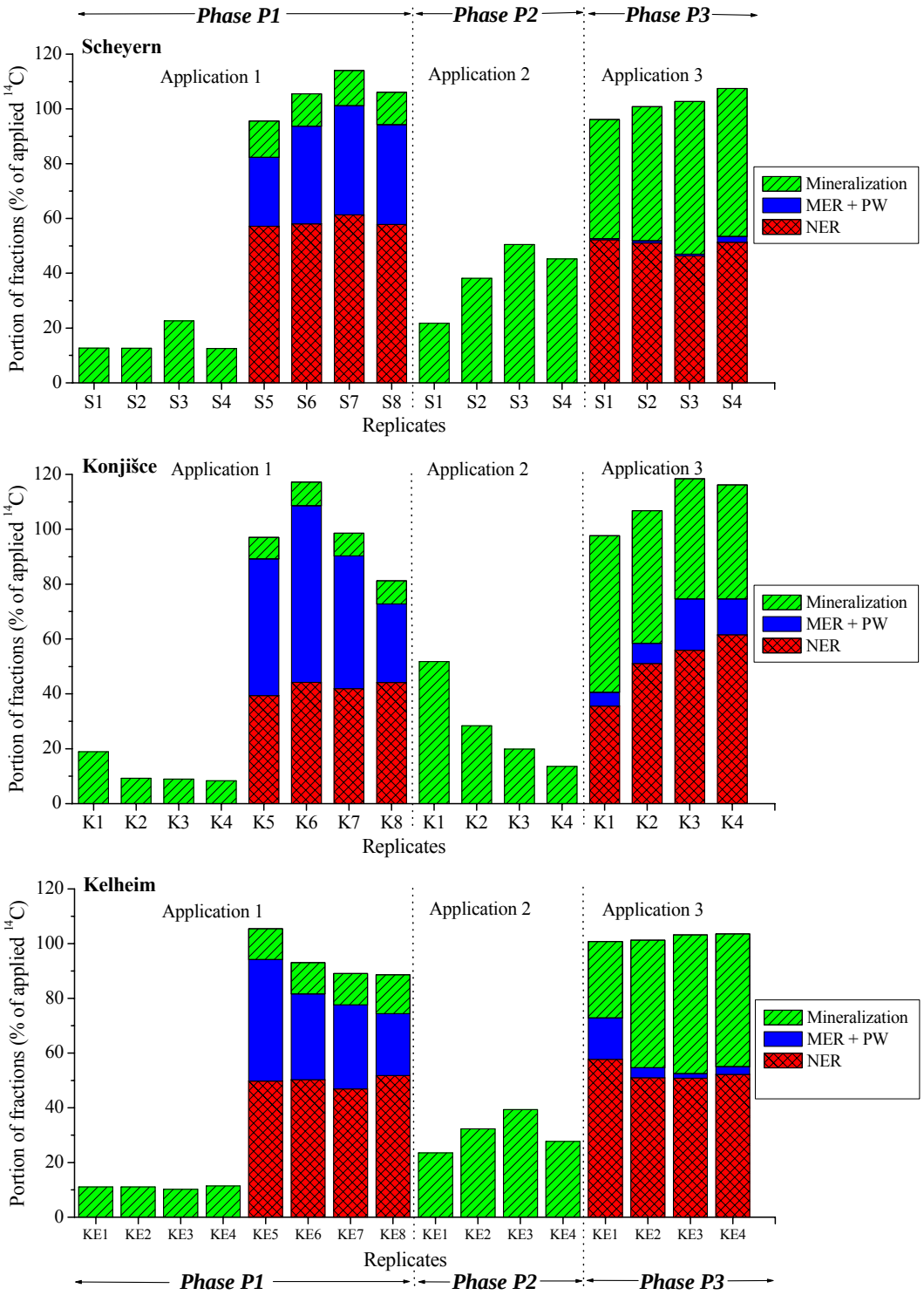


Figure 35: Development of the distribution of applied ^{14}C in soils Scheyern, Konjišče and Kelheim after 1, 2 and 3 applications of ^{14}C-IPU. The amount of NER, MER + IPU residues dissolved in pore water (PW) was determined at the end of phases P1 and P3. No replicates for analysis of IPU residues were stopped at the end of phase 2. Mineralization measurements were done continuously and are given as cumulative values. Replicates 1-4 passed through all phases.[2]

[2] NER = Non-extractable IPU residues; MER + PW = IPU residues extractable by Methanol and dissolved in pore water

Replicate KE1, which showed in phase P3 lower mineralization rates and a different mineralization dynamic compared to other replicates of soil Kelheim (chapter 3.5.2.1, Fig. 32), differed from other replicates also in the amount of extractable ^{14}C: a clearly higher amount of ^{14}C could be extracted from this replicate (Fig. 35).

At the end of phases P1 and P3, 95.6 % – 114.0 (soil Scheyern), 81.3 % – 118.4 (soil Konjišče) and 88.6 % – 105.4 % (soil Kelheim) of applied ^{14}C was recovered.

4 Discussion

The goal of this thesis was (1) to examine the capability of the microbial consortium IMMC and the IPU degrading microbial strain therein to mineralize IPU and some of its metabolites. IMMC was also investigated for its capability to mineralize the phenylurea herbicide Diuron; (2) to investigate to what extent the increased IPU mineralization, which is induced by inoculation of IMMC to agricultural soils, is conserved over a period of several months and (3) to investigate to what extent non-inoculated soils can be stimulated by repeated IPU applications towards accelerated IPU mineralization.

To examine the IPU degradation capabilities of the consortium IMMC and the IPU mineralizing bacterium, isolation and identification studies were performed along with degradation experiments in liquid culture. On basis of laboratory experiments with agricultural soils, conclusions could be drawn on the sustainability of IPU mineralization in inoculated soils as well as on the adaptability of non-inoculated soils to accelerated IPU degradation.

4.1 Isolation and identification of the IPU mineralizing bacterial strain from the consortium IMMC

The separation of microbes of IMMC on agar plates and subsequent tests in liquid culture for mineralization of ^{14}C-IPU led to the isolation of an IPU mineralizing strain that belongs to the genus *Sphingomonas* and was designated *Sphingomonas* sp. AK1 (chapter 3.1).

Bacteria of this genus are known to possess enormous metabolic capabilities and to degrade a broad spectrum of natural organic compounds (Sørensen et al., 2002; Balkwill et al., 2006; Stolz, 2009). The genus gains increasing importance in environmental microbiology as it is suspected that members of this genus adapt faster and more efficient to the degradation of new compounds than other bacterial genera (Basta et al., 2004; Stolz, 2009) and as this genus harbors an extraordinary high percentage of species that are able to degrade xenobiotics (Basta et al., 2004; Stolz, 2009). Several studies reported that strains of the family *Sphingomonadaceae*, which includes e.g. the genera *Sphingobium* and *Sphingomonas*, were involved also in the mineralization of IPU. Up to now, six IPU degrading isolates were reported, which belong to the family *Sphingomonadaceae*: *Sphingomonas* sp. SRS2 (Sørensen et al., 2001), *Sphingomonas* sp. F35 (Bending et al., 2003), *Sphingomonas* sp. strain SH (Hussain et al., 2011) and *Sphingobium* strains YBL1, YBL2 and YBL3 (Sun et al., 2009). Liquid culture studies with ^{14}C-IPU and the *Sphingomonas* sp. strains SRS2, F35 and SH, respectively, reported the formation of $^{14}CO_2$ which indicated the mineralization of IPU (Sørensen et al., 2001; Bending et al., 2003; Hussain et al., 2011). In studies

with *Sphingobium* strains YBL1, YBL2 and YBL3, no ^{14}C-labeled IPU was utilized. However, during 4IA degradation by all three strains catechol was detected, which was considered as key metabolite in the process of phenyl-ring cleavage of most aromatic hydrocarbons (Sun et al., 2009). These six IPU mineralizing strains were isolated from different geographical locations in UK (Sørensen et al., 2001; Bending et al., 2003), France (Hussain et al., 2011) and China (Sun et al., 2009). The isolates were extracted either from agricultural soils that had been treated with IPU regularly (Sørensen et al., 2001; Bending et al., 2003; Hussain et al., 2011) or from herbicide plants fields, which were also exposed to IPU previously (Sun et al., 2009).

Colonies of *Sphingomonas* sp. AK1 were yellow pigmented and cells rod shaped (chapter 3.1.2.2.) which is typical for the genus *Sphingomonas* (Basta et al., 2004; Balkwill et al., 2006; Stolz, 2009). Compared to other known IPU mineralizing isolates, cells of *Sphingomonas* sp. AK1 formed colonies on agar plates at a considerably lower speed: colonies of *Sphingomonas* sp. AK1 were detected on R2A plates after 4 to 5 weeks of incubation at +20 °C (chapter 3.1.2.2), while colonies of *Sphingomonas* sp. strain SRS2 were observed on R2A plates already after 5 to 6 days of incubation at +20 °C (Sørensen et al., 2001). Colonies of *Sphingomonas* sp. strain SH (Hussain et al., 2011) were visible after 5 (NA plates) and 15 days (MSA-IPU plates) of incubation at +20 °C. The *Sphingobium* sp. strains YBL1, YBL2 and YBL3 were observed already after 3 days of incubation at +30 °C (Sun et al., 2009).

4.2 Degradation of IPU, MD-IPU, DD-IPU and Diuron in liquid culture

4.2.1 Degradation of IPU and its metabolites MD-IPU and DD-IPU by IMMC and *Sphingomonas* sp. AK1, respectively

In the present thesis, liquid culture studies were performed with IMMC and *Sphingomonas* sp. AK1, respectively, in order to study their capability to degrade IPU and its metabolites MD-IPU and DD-IPU. Moreover, it was intended to analyze the metabolites which were formed during degradation. To do so, IPU, MD-IPU or DD-IPU (25 mg L^{-1}, 23.3 mg L^{-1} and 21.6 mg L^{-1}, respectively, which all corresponds to 121.19 µM) were applied to liquid medium and their disappearance as well as the formation of metabolites was measured in presence of IMMC (chapter 3.2) and *Sphingomonas* sp. AK1 (chapter 3.3), respectively.

For almost all reported IPU degrading isolates, the formation of metabolites during IPU degradation was examined, e.g. for strains *Sphingomonas* sp. SRS2 (Sørensen et al., 2001), *Sphingomonas* sp. SH (Hussain et al., 2011), *Methylopila* sp. TES (El-Sebai et al., 2004) and *Arthrobacter globiformis* strain D47 (Cullington and Walker, 1999). To detect and identify metabolites, the respective studies typically utilized the techniques of nuclear magnetic resonance and mass spectroscopy (MS) (Tixier et al., 2002), HPLC-MS (Sun et al., 2009) or HPLC (Roberts

et al., 1998; Cullington and Walker, 1999; Sørensen et al., 2001; El-Sebai et al., 2004; Hussain et al., 2011). Apart from the survey by Tixier et al. (2002), all studies proposed degradation pathways for IPU by the examined isolate based solely on the transient accumulation of the different metabolites. However, these conclusions drawn on the degradation pathway by this approach have to be regarded with suspicion for several reasons, e.g.:

(1) When investigating microbial transformations with whole cells, frequently a sequence of enzymes is available for degradation processes. Hence, a metabolite might not be detectable and misleadingly considered as not be formed (Hall et al., 2001). To overcome this difficulty, critical degradation steps or complete pathways may be elucidated by transformations performed by isolated microbial enzymes (Hall et al., 2001). However, it is not known which enzymes were active in IPU degradation by *Sphingomonas* sp. AK1 and IMMC, respectively. Furthermore, the enzymes identified in IPU degradation so far showed only a low activity for IPU (Turnbull, Cullington, et al., 2001; Turnbull, Ousley, et al., 2001; Khurana et al., 2009; Bers et al., 2011), preventing the use of isolated enzyme transformations in case of IPU.

(2) A microbial isolate may be capable to degrade a xenobiotic by different pathways. Kullman and Matsumura (1996), for example, investigated the enzymatic mechanism of Endosulfan degradation by the fungi *Phanerochaete chrysosporium* under nutrient deficient and nutrient rich culture conditions. The type of metabolites differed for the two media and the authors concluded that the fungi utilized an oxidative or hydrolytic pathway, respectively, depending on culture conditions. This finding was confirmed by results of elaborate inhibition studies in which the oxidative pathway was inhibited by addition of Piperonyl butoxide, a cytochrome P-450 inhibitor (Kullman and Matsumura, 1996). Two distinct pathways of P. *chrysosporium* were also reported for Phenanthrene: the metabolic pathway depended on the N concentration in the liquid medium (Hammel et al., 1992). For isolates degrading phenylurea herbicides, elaborate studies like the one of Kullman and Matsumura (1996) for Endosulfan are lacking. To the author´s knowledge, no information is available on (a) whether an IPU degrading isolate is capable to utilize different metabolic pathways for IPU degradation and (b) whether species of the genus *Sphingomonas* are able to utilize different pathways for the degradation of one pollutant.

Indications for the IPU degradation pathway exhibited by *Sphingomonas* sp. AK1 and IMMC

Based on the degradation of applied compounds and the detection of metabolites formed in the present study, some indications may arise on the IPU degradation pathway performed by *Sphingomonas* sp. AK1 and IMMC, respectively. However, for the reasons mentioned above, penetrative studies would be desirable to proof the proposed pathway.

The experiments with IMMC and *Sphingomonas* sp. AK 1, respectively, provided generally consistent results on the degradation pathway of IPU and are therefore considered in common in the following.

Sørensen et al. (2003) proposed different pathways for the microbial degradation of IPU (for pathways see chapter 1.2.1.2, Fig. 1). According to this scheme, the metabolism of IPU can be started by one of four different transformations (Fig. 1, steps 1, 4, 6, 7; 1 = N-demethylation, 4 = hydrolysis of the dimethylurea side chain, 6 & 7 = hydroxylation of the isopropyl side chain). In the present study, a transient accumulation of MD-IPU was measured (IMMC: chapter 3.2.2.; *Sphingomonas* sp. AK1: chapter 3.3.2), indicating that the degradation of IPU by *Sphingomonas* sp. AK1 and IMMC, respectively, was initiated by N-demethylation of the dimethylurea side chain (step 1), leading to MD-IPU. IMMC ($1.2 \pm 0.1 * 10^7$ CFUs applied per replicate) mineralized MD-IPU clearly faster than IPU (chapter 3.2.1, Fig. 11), while for *Sphingomonas* sp. AK.1 ($5.7 \pm 0.9 * 10^6$ CFUs applied per replicate) at least a tendency for a faster degradation could be observed (chapter 3.3.2, Fig. 19a,c). This implies that the initial N-demethylation of IPU to MD-IPU may be a rate and speed limiting step for the mineralization of IPU. This implication is supported by the fact that MD-IPU, which was formed during the degradation of IPU by IMMC and *Sphingomonas* sp. AK1, respectively, was detected only in traces in liquid cultures, i.e. MD-IPU seems to be degraded by microbes immediately after its formation. The transformation of IPU to MD-IPU was reported to be a rate limiting step in various pure cultures of soil bacteria (Roberts et al., 1998; Berger, 1999) and *Sphingomonas* species (Sørensen et al., 2001; Hussain et al., 2011). MD-IPU can be transformed further by steps 2, 5 or 8 (chapter 1.2.1.2, Fig.1; N-demethylation, hydrolysis of the urea side chain and hydroxylation of the isopropyl side chain). From these steps, the hydroxylation of MD-IPU leading to 2-OH-MD-IPU (step 8) seems unlikely as degradation of 2-OH-MD-IPU cannot, according to the scheme, result in the formation of 4IA, which was, however, detected during the degradation of IPU by IMMC (chapter 3.2.2, Fig.13a). Thus, steps 2 (N-demethylation of MD-IPU) and 5 (hydrolysis of the methylurea side chain of MD-IPU) remain as possible further transformation steps for MD-IPU. The metabolite DD-IPU was not detected during IPU degradation (IMMC: chapter 3.2.2, Fig. 13a; *Sphingomonas* sp. AK1: chapter 3.3.2, Fig. 19a). DD-IPU was also not measured when a high amount of MD-IPU was added to the medium (IMMC: Fig. 13c; *Sphingomonas* sp. AK1: Fig. 19c), which suggests that the lacking detection of DD-IPU during IPU degradation could not be attributed to an immediate transformation of DD-IPU to 4IA and thus leading to DD-IPU concentrations too low to be detected. Thus, N-demethylation of MD-IPU leading to DD-IPU (step 2) seemed not to take place. Results rather imply that MD-IPU was transformed directly to 4IA by hydrolysis of the methylurea side chain of MD-IPU (step 5), which does not involve the formation of DD-IPU. In liquid cultures with ^{14}C-IPU (IMMC: chapter 3.2.1; *Sphingomonas* sp. AK1: chapter 3.3.1, Fig. 17a),

mineralization was measured, which documents the further transformation of 4IA up to the ring cleavage and the formation of $^{14}CO_2$.

Of all known IPU degrading isolates, only the strains *Sphingomonas* sp. SRS2 (Sørensen et al., 2001), *Methylopila* sp. TES (El-Sebai et al., 2004), the *Sphingobium* sp. strains YBL1, YBL2 and YBL3 (Sun et al., 2009) as well as *Sphingomona*s sp. strain SH (Hussain et al., 2011) were capable to perform the ring cleavage of 4IA. From these isolates, *Sphingomonas* sp. SRS2 and *Sphingobium* sp. strain YBL2 were reported to mineralize IPU via two successive demethylations, leading via MD-IPU to DD-IPU. DD-IPU was then concluded to be transformed to 4IA, which was finally mineralized to $^{14}CO_2$ (Sørensen et al., 2001; Zhang et al., 2012) (chapter 1.2.1.2, Fig. 1, steps 1-3). In liquid cultures with *Sphingomonas* sp. strain SH, MD-IPU was detected during IPU mineralization, indicating demethylation from IPU to MD-IPU (Fig. 1, step 1) (Hussain et al., 2011). No further metabolites were detected. However, IPU mineralization was measured, implying that MD-IPU was further transformed until the ring structure was split and $^{14}CO_2$ was detected, although the respective metabolites could not be detected (Hussain et al., 2011). For *Sphingobium* sp. strains YBL1 and YBL3 as well as for *Methylopila* sp. TES, no metabolites were discovered at all during IPU mineralization (El-Sebai et al., 2004; Sun et al., 2009). Thus, no information is available on the possible underlying degradation pathways.

Implications for bioremediation

Sphingomonas sp. AK1 and IMMC, respectively, were capable to mineralize IPU (IMMC: chapter 3.2.1; *Sphingomonas* sp. AK1: chapter 3.3.1, Fig. 17) and to degrade its metabolites MD-IPU and DD-IPU (IMMC: chapter 3.2.2, Fig. 13c,e; *Sphingomonas* sp. AK1: chapter 3.3.2, Fig. 19c,e). Altogether, the degradation capabilities of IMMC and *Sphingomonas* sp. AK1, respectively, are assessed as beneficial for soil remediation for two reasons: (1) microbes were not only able to mineralize IPU itself, but also to transform effectively at least three of its metabolites (MD-IPU, DD-IPU, 4IA). Thus, degradation can be expected also in agricultural soils in which IPU was already degraded partially. And (2), microbes were able to transform IPU and its metabolites rapidly and only with a short-term accumulation of a few metabolites. As IPU and its metabolites were shown to have a toxic effect on e.g. fresh water animals (Mansour et al., 1999; Nitschke et al., 1999), short contact times are of considerable environmental interest. However, these experiments were conducted in liquid culture and it can be expected that the degradation efficiency might be different in soils, especially under outdoor conditions, when conditions may be not optimal for IPU degradation.

4.2.2 Degradation of Diuron by IMMC: specificity of the degradation enzymes that are responsible for IPU mineralization

By means of liquid culture studies it was investigated if the degradation enzymes that accounted for the mineralization of IPU can degrade also other phenylurea herbicides. Diuron was selected for this purpose as (1) microbial degradation is, like for IPU, the main mechanism for its removal from soil and water (Giacomazzi and Cochet, 2004; Sørensen et al., 2008) and (2) it is structurally similar to IPU (IPU has an isopropyl side chain and Diuron has instead two chlorine atoms on the benzene ring). Diuron belongs, like IPU, to the group of N,N-dimethyl phenylureas due to the presence of a dimethylurea side chain. Two concentrations of Diuron were utilized in liquid cultures (1 and 25 mg L^{-1}) that were inoculated with IMMC (for experimental setup see chapter 2.5.2).

When 25 mg L^{-1} Diuron were applied, no decrease in Diuron concentration was measured (chapter 3.2.3.1). Nevertheless, when 1 mg L^{-1} of Diuron was added, $^{14}CO_2$ was trapped in a quite low degree (chapter 3.2.3.2). Only 7.0 % of the applied Diuron was completely degraded to ^{14}CO (ring labeled Diuron), while a higher portion was partially degraded (15.0 % of applied side chain labeled ^{14}C).

The limited mineralization respectively degradation of Diuron might be caused by three factors:

(1) Degradation enzymes of *Sphingomonas* sp. AK1 and IMMC, respectively, were capable to degrade Diuron only to a limited degree. There are several bacterial and fungal isolates reported that degrade Diuron in considerable amounts (Turnbull, Cullington, et al., 2001; Widehem et al., 2002), but especially mineralization and thus ring cleavage at a high extent was observed rarely in environmental samples, although Diuron has been applied in agriculture since decades (Widehem et al., 2002; Sørensen et al., 2008). Just recently, *Variovorax* sp. strain SRS16 was shown to be the first strain able to mineralize the ring structure of Diuron (Sørensen et al., 2008). The lack of enzymatic degradation by IMMC might be attributed to the molecular structure of Diuron, as halogenated compounds like Diuron seem to be degraded more slowly in comparison to non-halogenated ones like IPU (Sørensen et al., 2003).

(2) During degradation of Diuron metabolites may have accumulated that had a toxic effect on IMMC and which thus limited further Diuron degradation. Diuron´s main metabolites 3,4-dichloroaniline, N-(3,4-dichlorophenyl)urea, and N-(3,4-dichlorophenyl)-N-methylurea are all chloroanilines which were shown to be clearly more toxic to non target organisms like soil microbes than Diuron itself (Tixier et al., 2002; Giacomazzi and Cochet, 2004; Sørensen et al., 2008). In the present study, degradation and mineralization, respectively,

were quantified by trapping $^{14}CO_2$. HPLC measurements, which might elucidate the formation of chloroanilines were not executed.

(3) IMMC might in principle be capable for Diuron degradation, but laboratory conditions may have provided unfavorable conditions for its degradation. Sørensen et al. (2008), who demonstrated first the ring cleavage of Diuron by a bacterial strain, compared the mineralization efficiency of the isolated *Variovorax* sp. strain SRS16 in several liquid media that were differing in their nutrient amendments. High mineralization was measured only in liquid cultures with appropriate growth substrates. In the present experiment, the liquid culture study with IMMC was only conducted in one type of liquid medium, the MS-medium (chapter 2.3.1). This medium was appropriate for IPU mineralization (chapter 3.2.1), but might not have allowed efficient Diuron degradation. The concentration of Diuron in liquid media also plays a decisive role for its degradation. In the liquid culture studies with *Variovorax* sp. strain SRS16, Sørensen et al. (2008) also tested the degradation of Diuron in MS-medium at different concentrations of Diuron. Lowering the Diuron concentration in the MS-medium from 10 mg L^{-1} to 38.9 µg L^{-1} resulted in an enhanced mineralization of Diuron by the isolated strain. The higher degradation response of IMMC to the Diuron concentrations of 1 mg L^{-1} (chapter 3.2.3.2, Fig. 16) than of 25 mg L^{-1} (chapter 3.2.3.1, Fig. 15) in the present study suggests also toxic effects at 25 mg L^{-1} for IMMC. Toxic effects of high Diuron concentrations were previously described for both soil bacteria (Tixier et al., 2002; Sørensen et al., 2008) and fungi (Tixier et al., 2000). Nevertheless, Diuron mineralization at 1 mg L^{-1} was still on a low level, especially in comparison to IPU, suggesting that the concentration might not be the main reason for limited degradation by IMMC.

For the isolates known so far that were able to perform the ring cleavage of IPU, different enzymatic capabilities regarding degradation were reported: degradation experiments with different phenylurea herbicides suggested enzymatic specificity of *Sphingomonas* sp. SRS2 and *Sphingobium* sp. strains YBL2 and YBL3 for the group of dimethyl-substituted phenlyurea herbicides (Sørensen et al., 2001; Sun et al., 2009). In contrast, catabolic activities of *Sphingomonas* sp. strain SH and *Methylopila* sp. TES seemed to be highly specific for the metabolism of IPU as no other phenylurea herbicides were degraded by these isolates (El-Sebai et al., 2004; Hussain et al., 2011).

4.2.3 The importance of amino acids for the degradation of IPU, MD-IPU and DD-IPU by *Sphingomonas* sp. AK1

In the course of liquid culture experiments evidence grew that the IPU mineralizing strain *Sphingomonas* sp. AK1 benefited from the presence of other microbial members of IMMC regarding pesticide degradation.

Pure culture studies with IPU and its metabolites MD-IPU and DD-IPU, respectively, showed a fast disappearance of the applied compound when amino acids were added to the MS-medium (no IPU detected after day 11, MD-IPU up to day 28; recovery of 31.5 ± 3.0 % of DD-IPU at end of experiment at day 36; chapter 3.3.2, Fig. 19a,c,e). In MS-medium alone, only a minor disappearance was measured (recovery at day 30: 88.2 %, 54.1 % and 78.6 % of applied IPU, MD-IPU and DD-IPU, respectively; chapter 3.3.2, Fig. 19b,d,e). The same pattern was observed in liquid cultures with ^{14}C-IPU (MS-medium: maximum mineralization rate of 0.1 ± 0.0 % d^{-1}; MS-medium + amino acids: 3.3 ± 0.7 % d^{-1}; chapter 3.3.1, Fig. 17). This indicates that *Sphingomonas* sp. AK1 was auxotrophic and that its IPU mineralization ability depended on the provision of amino acids.

The important role that the presence of amino acids played for the degradation of IPU, MD-IPU and DD-IPU by *Sphingomonas* sp. AK1 in liquid culture might be attributed to a mechanism that was described in literature previously: the pollutant degrading bacterium within an bacterial consortia is reported as fastidious and depends, due to own metabolic deficiencies, on secondary strains that provide essential growth factors or nutrients (Sørensen et al., 2002; Dejonghe et al., 2003). This seems to be the case for *Sphingomonas* sp. AK1 and its supply with at least one amino acid. Only a few studies reported on this mechanism. For example, Maymo-Gatell et al. (1997) isolated an anaerobic bacterium from groundwater that was able to degrade Tetrachloroethene only in presence of either cell components from other specific bacteria or a mixture of amino acids. Metabolic deficiencies and thus a dependency on other microbial species and their metabolic products are also known for representatives of the genus *Sphingomonas*: a *Sphingomonas* sp. that was isolated by Hay et al. (2001) mineralized the biocidal product Triclosan effectively only in presence of other specific bacteria or on a complex medium. Pure culture studies with the IPU mineralizing strain *Sphingomons* sp. SRS2 and an unidentified bacterial strain SRS1 indicated that strain SRS1 provided amino acids to *Sphingomons* sp. SRS2, thereby enabling a significant increase in IPU mineralization by strain *Sphingomons* sp. SRS2 (Sørensen et al., 2002).

In case of *Sphingomonas* sp. AK1, the supply with amino acids played a decisive role for the degradation of IPU, MD-IPU and DD-IPU. Results indicate that at least one amino acid may be lacking thereby in multiple respect:

(1) For the formation of IPU degradation enzymes:

During microbial degradation of xenobiotics, all degradation steps are controlled by specific enzymes which are produced by the degrading cells. When no (more) adequate enzymes are present, the degradation process usually stops (Maier, 2009). As enzymes are proteins and are thus consisting of amino acids (Lim, 1998; Singleton and Sainsbury, 2001), the formation of enzymes is highly depending on the presence of amino acids. In case of *Sphingomonas* sp. AK1, a considerable disappearance of IPU was only measured when amino acids were present in the liquid medium (no more detection of IPU after 11 days; chapter 3.3.2, Fig. 19a). When no amino acids were applied to the medium, only minor IPU disappearance was detected (recovery of 88.2 % of applied IPU at day 30; chapter 3.3.2, Fig. 19b). The main disappearance in the latter case was observed immediately after the start of the experiment. This pattern was also observed when MD-IPU or DD-IPU was applied instead of IPU (chapter 3.3.2, Fig. 19c-f). This decrease in pesticide concentration at start of the experiment may be attributed to the procedure for preparing the bacterial inoculum: colonies of *Sphingomonas* sp. AK1 were detached from R2A plates, which contained amino acids, and mixed with 1x PBS buffer. Then, 1 mL of this solution was used for inoculation of liquid cultures. As it was shown that a centrifugation of liquid cultures leads only to a fragmentary pelletizing of cells from *Sphingomonas* sp. AK1 (chapter A1.2, Appendix), a washing of cells was not possible. Thus, small amounts of amino acids might have been transferred to the flasks and were then available for enzyme production at least at the beginning of the experiment.

(2) For the synthesis of new bacterial cells:

Cell counts showed that a proliferation of *Sphingomonas* sp. AK1 was only possible when IPU liquid cultures contained amino acids: a maximum of $61.1 \pm 10.9 * 10^7$ CFUs was measured in liquid medium containing amino acids (applied: $0.6 \pm 0.1 * 10^7$ CFUs; chapter 3.3.1, Fig. 17a), while cell numbers decreased immediately in MS-medium alone (i.e. at most $0.6 \pm 0.1 * 10^7$ CFUs; Fig. 17b). No cell counts are available for liquid cultures to which MD-IPU and DD-IPU were applied.

In general, many organisms require for the buildup of new biomass not only minerals and sources of carbon and energy, but also supplementary compounds, the so-called growth factors. Amino acids are attributed to the growth factors if they cannot be synthesized by the organism itself. Amino acids are components of proteins and nucleic acids and are thus needed in high amounts for the buildup of cells (Schink, 2007a). Proteins are typically the most abundant components (55 % of dry weight) of bacterial cells (Lim, 1998).

However, when amino acids but no IPU, MD-IPU or DD-IPU was applied to liquid cultures, basically stable cell numbers and no proliferation of *Sphingomonas* sp. AK1 was observed for 31 days (chapter 3.3.1, Fig. 18). This suggests that amino acids, which typically contain also C

and nitrogen (N) (Madigan and Martinko, 2006), provide these substances for the buildup of microbial cells and prevent a decrease in cell numbers.

(3) For the survival of applied bacterial cells:

The cell numbers of *Sphingomonas* sp. AK1 decreased rapidly especially within the first two days of the experiment when no amino acids but IPU was present in the liquid medium (day 0: 57.3 ± 9.3 $*10^5$ CFUs, day 2: 24.7 ± 8.0 $*10^5$ CFUs; chapter 3.3.1, Fig. 17b). This reduction in cell numbers suggests that compounds, that guarantee the functioning of elementary processes in cell metabolism, were lacking. As in replicates that contained amino acids but not IPU, basically stable cell numbers were observed (chapter 3.3.1, Fig. 18), amino acids might support the functioning of such processes in cell metabolism.

For *Sphingomonas* sp. AK1, the supply with amino acids played a decisive role regarding the degradation of IPU, MD-IPU and DD-IPU. The amino acid mix that was applied to the MS-medium contained, according to the manufacturers´ instruction leaflet, 18 different amino acids in varying amounts (chapter 2.3.1). From the microbial genera and species, respectively, that were identified from IMMC (chapter A1.2, Appendix, Table A1), especially those of the genus *Pseudomonas* are known for the biosynthesis of amino acids and might come into consideration for the support of *Sphingomonas* sp. AK1 with these compounds: for example, *P. dacunhae* was reported to synthesize L-alanine (Kumagai, 2006) while *P. aeruginosa* and *P. putida* were observed to form arginine (Voellmy and Leisinger, 1975) and methionine (Picardeau et al., 2003). Regarding the biosynthesis of amino acids by members of IMMC, attention should be paid to the fact that only 25 clones of IMMC were examined in this thesis. Thus, IMMC might yet contain several other species that could provide amino acids for *Sphingomonas* sp. AK1 than the ones identified in this study. For IPU degrading strains, a dependency on the presence of amino acids was only reported for *Sphingomonas* sp. SRS2: a test of various amino acids showed that exclusively the application of the amino acid L-methionine gave rise to a significant increase of IPU mineralization by *Sphingomonas* sp. SRS2 (Sørensen et al., 2002). However, it cannot be excluded that members of IMMC also provide other growth factors or nutrients to *Sphingomonas* sp. AK1.

In contrast to pure culture studies with *Sphingomonas* sp. AK1, no addition of amino acids was necessary for an effective IPU mineralization by the consortium IMMC. The maximum mineralization rate of IMMC in MS-medium for IPU (4.4 ± 2.3 % d^{-1}, chapter 3.2.1, Fig. 12, with application of 0.6 ± 0.0 $*10^7$ CFUs into 25 mL medium) was similar to the one of *Sphingomonas* sp. AK1 in MS-medium provided with the amino acid mix (3.3 ± 0.7 % d^{-1}, chapter 3.3.1, Fig. 17a, with application of 0.6 ± 0.0 $*10^7$ CFUs into 20 mL medium). This suggests that one or several microbial species of IMMC may have provided the amino acid(s) that were essential for *Sphingomonas* sp. AK1 and were thus contributing indirectly to the mineralization of IPU.

Although the supply with amino acids by members of IMMC played a decisive role for an effective mineralization of IPU by *Sphingomonas* sp. AK1, this pattern was not observed in liquid cultures with colony C1, from which *Sphingomonas* sp. AK1 was purified from: high mineralization was measured in MS-medium independent from the presence of amino acids (MS-medium: 66.3 % of applied ^{14}C-IPU; MS-medium + amino acids: 65.1 %; each in 44 days; chapter 3.1.2.2, Fig. 9a). The different response of colony C1 and the purified strain *Sphingomonas* sp. AK1 to amino acids might be an effect of the purification of colony C1: it is assumed that colony C1, which was detached from plate and tested for IPU mineralization, actually consisted of at least two different microbial species, the IPU mineralizing strain *Sphingomonas* sp. AK1 as well as an unidentified microbial species that was delivering amino acids to *Sphingomonas* sp. AK1. As soon as the pure culture solely with *Sphingomonas* sp. AK1 was present, *Sphingomonas* sp. AK1 could no more be supported with amino acids by this strain and became thus strongly depending on the external addition of amino acids.

It is assumed that the presence of amino acids played an important role already at the very beginning of the isolation process when microbes of IMMC were separated on agar plates, as the plates differed in their content of amino acids. After obtaining the purified strain *Sphingomonas* sp. AK1, several dilutions with it were spread on the three types of agar plates (MS-IPU, LB and R2A plates) that were utilized during isolation. Growth of colonies was only detected on R2A plates (chapter 3.1.2.2), which included several sources of amino acids like yeast extract (chapter 2.4.1.1). MS-IPU plates did not contain any amino acids (chapter 2.4.1.1) which is a major component of bacterial cells, thus preventing the synthesis of new cells, i.e. colonies. LB plates contained, like R2A plates, several sources of amino acids (chapter 2.4.1.1). Nevertheless, *Sphingomonas* sp. AK1 was not able to form colonies on LB plates. This may be attributed to an excess of nutrients provided by the rich LB plates to species of the genus *Sphingomonas* that are typically used and adapted to oligotrophic conditions (Balkwill et al., 2006).

4.3 Sustainability of the enhanced, metabolic IPU mineralization function induced by inoculation of IMMC into soil

Aim of the present experiment was to examine how long and to what degree the enhanced IPU mineralization, which is induced by inoculation of IMMC into soil, is maintained in soil when no IPU is detected in soil for several months.

4.3.1 IPU mineralization in soil

IPU mineralization after inoculation with IMMC and a single IPU application

The transfer of IMMC attached on Seramis® clay particles into soil Scheyern, supplied with 5 mg kg^{-1} of IPU (= 250 µg in 50 g dry soil), had a considerable effect on IPU mineralization: while only 34.4 µg (= 13.8 % of applied ^{14}C) were mineralized within 27 days in the non-inoculated soil (chapter 3.5.2.1), 133.6 µg (= 53.4 %) were mineralized in the same period when the soil was inoculated with IMMC (chapter 3.4.1.1). A clear sigmoid-shaped pattern was observed for cumulative IPU mineralization (chapter 3.4.1.1, Fig. 20), indicating a metabolic and thus cell growth induced mineralization of IPU by IMMC. Enhanced IPU mineralization was also reported by Grundmann et al. (2007) when inoculating IMMC into various soils that were supplemented once with IPU (5 mg kg^{-1}).

IPU mineralization after IPU application 2

To test if inoculated microbes of IMMC survived in soil and were still capable to mineralize IPU, IPU was applied for a second time to soil after different time spans (1.5, 6 and 7.5 months after application 1 as well as after a simulated winter period 10 months after application 1; chapter 2.8.1).

When IPU was added a second time to soil at different time intervals after the first IPU application, clear peaks in mineralization rates were detected also for all reapplications conducted (chapter 3.4.1.2, Fig. 21b-e), confirming that IPU was still mineralized metabolically in soil. However, the maximum mineralization rates in phases 2a, b and d (31.3, 27.3 and 23.8 µg d^{-1}, respectively; Fig. 21b-e) were significantly higher than the one in phase 1 (19.8 µg d^{-1}; for statistical significance see chapter 3.4.1.3, Fig. 23a). During metabolic degradation, pollutant degrading microbes can utilize the organic contaminant for the synthesis of new microbial biomass (Blume, 1990; Scheunert, 1994; Fomsgaard, 1997), thus, the number of pollutant degrading microbes affects the metabolic degradation rate (Becker, 2010). A significant higher maximum mineralization rate in phases 2a, b and d than in phase 1 therefore suggests that a higher number of IPU degrading microbes were present in soil incubators at the beginning of phases 2a, b and d than during phase 1 and degraded IPU more rapidly than after IPU application 1.

4.3.2 Establishment of IPU degrading microbes after inoculation

Survival of IMMC on Seramis® clay particles

To investigate indirectly if microbes of IMMC may have survived at all until reapplication of IPU and could thus be responsible for IPU mineralization in phase 2, Seramis® clay particles were

removed from soil and transferred to new soil incubators (containing 5 mg kg^{-1} IPU) 322 days after IPU application 1 (chapter 2.8.5.4).

The transfer of the Seramis® clay particles resulted in enhanced IPU mineralization: while only 34.4 µg (= 13.6 % of applied ^{14}C-IPU) were mineralized in the non-inoculated soil within 27 days (chapter 3.5.2.1), 101.5 µg (= 40.6 %) were measured in the same period when the Seramis® clay particles from phase 1 were introduced (chapter 3.4.4, Fig. 27a) into newly equilibrated soil supplemented with 5 mg IPU kg^{-1}. The sigmoid patterns of cumulative mineralization (Fig. 27a) as well as enhanced mineralization rates (Fig. 27b) indicate a metabolic mineralization of IPU. Thus, it can be concluded that microbes of IMMC survived on the Seramis® clay particles for at least 322 days and were still able to mineralize IPU efficiently.

However, IPU was less efficiently mineralized than in phase 1, where a considerably higher mineralization rate (19.8 µg d^{-1}, chapter 3.4.1.2, Fig. 21a; transfer: 5.7 µg d^{-1}, chapter 3.4.4, Fig. 27b) and a higher cumulative mineralization were measured (133.6 µg = 53.4 % of applied ^{14}C-IPU; day 27). The decreased mineralization efficiency of IMMC after the transfer of Seramis® clay particles may be attributed to (1) a potential impairment of microbes when they were removed from the bulk soil, as Seramis® clay particles dried a bit and may also be mechanically damaged by the tweezers with which they were held while the bulk soil was removed; (2) microbes of IMMC may have also been impaired during phase 1, e.g. by a lack of nutrients: from IPU application 1 on, no fertilizers were applied to the soil that could serve as a source of nutrients for microbes for more than 10 months. The continuous decrease in mineralization rates for phases 2a to 2d (chapter 3.4.1.2, Fig. 21b-e) may reflect this circumstance.

However, the loss in IPU mineralization efficiency appears to be only temporary: when soil incubators were stopped 51 days after transfer of Seramis® clay particles, a similar amount of ^{14}C-IPU was mineralized as in phase 1 in the same period (cum. mineralization after transfer: 137.9 µg, chapter 3.4.4, Fig. 27a; at day 51 of phase 1: 141.5 ± 7.3 µg, chapter 3.4.1.1, Fig. 20).

Metabolic IPU mineralization in bulk soil?

Enhanced IPU mineralization after a transfer of Seramis® clay particles into new soil incubators showed indirectly that inoculated IPU degrading microbes established and proliferated on Seramis® clay particles after inoculation.

However, it is assumed that microbes of IMMC did, besides this, not migrate from the Seramis® clay particles into the surrounding bulk soil and establish and proliferate also there. Two facts are considered as crucial in here:

(i) The survival and thus dispersal of external microbial cells in soils depends clearly on biotic factors like the predation by protozoa, which were frequently reported to prevent the establishment of external cells in soil (Gentry et al., 2004; Owsianiak et al., 2010).

If cells of IMMC disperse from the Seramis® clay particles into bulk soil, they would leave a comparatively protected area for a hostile environment, probably impairing their chances to survive. Van Dyke and Prosser (2000) could confirm that the use of carrier material has a protective effect on inoculated microbes: pre-incubation of *Pseudomonas fluorescens* on a sterile soil carrier resulted in a greater survival of microbes than application as a liquid cell suspension. Likewise, Wang et al. (2010) could show that a 1,2,4-TCB mineralizing microbial community that was inoculated on Seramis® clay particles into soil with 5 mg kg^{-1} 1,2,4-TCB did hardly migrate into bulk soil: 30 days after inoculation more than 97 % of the cells of the mineralizing strain *Bordetella* sp. F2 were detected on the carrier material.

(ii) The survival of introduced microbes also depends on the contaminant´s concentration and thus on a selective advantage towards native microbes that are not able to utilize the contaminant as a substrate (Pritchard, 1992; van Veen et al., 1997). Colores and Schmidt (1999), for example, investigated the survival of the Pentachlorophenol (PCP) mineralizing bacterium *Sphingomonas chlorophenolica* strain RA2 when inoculated into a soil containing different PCP concentrations (0, 10, 50, 100 and 300 mg kg^{-1}) for a period of 170 days. The long-term survival of S. *chlorophenolica* strain RA2 was clearly dependent on the initial PCP concentration: when 300 mg PCP kg^{-1} were applied to soil, the population size of S. *chlorophenolica* strain RA2 at day 0 (about $7*10^5$ CFUs g^{-1} soil) was not significantly different from the one at day 170 (about $1*10^6$ CFUs g^{-1} soil); when intermediate concentrations (10, 50 and 100 mg kg^{-1}, respectively) were added, cell numbers at day 170 were significantly lower than those at day 0 (e.g. variant with 10 mg kg^{-1}: about $3*10^7$ CFUs g-1 at day 0, $1*10^4$ CFUs g^{-1} at day 170). No cells of the strain were detected after 170 days when no PCP was added to soil (0 mg kg^{-1}).

In the present experiment, only a low amount of IPU (5 mg kg^{-1}) was applied to soil. Furthermore, IPU concentration in bulk soil decreased rapidly in the course of the experiment: the amount of bioavailable ^{14}C in soil pore water decreased from 67.3 µg at day of IPU application 1 to 1.8 ± 0.1 µg within 30 days (chapter 3.4.2.1, Fig. 24). Also the amount of MER in bulk soil declined fast (182.8 µg at day of IPU application 1, 7.3 ± 0.3 µg at day 30; Fig. 24), suggesting that only small amounts of IPU might be delivered to pore water from about 4 weeks after application 1 on. Given these marginal IPU concentrations in soil Scheyern, *Sphingomonas* sp. AK1 had probably only a low or no selection advantage at all towards native soil microbes for extensive periods of time. However, Seramis® clay particles and thus introduced IPU degraders were spread heterogeneously in soil. Despite this heterogeneous distribution it can be assumed that microbes on the carrier material were supported to a higher degree with

IPU than microbes in bulk soil: an inoculation experiment in soil with IPU and IMMC attached to Seramis® clay particles demonstrated a fast diffusion of IPU towards IPU degrading microbes on the carrier material, thus enabling rapid IPU mineralization in soil (Grundmann et al., 2007). Given this strong afflux of IPU towards the Seramis® clay particles, a spread of IMMC into bulk soil seems to misspend valuable energy resources. However, experiments on the dispersal of xenobiotic-degrading bacteria might confirm this assumption: resource availability was shown to play a decisive role in such a way that bacteria dispersed less when resources were abundant (Banitz et al., 2012).

It is also considered as unlikely that IPU degrading genes from *Sphingomonas* sp. AK1 were transferred towards native soil microbes, which would then have attributed to an enhanced IPU mineralization in phase 2. A survey of different xenobiotic-degrading Sphingomonads revealed that almost all strains contained two to five circular plasmids (Basta et al., 2004), i.e. an emission of genes may be in principal possible. These plasmids were shown to play a decisive role in the degradation of various xenobiotics, e.g. Naphthalene (Romine et al., 1999), Carbofuran (Ogram et al., 2000), Mecoprop (Lim et al., 2004) and Lindane (Nagata et al., 2007). However, there are no reports to the author´s knowledge confirming that degradation genes for IPU may also be situated on plasmids of *Sphingomonas´* species. However, even if *Sphingomonas* sp. AK1 may possess a plasmid and additionally may have the IPU degradation genes on that plasmid, it seems unlikely that these genes would be transferred under the conditions of the present experiment. A transfer of degradation genes from inoculated donor cells to native soil microbes was demonstrated to take place at medium or high contaminant concentrations, e.g. at 2.000 mg kg^{-1} for Naphtalene (Gomes et al., 2005) or at 100 mg kg^{-1} for 2,4-D (Dejonghe et al., 2000), thereby providing the recipient cell with a selective advantage towards other native microbes. There is no evidence that HGT may also occur at much lower concentrations (Top et al., 2002; Top and Springael, 2003), e.g. the 5 mg kg^{-1} of IPU which were applied in the present study. Furthermore, the amount of bioavailable IPU was shown to decrease rapidly in soil (chapter 3.4.2.1, Fig. 24 & Fig. 25), therefore microbes were provided with much less than 5 mg kg^{-1} of IPU for extensive periods of time until IPU was applied for the second time to soil, e.g. in case of application 2b for 6 months. And even if IPU degradation genes may have been transferred in the early phase of application 1 when IPU concentration was highest (5 mg $kg^{-1}$of IPU), the recipient cells would probably have lost this gene until application 2 as genes that are acquired by HGT were observed to get frequently lost when the selective advantage decreased (Madigan and Martinko, 2006).

Possible reasons for the long sustainability of the utilized inoculation approach

Mineralization results (chapter 3.4.1.2) in the present experiment demonstrated that IPU was efficiently mineralized 10 months after inoculation of IMMC into soil although only minimal amounts of IPU were available for microbes for extensive periods of time (chapter 3.4.2.1). Furthermore, the soil in the present study was inoculated only with a low number of microbial cells: the total of Seramis® clay particles, which were heterogeneously distributed in 50 g dry soil, contained $5.0 \pm 1.9 * 10^5$ CFUs when applied (chapter 3.4.1.1).

Typically, high cell numbers are applied to soil in both short- and long-term inoculation studies in order to attain a population size that is sufficient for pollutant degradation and to optimize survival and establishment of inoculated cells in soil:

E.g. Goux et al. (2003) inoculated a microbial consortium (COM15) that was able to degrade Atrazine into two agricultural soils (application of 10^5 cells g^{-1} soil). Microbial cells were applied as suspension to soil which was then incubated at 80 % of the maximum water holding capacity in laboratory. Nine months after inoculation rapid dissipation of Atrazine was measured and quantification of Atrazine degraders by the Most Probable Number (MPN) technique confirmed that inoculated cells were established in soil at time of Atrazine reapplication: at least $1*10^4$ cells g^{-1} soil were determined. However, the high contaminant concentration of 10,000 mg kg^{-1} may anyhow provided favorable conditions for the survival of introduced microbes.

In the previously mentioned study by Colores and Schmidt (1999), the PCP mineralizing bacterium *Sphingomonas chlorophenolica* strain RA2 was introduced into a soil to which a low level of PCP was added (10 mg kg^{-1}; unlabeled PCP). Bacteria were applied as suspension to soil (application of about $3*10^7$ cells g^{-1}) which was then incubated in laboratory at a water content equivalent to 70 % of the gravimetric water holding capacity. 170 days after inoculation, about 10^4 cells g^{-1} were detected in soil by MPN technique and, after reapplication of ^{14}C-PCP (100 mg kg^{-1}), enhanced mineralization was observed. At time of PCP-reapplication, cells of *S. chlorophenolica* strain RA2 were not only detected in this variant, but also in those which received less PCP (10 and 50 mg kg^{-1}). Unfortunately, no PCP was reapplied to these variants to examine PCP-mineralization. Also, the supply of microbes with PCP after application 1 was not reported for any variant. However, the soil used was sampled from a meadow and was very fertile (5.0 % SOM) and applied cell numbers were very high in all variants (minimum about $6*10^5$ g^{-1} cells g^{-1} soil), which both may favored the establishment and survival of inoculated microbes in soil.

When referring to a mass of 50 g dry soil, the cell numbers that were inoculated in the present experiment on the surface of Seramis® clay particles corresponded only to 10.0 % of cells that were applied by Goux et al. (2003) and less than 1 % of the minimum cell numbers applied by Colores and Schmidt (1999). Moreover, the applied pesticide concentration was much lower

(5 mg kg^{-1} of IPU). Furthermore, soil Scheyern was less fertile than the soil utilized by Colores and Schmidt (1999), with only 1.5 % total organic carbon (TOC) (Table 10). As SOM contains on average 50 % carbon (Kuntze et al., 1994; Blume et al., 2002), this corresponds to approximately 3.0 % SOM. However, accelerated IPU mineralization was detected still 10 months after inoculation of IMMC to soil.

But what are the reasons for the long sustainability of the present inoculation approach? The long-standing survival of introduced microbes of *Sphingomonas* sp. AK1 and the stable enhanced IPU mineralization might be attributed to several mechanisms:

(1) The **establishment of IMMC on autoclaved Seramis® clay particles** has probably contributed to the long-term survival of *Sphingomonas* sp. AK1 in soil.
It was reported previously that preincubation of inoculants on a sterile carrier material can enhance their survival in soil by providing a protective niche e.g. against grazing (Gentry et al., 2004; Owsianiak et al., 2010; Lebeau, 2011). This was confirmed also for the Seramis® clay particles that were used as carrier material in the present study: different techniques were tested for the introduction of microbes to soils with low pesticide concentrations e.g. of IPU (Grundmann et al., 2007) and 1,2,4-TCB (Schroll et al., 2004; Wang et al., 2010) (each soil with 5 mg kg^{-1} of the pollutant; incubation conditions were like in the present experiment (water potential of -15 kPa, soil density of 1.3 g cm^{-3}; chapter 2.7.2)). It was shown that the most effective and sustainable approach was to extract a microbial consortium from pesticide degrading soils and establish it on Seramis® clay particles as carrier material (Wang et al., 2010). This approach was more effective regarding pollutant degradation than an introduction of the microbial consortium via liquid culture or the application of the isolated single strain, either established on Seramis® clay particles or via liquid culture (Wang et al., 2010): e.g. when applying the consortium attached on the carrier material, a maximum mineralization rate for 1,2,4-TCB of $53.5 \pm 4.4 * 10^{-14}$ g d^{-1} per applied cell was measured, while the maximum rate was much lower when applying the microbes as cell suspension ($16.6 \pm 0.2 * 10^{-14}$ g d^{-1} per cell). The inoculation of microbial consortia that were previously attached to Seramis® clay particles was shown to be effective not only in laboratory studies (Grundmann et al., 2007; Wang et al., 2010) but also under outdoor conditions in a lysimeter system (Grundmann et al., 2007).

(2) Several results in this thesis indicate that *Sphingomonas* sp. AK1 benefited highly from the presence of other species of IMMC, e.g. by the supply with amino acids (e.g. chapter 3.3.1). When inoculating *Sphingomonas* sp. AK1 to liquid cultures with IPU, MD-IPU or DD-IPU, degradation was low and cell numbers decreased rapidly when just using MS-medium (chapter 3.3.2, Fig. 19b,d,f). When adding an amino acid mix to this liquid

medium, fast degradation and a considerable proliferation of microbial cells were observed (Fig. 19a,c,e). No addition of amino acids was necessary for liquid cultures containing IMMC instead of *Sphingomonas* sp. AK1 (chapter 3.2.2, Fig. 13a,c,e), indicating that *Sphingomonas* sp. AK1 was supported by other microbes of IMMC with amino acids. **Possibly, IMMC even formed a microbial biofilm on the Seramis® clay particles** on which they were inoculated into soil, contributing to a better survival of microbes. In general, biofilms are defined as aggregates of microbial cells which reside in a complex matrix that attaches them to a solid surface (Madsen, 1998; Flemming and Wingender, 2001). Such biofilms constitute an important strategy of microbes to survive also in harsh environments like agricultural soils as interactions can evolve easily (Flemming and Wingender, 2001). Due to these microbial interactions, microbes in biofilms can benefit from several advantages that individual cells can not enjoy (Schink, 2007b; Ottow, 2011): e.g. microbes within biofilms are to a lower extent exposed to hydraulic stresses, nutrient deprivation, pH changes and dehydration (Flemming and Wingender, 2001; Madsen, 2008; Lebeau, 2011). If microbes of IMMC formed a microbial biofilm on Seramis® clay particles, advantages like these have probably contributed to a better survival of *Sphingomonas* sp. AK1 after inoculation into soil.

The recent study by Wang et al. (2013) aimed to reveal the reasons for the efficient degradation of 1,2,4-TCB that was observed by Wang et al. (2010) when introducing a microbial consortium attached to Seramis® clay particles to soil. They demonstrated a more efficient cell-to-cell communication of mineralizing microbes when attached to the carrier material compared to a non-attached community, resulting in higher pesticide mineralization. This finding was considered as a hint for the development of a microbial biofilm on the carrier material (Wang et al., 2013).

4.3.3 Extractable IPU residues in soil

4.3.3.1 Extractable IPU residues after inoculation with IMMC and a single IPU application

After IPU application 1, extractable IPU metabolites were identified and quantified regularly in order to determine the supply of microbes with IPU.

Amount of extractable IPU residues

The inoculation of soil Scheyern with IMMC resulted in a rapid decrease of extractable ^{14}C in soil (day 0: 26.9 % of applied ^{14}C were dissolved in pore water and 73.1 % extractable by MeOH; day 30: 0.7 % in pore water and 3.0 % MER; chapter 3.4.2.1), therefore only marginal amounts were available for microbial degradation for extensive periods of time.

A rapid reduction of extractable ^{14}C was previously also reported by Grundmann et al. (2007) for other soils (each with an application of 5 mg kg^{-1} IPU) that were inoculated with IMMC, e.g. for soil Kelheim (day 44: PW + MER = 3.2 ± 0.2 % of applied ^{14}C in inoculated soil and 11.5 ± 0.8 % in non-inoculated soil, respectively) as well as an Urbic Anthrosol (day 44: PW + MER = 4.3 ± 0.2 % of applied ^{14}C in inoculated soil and 37.0 ± 5.5 % in non-inoculated soil, respectively).

Identified extractable IPU residues

The number and type of metabolites depended strongly on the date of sampling, both in pore water and in the MeOH-extractable fraction: while the number of metabolites reduced quite fast in pore water (IPU and 8 metabolites were distinguished; detection only within 24 days after IPU application 1; chapter 3.4.2.1, Fig. 25), no clear reduction in the number of metabolites with time was observed in the MeOH-extractable fraction (IPU and 15 metabolites were distinguished; metabolites were detected until day 322; chapter 3.4.2.1, Table 16). Metabolites of IPU were continuously formed and further metabolized. There are only three long-term inoculation studies in soil known to the author that exceed a period of 60 days, namely for Atrazine (Struthers et al., 1998; Goux et al., 2003) and a undefined mixture of oil products at a depository area for chemical industry solid waste (Juhanson et al., 2009). None of them determined metabolites and their dynamics with time. For IPU, only Kühn (2003) identified extractable metabolites in inoculated soil, but this was done only once at the end of the experiment at day 46.

In the course of the present experiment, MD-IPU was the metabolite accumulating in highest concentration in the MeOH-extractable fraction (except at days 20 and 24; chapter 3.4.2.1, Table 16), indicating that the demethylation that transforms IPU to MD-IPU was a rate-limiting step in IPU mineralization by IMMC. This finding is in agreement with liquid culture studies with IMMC, where MD-IPU was degraded considerably faster than IPU: MDIPU was below the LOQ from day 14 on, while IPU was detected in liquid culture until the end of the liquid culture experiment at day 21 (chapter 3.2.2, Fig. 14). The accumulation of MD-IPU in the present soil study is also in accordance with other soil studies: MD-IPU was observed as main metabolite in soil Feldkirchen, from which IMMC was extracted years ago (Schroll and Kühn, 2004); furthermore, when inoculating another agricultural soil with IMMC, the share of MD-IPU was reported to increase in comparison to the non-inoculated soil (Kühn, 2003).

According to liquid culture studies, also the metabolite 4IA was formed during IPU degradation by IMMC. It was detected at 7 samplings until the end of the study at day 21 (chapter 3.2.2, Fig. 13a). In inoculated soil, the metabolite 4IA was measured only at two samplings (15 and 20 days, respectively, after application of IPU; chapter 3.4.2.1, Table 16) and only in minimal amounts. It is assumed that 4IA in soil was, parallel to its degradation, adsorbed to soil particles as 4IA was reported to be rapidly bound to soil particles in a non-extractable form (Bollag et al., 1978; Reuter

et al., 1999). 4IA has been previously detected in various agricultural soils (Mudd et al., 1983; Lehr et al., 1996a; Sørensen and Aamand, 2001), but not in soil Feldkirchen (Schroll and Kühn, 2004), where IMMC originated from.

4.3.3.2 Extractable IPU residues after IPU application 2

At the end of phases 2a to 2d, also the amount of extractable IPU residues was determined. As the application standard of ^{14}C-IPU, which was applied to these replicates, had a low specific radioactivity due to financial reasons (chapters 2.8.4.1 & 2.8.4.2), extractable metabolites could not be identified.

4.3.4 Distribution of ^{14}C between the fractions mineralization, IPU residues in PW, MER and NER

Distribution of ^{14}C after inoculation of IMMC and a single IPU application

The distribution of ^{14}C, which was regularly determined after IPU application 1 in four fractions (mineralization, residues dissolved in pore water, MeOH-extractable and non-extractable IPU residues), was strongly dependent on sampling time (chapter 3.4.3.1): immediately after the start of the experiment, the extractable fraction decreased quickly, indicating that IPU degradation was not limited by the degradation capabilities of microbes. Parallel to the decrease in the extractable fraction, the portions of mineralization and NER increased in about equal portions. Over the months, the portion of NER decreased while ^{14}C was mineralized more and more.

Previous long-term inoculation studies in soil (Colores and Schmidt, 1999; Goux et al., 2003; Juhanson et al., 2009) focused on the survival of introduced microbes rather than on the kinetics of pesticide residues, thus no comparison could be drawn to other inoculation studies concerning the dynamics of pesticide residues. However, a time-dependent distribution of applied ^{14}C between individual fractions was reported previously for non-inoculated agricultural soils that received IPU: Mordaunt et al. (2005) applied 2.7 mg kg^{-1} of ^{14}C-IPU to microcosms with an agricultural soil and examined the amount of ^{14}C in the individual fractions over a period of 91 days. Pieuchot et al. (1996) did so for a period of 120 days in three soils under laboratory conditions that had received 1000 g ha^{-1} of ^{14}C-IPU. As observed for the inoculated soil in the present study, the distribution of applied ^{14}C changed especially in the first month after application of IPU to soil, with decreasing portions of extractable ^{14}C and increasing shares of mineralization and NER. However, the distribution between the individual fractions was different for each type of soil, depending e.g. on pH and thus activity of microbes (Pieuchot et al., 1996; Mordaunt et al., 2005).

The ^{14}C distribution between inoculated (chapter 3.4.3.1) and non-inoculated soil Scheyern (chapter 3.5.2.2) differed considerably after one application of IPU: in inoculated soil about equal shares of ^{14}C were mineralized (46.3 % of applied ^{14}C) and detected as NER (51.9 %), respectively,

after 24 days, while only a minimal portion was extractable (3.2 %). In contrast, only 13.8 % of applied ^{14}C was mineralized in the non-inoculated soil after a similar period of time (27 days), while 58.6 % were measured to be non-extractable and 34.3 % extractable residues. Thus, inoculation resulted in considerably enhanced mineralization and a decreased amount of extractable IPU residues compared to non-inoculated soil. The same trend was reported also for several other soils that were inoculated with IMMC and received 5 mg kg^{-1} of IPU (Grundmann et al., 2007).

Distribution of ^{14}C after IPU application 2

The distribution of ^{14}C in inoculated soil at the end of phases 2a to 2d was characterized by noticeable similar portions for the individual applications (chapter 3.4.3.2, Table 17): a nearly identical portion (61.9 - 64.1 %) of the so far applied ^{14}C was mineralized in phases 2a to 2d, even though the duration of phase 1 (e.g. applications 2a and 2d were performed 1.5 and 7.5 months after application 1) and thus the amount mineralized in this period differed to some extent considerably. The portion of NER at the end of applications 2a to 2d was also in a quite narrow range (26.3 - 33.3 %) and thereby in a dimension that was achieved in phase 1 only after several months, while phase 2 lasted just 21 to 27 days. Altogether, IPU application 2 resulted in a proportional smaller fraction of NER and higher percentage of mineralized ^{14}C if application 2 was done within about 6 months after application 1. When application 2 was done later, the percentage detected as NER or mineralized, respectively, was similar to the ones in phase 1. However, at the end of all phases 2 a characteristic proportion of about 1/3 NER and 2/3 mineralization was measured.

As previously mentioned there are no studies to the author´s knowledge in which ^{14}C-labeled pesticides were applied to soil and the effect on pesticide residues were determined.

4.4 Adaptation of microbes in non-inoculated soils to IPU mineralization by repeated IPU applications

Aim of the following experiment was to investigate if IPU mineralization could be increased by repeated applications of IPU at low concentrations (5 mg kg^{-1}) in soils that degrade IPU cometabolically after a single IPU application, as it was previously observed for soils Feldkirchen, Scheyern, Kelheim and Konjišče (Folberth et al., 2009).

4.4.1 Effect of repeated IPU applications on soil Feldkirchen

4.4.1.1 Effect of repeated IPU applications on IPU mineralization

The pattern for IPU mineralization in soil Feldkirchen changed in the course of the experiment: After IPU application 1, constant mineralization rates at low level (mean 0.9 ± 0.0 µg d^{-1}; chapter 3.5.1.1, Fig. 28) indicated a cometabolic degradation of IPU. With further applications of IPU,

maximum mineralization rates increased, suggesting a change from a cometabolic to a metabolic IPU mineralization pattern (e.g. phase C2: 3.3 ± 2.0 µg d^{-1}, phase C4: 8.3 ± 1.2 µg d^{-1}). The portion of IPU that was mineralized in phase C1 (10.9 ± 0.3 % of applied ^{14}C within 29 days; chapter 3.5.1.2, Table 18) was in the same range as the amount reported for the soil by Folberth et al. (2009) (< 15 % of total ^{14}C applied within 46 days) after a single IPU application and increased considerably until phase C4 (33.9 ± 3.5 % of applied ^{14}C; Table 18).

A change in the IPU degradation pattern was also confirmed by the fact that the portion of extractable ^{14}C decreased clearly from phase C1 to C4 (C1: 42.7 ± 3.4 % of applied ^{14}C; C4: 13.4 ± 3.5 %; Table 18). A continuous decrease in the lag phase (phase C2: 12.2 ± 2.6 days after application 2, 3; phase C4: 0.8 ± 0.0 days after application 4; chapter 3.5.1.1, Fig. 29b) indicates an increasing IPU degradation efficiency of the soil.

Mineralization rates of **replicate F1** were at a similar level as in replicates F2-F6 (phase C2: 1.6 µg d^{-1}; C3: 6.2 µg d^{-1}; C4: 13.4 µg d^{-1}; chapter 3.5.1.1, Fig. 28), but the time period to the maximum rate was clearly longer, especially in phases C3 and C4 (C3: 21.8 and 17.8 days after applications 3 and 4, respectively; Fig. 28).

4.4.1.2 Adaptation to IPU mineralization or recovery of the specific soil function IPU mineralization?

Soil Feldkirchen was reported to mineralize IPU cometabolically after a single application of IPU (Folberth et al., 2009) and showed a metabolic IPU mineralization pattern when treated repeatedly with IPU in the present experiment. However, a degradation study that was conducted in 2001/2002 reported that this soil was able to degrade IPU metabolically before 2003 (Grundmann et al., 2007). Investigations by Levy et al. (2007) showed that the IPU mineralization capability of the soil was impaired by the hot and dry summer of 2003. Thus, no conclusions can be drawn on whether the metabolic mineralization pattern observed in the present study was induced by (1) the recovery of the metabolic IPU mineralization function that the soil exhibited before summer 2003 or by (2) adaptation of the soil to accelerated IPU mineralization.

However, did soil Feldkirchen at the end of the present experiment show an IPU degradation capability similar to that before its impairment during summer 2003? Kühn (2003) conducted degradation experiments with IPU in soil Feldkirchen in 2001/2002 and reported after a single IPU application (5 mg kg^{-1}) a maximum mineralization rate of 3.3 % of the applied ^{14}C per day, which corresponds to 8.3 µg d^{-1}; according to the exhibited graph, standard deviations seemed to be small and are estimated to less than 0.2 µg d^{-1}. Similar rates were achieved in the present thesis in phases C3 and C4 (C3: 9.1 ± 2.8 µg d^{-1}, C4: 8.3 ± 1.2 µg d^{-1}; chapter 3.5.1.1, Fig. 28), but standard deviations were much higher. However, finally 33.9 ± 3.5 % of the applied ^{14}C was mineralized until the end of phase C4 (chapter 3.5.1.2, Table 18), while the cumulative mineralization before

summer 2003 was 48.6 % after a single application (Kühn, 2003), indicating a weaker mineralization capability of soil Feldkirchen in the present study. Mineralization results indicate that the increase in IPU mineralization induced by repeated applications of IPU may be largely finished already after three IPU applications as no further significant increase in the maximum mineralization rate was observed between IPU applications 3 and 4 (chapter 3.5.1.1, Fig. 29a).

Levy et al. (2007) simulated the water and heat stress of summer 2003 in the laboratory with soil Feldkirchen that was sampled from the deeper soil of the lysimeter and confirmed that these environmental conditions resulted in reduced IPU mineralization in the upper soil layer. The effect of disturbance and stress, respectively, on ecosystem stability can be classified into two components, namely resistance and resilience. While resistance is defined as the capacity to withstand disturbances, resilience can be defined as the capacity to recover after a disturbance (Pimm, 1984; Seybold et al., 1999). Disturbances can lead to a reduced biodiversity and changes in microbial community structure, especially at high stress levels and/or disturbance over prolonged time periods, as microbes are to a different extent tolerant to particular stress factors (Giller et al., 1998; Tobor-Kaplon et al., 2005). A change in the microbial community structure due to drought and heat of summer 2003 was also demonstrated in soil Feldkirchen (Levy et al., 2007). Some studies suggested that reduced microbial diversity in soils or changes in their microbial community structure can reduce resilience after disturbances (Griffiths et al., 2000; Girvan et al., 2005; Banning and Murphy, 2008). Levy et al. (2007) showed that four weeks of re-equilibration for the impaired soil Feldkirchen and a subsequent single IPU application did not result in metabolic IPU mineralization as it was observed before summer 2003. The repeated applications of IPU to soil Feldkirchen in the present experiment led to a higher IPU mineralization as observed by Levy et al. (2007), but results also indicated that the increase in IPU mineralization may probably be finished. The observation that IPU mineralization after repeated IPU applications in the present study was still lower than mineralization before summer 2003 may be attributed to (1) a permanent impairment of IPU degrading microbes, enabling only a limited resilience or adaptation, respectively, of soil microbes with respect to IPU mineralization and/or (2) deficiencies in compounds that are essential for efficient IPU degradation; this may concern e.g. amino acids that were shown to be crucial for survival and IPU mineralization by *Sphingomonas* sp. AK1 (chapter 4.2.3) as IPU mineralizing microbe in soil Feldkirchen. The amino acid mix used for liquid culture studies with *Sphingomonas* sp. AK1 and essential for its IPU mineralization contained also the amino acid methionine that was reported to improve bacterial tolerance to environmental stressful conditions (Andersen et al., 1998). As soil microbes were shown to be to a different degree tolerant to heat (Hart et al., 2005), possibly one or several microbial species were harmed during summer 2003 that formed compounds essential for survival of *Sphingomonas* sp. AK1 and its IPU mineralization.

The maximum mineralization rate in replicate F1 was measured after a considerably longer time period in phases C3 and C4 than in replicates F2-F6 (chapter 3.5.1.1, Fig. 28). As IPU degradation rates in soils were frequently observed to vary strongly even at millimeter scale (Gonod et al., 2003) and microbes of soil Feldkirchen were maybe not impaired uniformly by the climatic conditions in summer 2003, it is assumed that this replicate had different metabolic characteristics than replicates F2-F6.

Repeated applications of IPU to soil Feldkirchen enhanced IPU mineralization to a lower extent as in the soils Scheyern, Kelheim and Konjišče (chapter 3.5.2), thereby illustrating the limited IPU degradation capabilities of microbes in soil Feldkirchen. In soil Feldkirchen, the portion of mineralized ^{14}C-IPU increased significantly from phase C1 (10.9 ± 0.3 % of applied ^{14}C; chapter 3.5.1.2, Table 18) to C4 (33.9 ± 3.5 %; Table 18), while the amount of extractable ^{14}C decreased significantly (phase C1: 42.7 ± 3.4 %; phase C4: 13.4 ± 3.8 %; Table 18), both confirming a change from cometabolic to metabolic IPU mineralization. Nevertheless, this change in soil Feldkirchen was weaker and slower developed than in soils Scheyern, Kelheim and Konjišče whose soil microbes were previously not impaired: from phase C1 to C3, the amount of ^{14}C-IPU mineralized in soil Feldkirchen increased by 255.1 % while the amount of extractable residues decreased by 48.4 % (data Table 18). In contrast to this, mineralization in soils Scheyern, Kelheim and Konjišče increased by 367.1, 377.2 and 484.3 % from phases P1 to P3, while extractable residues declined by 97.0, 81.9 and 76.9 %, respectively (chapter 3.5.2.2).

4.4.2 Effect of repeated IPU applications on soils Scheyern, Kelheim and Konjišče

In the previous experiment an increase of IPU mineralization was observed in soil Feldkirchen after repeated applications of IPU. However, it could not be ascertained whether this enhancement was induced by (1) the recovery of the metabolic IPU mineralization function that was detected in the soil before its impairment during summer 2003 or by (2) adaptation of the soil to accelerated IPU mineralization. To test if a change from cometabolic to metabolic IPU mineralization pattern may be induced by adaptation of soil microbes, three further soils were investigated which did not mineralize IPU metabolically before.

While IPU was added to soil Feldkirchen in total four times, it was applied three times to soils Scheyern, Kelheim and Konjišče. Moreover, phase lengths differed slightly between soil Feldkirchen (chapter 3.5.1.1, Fig. 28) and the other three soils (chapter 3.5.2.1, Fig. 32). Therefore, phases for soils Scheyern, Kelheim and Konjišče (phases P1, P2 and P3) were designated different from phases of soil Feldkirchen (phases C1, C2, C3, C4).

4.4.2.1 Effect on IPU mineralization

After IPU application 1 constant mineralization rates at low level were detected in all three soils (1.2 ± 0.5, 1.0 ± 0.2 and 0.9 ± 0.6 µg d^{-1} for soils Scheyern, Kelheim and Konjišče, respectively; chapter 3.5.2.1, Fig. 32), indicating a cometabolic degradation of IPU. In phase P2, elevated mineralization rates and clear variations between replicates were observed, especially in soil Scheyern (15.2 ± 8.9, 8.0 ± 2.5 and 8.2 ± 6.5 µg d^{-1} for soils Scheyern, Kelheim and Konjišče, respectively). This change in mineralization dynamics suggests a change in the metabolic pattern from cometabolic to metabolic IPU degradation. In phase P3, high mineralization rates at a similar level and with similar lag phases were measured for all replicates of soil Scheyern and also for those of soils Kelheim (except replicate KE1) and Konjišče (21.6 ± 3.3, 28.7 ± 7.5 and 24.7 ± 8.9 µg d^{-1} for soils Scheyern, Kelheim (without replicate KE1) and Konjišče, respectively), indicating a distinct metabolic mineralization of IPU and confirming a change in degradation pattern within three applications of IPU for all three soils.

The maximum mineralization rate in soil Scheyern was in a similar range when the non-inoculated soil was treated three times with IPU (21.6 ± 3.3 % d^{-1}; chapter 3.5.2.1, Fig. 32) and when the soil was inoculated with IMMC and treated with IPU once (19.8 ± 2.0 % d^{-1}; chapter 3.4.1.2, Fig. 21a). Thus, IPU mineralization in the non-inoculated soil may be highly efficient already after three applications of IPU, when low IPU concentrations (5 mg kg^{-1}) are applied at laboratory conditions optimal for IPU degradation (water potential of -15 kPA, soil density of 1.3 g cm^{-3}; chapter 2.7.2).

4.4.2.2 Distribution of ^{14}C at the end of phases P1, P2 and P3

The change in IPU mineralization dynamics was reflected in the distribution of ^{14}C (chapter 3.5.2.2, Fig. 35): At the end of phase P1 low amounts of IPU were mineralized while considerable amounts were extractable in all soils and their replicates, indicating that IPU degradation was not limited by the bioavailability of the compound but rather by the number of IPU degrading cells and/or their degradation capabilities, respectively. At the end of phase P3, the ^{14}C was again distributed in a similar pattern in the three soils: the share in mineralization was considerably higher than in phase P1and portions of extractable ^{14}C were clearly lower, illustrating that degradation was in contrast to phase P1 now limited by the bioavailability of ^{14}C-IPU.

4.4.2.3 Adaptation of microbes to IPU mineralization

The change in mineralization degree and dynamics (chapter 3.5.2.1, Fig. 32) as well as the distribution of ^{14}C at the end of phases P1 and P3 (chapter 3.5.2.2, Fig. 35) suggest that microbes of all three soils adapted to IPU mineralization within three applications of IPU at intervals of about four weeks. The mechanisms that can lead to the adaptation of microbes to contaminant degradation are versatile and may in general involve (1) the induction of degradation enzymes, (2)

the proliferation of cells that are able to degrade the respective compound and that are already present in soil, but in amounts below a threshold size sufficient for IPU degradation, and (3) genetic changes within the microbial population, e.g. mutation, DNA recombination and HGT (Spain et al., 1980; Sørensen and Aamand, 2003; Top and Springael, 2003; van der Meer, 2006). At the end of phase P3, all replicates of soil Scheyern showed similar mineralization rates and lag phases, also the distribution of ^{14}C between the individual fractions had a similar pattern. The same trend was observed in soils Konjišče and Kelheim (except replicate KE1). These similarities strongly suggest that the adaptation of the microbial communities in soils Scheyern, Kelheim, and Konjišče, respectively, was not a result of genetic changes as these are reported to be random and hardly controllable incidents (Munk, 2000). Also a transfer of IPU degrading genes towards other microbial species by HGT seems unlikely as the HGT rate depends strongly on the pesticide concentration (Rensing et al., 2002; Top et al., 2002) and seems unlikely to occur at low pesticide concentrations (Top et al., 2002; Top and Springael, 2003) like the ones that prevailed in the present experiment (3 applications of each time 5 mg kg^{-1}). It is rather assumed that soil microbes which were able to mineralize IPU needed either to induce IPU degradation enzymes or to proliferate to a threshold size sufficient for IPU degradation. Thus, microbes that were able to degrade IPU had to be present in soil already at time of IPU application 1 in the present experiment. Two factors may have contributed to the fast adaptation of microbes in investigated soils to accelerated IPU mineralization: (1) the soils may have contained one or several microbial populations with broad metabolic capabilities which were able to degrade a multitude of natural organic compounds and which could adapt fast to applications of xenobiotics as e.g. reported for species of the genus *Sphingomonas* (Sørensen et al., 2002; Balkwill et al., 2006; Stolz, 2009) and/or (2) the application history of soils regarding IPU may have played a role: all investigated soils had received several applications of IPU before they were sampled. When the soils Scheyern, Kelheim and Konjišče were utilized for this study, they had not received any IPU application for at least 19, 12 and 2 years, respectively (chapter 2.6). However, it is not known if soils degraded IPU metabolically before sampling. In case of a metabolic IPU mineralization pattern before sampling, IPU degrading microbes may have survived for prolonged time periods without further applications of IPU and/or were capable to utilize other sources of C than IPU. It is reported in literature that accelerated pollutant degradation in soil can be preserved over prolonged time periods also in the absence of further pollutant applications, e.g. for at least 5 years in case of Carbofuran (Karpouzas et al., 1999) and 4 years with Fenamiphos (Ou, 1991).

Walker and Austin (2004) investigated the stability of accelerated IPU degradation in soils with different pesticide history and detected enhanced IPU mineralization only in soils that had received IPU applications within the last six years. Sørensen and Aamand (2003) examined IPU mineralization in soil slurries (10 mg L^{-1} of IPU) of agricultural soils that were either treated with

IPU for 9 consecutive years before soil sampling or that previously never received IPU or other phenylurea herbicides. The results of this study suggested, similar to the ones by Walker and Austin (2004), that rapid IPU mineralization in investigated agricultural soils was related to the previous exposure to that compound. The study of Cox et al. (1996) reported contrary results: they applied unlabeled IPU to 10 agricultural soils (each 10 mg kg^{-1}) in intervals of 25 days and showed an enhancement of IPU dissipation regardless of previous treatment history. However, this study examined solely the disappearance of IPU, thus, formed metabolites may have been overlooked. Although soils Scheyern and Kelheim did not receive IPU applications for at least 19 and 12 years, respectively, it cannot be excluded that application history regarding IPU may have contributed to an accelerated the adaptation of microbes in soils in the present experiment.

The quite large variation in mineralization rates in phase P2 (chapter 3.5.2.1, Fig. 32), especially in soil Scheyern, is attributed to variations in population size of IPU mineralizing microbes: several studies demonstrated a strong variation in pesticide degradation on different spatial scales, in fact on field (Walker et al., 2001; Bending et al., 2003), plot (Walker and Brown, 1983) and even millimeter scale (Gonod et al., 2003). This spatial variation in pesticide degradation is explained by spatial differences in physical, chemical and biological processes in soil as well as to variations in application rates (Price et al., 2009). Hence, it can be assumed that IPU mineralizing microbes were distributed heterogeneously in the three investigated soils.

One replicate of soil Kelheim (replicate KE1) was, in contrast to all other replicates and soils, still characterized by a low maximum mineralization rate even after three applications of IPU (KE1: 12.0, KE2: 23.0, KE3: 25.8 and KE4 37.2 µg d^{-1}; chapter 3.5.2.1, Fig. 32), indicating that no alteration from cometabolic to metabolic degradation occurred in this replicate. At the end of phase 3, still considerable amounts of extractable ^{14}C were measured (KE1: 15.2 % of applied ^{14}C; mean of replicates KE2-KE4: 2.7 ± 1.0 %; chapter 3.5.2.2, Fig. 35), suggesting that IPU degradation was not limited by the bioavailability of IPU and its metabolites to microbes but rather by the amount and capabilities of IPU degrading microbes. This may be induced e.g. by a local low soil pH which was frequently reported to strongly affect IPU degradation in liquid cultures (Bending et al., 2003; Hussain et al., 2009; Sun et al., 2009) and soil (Houot et al., 2000; El-Sebai et al., 2005).

4.4.2.4 Implications for bioremediation

The present experiment demonstrated that the threefold application of IPU at low concentration (5 mg kg^{-1}) in intervals of four weeks was a suitable strategy to accelerate IPU mineralization in the three investigated agricultural soils that had only shown cometabolic IPU mineralization after a single application. This may enable subsequently the extraction of the respective microbial communities and their later use for inoculation purposes. This strategy may also work for other biodegradable pesticides than IPU.

The present experiment was conducted in laboratory (1) under conditions that were determined as optimal for microbial pesticide degradation (water potential of -15 kPa and soil density of 1.3 g cm $^{-3}$; chapter 2.7) and (2) IPU was applied in short intervals of about four weeks, resulting in a more or less continuous supply of soil microbes with IPU. Conditions in agricultural practice vary from the laboratory conditions in the present study in some respects, e.g. (a) soil moisture fluctuates, which reduces time periods with a water supply optimal for microbial pesticide degradation (-15 kPa) and (b) pesticides in agricultural practice are typically applied less frequently and time intervals between applications are longer, resulting in longer time periods with lower pesticide concentrations. However, a high selection pressure, which is in this case the contaminants´ concentration, and a preferably long contact time favor (i) the survival and proliferation of cells that are able to degrade the pollutant in soil (Spain and van Veld, 1983; Top et al., 2002; Macleod and Semple, 2006) and (ii) the transfer of pesticide degrading genes to receptor cells (Rensing et al., 2002). Therefore it is expected that adaptation of microbes to pesticide degradation takes a longer period at field conditions than under laboratory conditions.

4.4.2.5 Adaptation of native soil microbes to metabolic IPU mineralization in inoculated soil Scheyern?

When investigating the sustainability of enhanced IPU mineralization in soil Scheyern that was inoculated with IMMC, IPU was applied a second time to inoculated soil after different time intervals in order to test to what extent IPU mineralization was still increased (chapter 3.4). In this phase 2, IPU was mineralized with high rates that were similar to the ones after a single IPU application (chapter 3.4.1.2, Fig. 21b-e). Results suggest indirectly that inoculated microbes survived on the carrier material after inoculation and were still able to mineralize IPU efficiently in phase 2 (chapter 4.3.2).

As enhanced IPU mineralization rates were observed in the non-inoculated soil Scheyern due to adaptation of soil microbes already after two applications of IPU (chapter 4.4.2.3), it may be conceivable that adapted native soil microbes may have contributed to accelerated IPU mineralization also in the inoculated soil after two applications of IPU.

However, several facts argue for the scenario that an adaptation of native soil microbes to increased IPU mineralization could not have contributed considerably to increased IPU mineralization in phase 2 in the inoculated soil Scheyern:

(a) <u>IPU mineralization by native soil microbes:</u>

When IPU was reapplied to the non-inoculated soil 27 days after IPU application 1, the maximum mineralization rate was 8.2 ± 13.3 µg d^{-1} (chapter 3.5.2.1) and thus considerably lower than in the inoculated soil, where after IPU application 2a (conducted 1.5 months

after application 1) a maximum rate of 31.1 ± 2.1 µg d^{-1} was measured (chapter 3.4.1.2, Fig. 21b-e).

The period of time until the maximum mineralization rate was detected, was also different between the non-inoculated and the inoculated soil Scheyern: in the non-inoculated soil, these time periods differed clearly between replicates, with an average period of 14.3 ± 9.0 days (chapter 3.5.2.1). In contrast, the maximum mineralization rates in inoculated soil were measured much earlier and varied less (application 2a: 5.8 ± 0.0, 7.8 ± 0.0, 8.3 ± 0.6 and 9.3 ± 1.0 days for applications 2a to 2d (chapter 3.4.1.2, Fig. 21b-e)).

Given these clear differences (1) in maximum mineralization rates and (2) the time period when these rates were measured between non-inoculated and inoculated soil Scheyern, the adaptation of native microbes to IPU mineralization cannot explain the stable and high mineralization in inoculated soil.

(b) Conditions for adaptation of native microbes to IPU mineralization:

When 5 mg kg^{-1} of IPU were applied for the second time to the non-inoculated soil, still considerable amounts of extractable ^{14}C-IPU were detected in the soil from application 1 (on average 85.7 µg = 34.3 % of applied ^{14}C; chapter 3.5.2.2; extracts were not analyzed for IPU metabolites). Thus, soil microbes were supported continuously with a moderate IPU concentration for at least 1 month. The high amounts also indicate that mineralization of IPU was not limited by the bioavailability to microbes but rather by the mineralization capability of microbes. An increase in mineralization rates and thus an adaptation of microbes was first observed after application 2 (chapter 3.5.2.1, Fig. 32), suggesting that microbes´ adaptation to IPU degradation required at least four weeks of continuous and moderate IPU supply in phase P1 at optimal water-conditions (-15 kPa) in soils (which are only seldom realized under real environmental conditions) and under a lack of other easily degradable microbial carbon sources which e.g. could be provided by plant roots in form of exudates under real field conditions.

When IMMC was inoculated into soil Scheyern, the amount of extractable ^{14}C decreased rapidly and after 1 month only 9.1 µg were detected (chapter 3.4.2.1, Fig. 24), which is only 10.6 % of the amount that was measured in the non-inoculated soil after this period of time. From the 9.1 µg of extractable ^{14}C (pore water and MeOH-extracts), only 2.7 ± 1.2 µg were available in the form of IPU (chapter 3.4.2.1, Fig. 25 & Table 16). Furthermore, results by Grundmann et al. (2007) indicated a fast mass transfer of IPU towards IMMC attached to Seramis® clay particles (6 µg d^{-1}), which was in the same order of magnitude as the ability of inoculated microbes to mineralize IPU (about 5 µg d^{-1}). The authors therefore concluded that high mass transfer rates enabled the high IPU mineralization rates.

As concentration and contact time are decisive factors for the adaptation of native microbes (Spain and van Veld, 1983; Top et al., 2002; Macleod and Semple, 2006), it can be assumed that adaptation of native microbes in soil inoculated with IMMC occurred slower and to a lower degree than it was observed by repeated IPU application in the non-inoculated soil Scheyern. Maybe, the extractable amount of IPU in soil was even below a threshold concentration for adaptation as it was reported previously e.g. for *p*-nitrophenol (Spain and van Veld, 1983).

All in all, it is considered as unlikely that native microbes were able to adapt to IPU mineralization and contributed to a significant extent to the enhanced IPU mineralization that was observed after reapplications 2a-d in soil Scheyern when inoculated with IMMC. Results from Gomes et al. (2005) confirm this conclusion: when inoculating the naphthalene degrading strain *Pseudomonas putida* KT2442 into soil, pollutant degradation was enhanced and the enrichment of indigenous Naphthalene degraders was suppressed by the inoculants strain.

4.5 Summary

Microbial degradation processes play a decisive role in the removal of organic pollutants such as pesticides from soils. However, when pesticide degrading microbes are introduced into soil the size of these microbial populations typically declines rapidly to a level no more sufficient for pollutant degradation. This phenomenon was observed especially at low contaminant concentrations (< 50 mg kg^{-1}) which are typical for agricultural soils. However, successful inoculations were also reported at such low concentration levels but no long-term studies are available up to now.

To enable enhanced pollutant degradation by inoculation of specific microbes, microbes capable to degrade this pollutant need to be available in the first place. As the adaptation of native soil microbes to pollutant degradation can take decades, it is desirable to accelerate the adaptation of native soil microbes to the degradation of new pesticides under laboratory conditions, and then to extract the respective microbes from soil for their use in bioremediation.

In the present thesis, two laboratory experiments with agricultural soils were conducted to investigate (1) how long inoculation-induced pollutant degradation is maintained in a soil supplied with low contaminant concentrations and (2), to what extent native microbial communities can adapt to accelerated pesticide degradation. These experiments were performed with the herbicide Isoproturon (IPU). In the first soil experiment, an IPU mineralizing microbial community (IMMC) was inoculated into soil. The properties of IMMC were investigated in a set of isolation and identification studies along with degradation experiments in liquid culture. The objective was to determine the degradation capabilities of the microbial consortium IMMC for IPU and some of its

metabolites and to isolate and identify the microbial species within IMMC that is capable to mineralize IPU.

As a result of the isolation and identification studies, the microbial species within IMMC that is responsible for IPU mineralization was identified as a member of the genus *Sphingomonas* and was designated *Sphingomonas* sp. AK1. Members of this genus are known to degrade a broad spectrum of xenobiotics and are suspected to adapt faster and more efficiently to the degradation of new compounds than other members of other microbial genera.

Further, liquid culture studies with IMMC or *Sphingomonas* sp. AK1, respectively, revealed that microbes could not only degrade IPU but also its metabolites MD-IPU and DD-IPU, thus a beneficial effect of inoculation may be expected also in soils in which IPU was already partially degraded. During IPU degradation, the transient accumulation of MD-IPU was observed, indicating that the degradation of IPU was initiated by N-demethylation of the dimethylurea side chain. Results further suggest that this step was a speed limiting transformation during IPU mineralization.

The consortium IMMC led only in a manageable extent to the degradation of the phenylurea herbicide Diuron in liquid culture. The amount of mineralization could be enhanced by lowering the Diuron concentration, but was still on a low level. This may indicate a specificity of IMMC for IPU.

Isolation and liquid culture studies demonstrated that *Sphingomonas* sp. AK1 benefited to a high extent from being included within the microbial consortium of IMMC: both survival and ability of *Sphingomonas* sp. AK1 to mineralize IPU were to a high extent dependent on the supply with amino acids which were probably formed by other members of IMMC. A goal of continuative activities should be to further elucidate the cooperation of microbes within IMMC and its importance for IPU mineralization. It may be demonstrated that microbes of IMMC are possibly organized as a microbial biofilm.

In case of the first soil experiment, IPU mineralizing microbes were introduced into one soil (homogenized top soil Scheyern) under laboratory conditions and 5 mg kg^{-1} of IPU were applied, in order to investigate the long-term sustainability of accelerated IPU mineralization at low IPU concentrations. Microbes were inoculated as the microbial consortium IMMC and attached to Seramis® clay particles as carrier material, leading to a heterogeneous spreading of introduced IPU degraders in soil (microbial “hot spots”). In comparison to the majority of inoculation studies, cell numbers introduced to soil were very low. By applying IPU for a second time to soil it was demonstrated that accelerated IPU mineralization was maintained in soil without any further IPU applications at least to the next growing season even though only marginal amounts of IPU were available for microbes for extensive time periods. Mineralization results suggested indirectly that

IMMC was able to survive on Seramis® clay particles for at least 322 days and was still able to mineralize IPU efficiently. A direct proof for the survival and dispersion of *Sphingomonas* sp. AK1 in soil with an adequate technique like Fluorescence *in situ* hybridization (FISH) was not possible as the required DNA probes specific for *Sphingomonas* sp. AK1 were not established at the time of this experiment. The development and establishment of such specific DNA probes to provide direct evidence and trace *Sphingomonas* sp. AK1 in soil is an important step for future investigations, especially under outdoor conditions, which do not provide optimal conditions for pesticide degradation compared to the present laboratory experiment.

In the second soil experiment it was investigated to what extent soil microbial communities can adapt to accelerated IPU degradation. For this purpose, four non-inoculated soils (homogenized top soils Scheyern, Kelheim, Konjišče and Feldkirchen) were treated with low doses of IPU (3 applications to soils Scheyern, Kelheim, Konjišče and four application to soil Feldkirchen, each time application of 5 mg kg^{-1} IPU). IPU was applied in intervals of about four weeks and thus in intervals much shorter than commonly done in agricultural practice. Experiments with soils Scheyern, Kelheim and Konjišče demonstrated that accelerated IPU mineralization developed under optimized laboratory conditions already within these three applications of IPU. All soils had shown a cometabolic mineralization pattern after a single application. The strategy utilized may also facilitate the adaptation of microbial communities to an accelerated degradation of other biodegradable pollutants. Four applications of IPU led to enhanced mineralization also in soil Feldkirchen, which had previously mineralized IPU metabolically but had been impaired by heat and water stress in summer 2003. However, the original extent in IPU mineralization was not achieved.

Altogether, the results in this thesis underline the relevance and realistic applicability of the investigated “hot spot” inoculation approach as *in situ* decontamination technique for agricultural soils with a specific microbial consortium.

4.6 Excursion: Further records for a possible commercial use of the investigated inoculation approach

In the framework of my PhD I was also working on a project with an industrial partner. The goal of this project was to assess the potential of the inoculation approach that I investigated in my PhD for its commercial use in agriculture. Within the scope of this project, we focused on several aspects that are essential for bringing this inoculation approach as an *in situ* decontamination procedure for IPU to the market. Several aspects on e.g. product optimization or the delivery system were discussed and investigated in the laboratory, for example: How long does the IPU mineralizing microbial community, established on Seramis® clay particles, maintain its degradation function for IPU during storage? This would support the development of a commercial manufacturing process

and guarantee an effective working product for the farmer. Results showed that the microbe-carrier-complexes could be stored with a quite simple technique for several weeks but also storage for several months was feasible by optimized standard methods.

The output of this cooperation project further affirmed the high potential of the investigated inoculation approach for commercial use.

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6 Appendix

A1.1 Identification of IMMC: clone sequences of Plasmid DNA

Table A1: Identified clone sequences of Plasmid DNA obtained from IMMC. See next pages for clones 7-25.

	Sequenced clones of Plasmid DNA					
	Plasmid DNA 1	Plasmid DNA 2	Plasmid DNA 3	Plasmid DNA 4	Plasmid DNA 5	Plasmid DNA 6
Next relative	Uncultured bacterium clone S30 16S ribosomal RNA gene, partial sequence	Uncultured bacterium clone 0p8_515 16S ribosomal RNA gene, partial sequence		Uncultured bacterium clone MS92 16S ribosomal RNA gene, partial sequence	Uncultured bacterium clone 1000N-10 16S ribosomal RNA gene, partial sequence	Uncultured bacterium clone Anxy21 16S ribosomal RNA gene, partial sequence
Accession number	JF775624.1	FJ934170.1		JN869173.1	JF772743.1	HQ343226.1
Max. identity (%)	99	90		99	99	99
Taxonomy						
Next related cultured species	*Pseudomonas* sp. RB5 16S ribosomal RNA gene, partial sequence	*Pseudomonas* sp. 0704CCL 16S ribosomal RNA gene, partial sequence	*Pseudomonas putida* strain MC4 16S ribosomal RNA gene, partial sequence	*Pseudomonas* sp. R8 partial 16S rRNA gene, isolate R8	*Acidobacteria* bacterium enrichment culture clone UCLd35 isolate DGGE gel band A 16S ribosomal RNA gene, partial sequence	*Variovorax ginsengisoli* partial 16S rRNA gene, strain R-38535
Accession number	GU232769.2	EU335084.1	JF825523.2	AM076674.1	JF731353.1	FR682712.1
Max. identity (%)	99	89	99	99	98	99
Taxonomy: phylum	Gamma-*Proteobacteria*	Gamma-*Proteobacteria*	Gamma-*Proteobacteria*	Gamma-*Proteobacteria*	*Acidobacteria*	Beta-*Proteobacteria*
Genus	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	environmental samples	*Variovorax*
Number of sequenced base pairs	753	750	826	669	489	777

Continuation of Table A1: Identified clone sequences of Plasmid DNA obtained from IMMC.

	Sequenced clones of Plasmid DNA					
	Plasmid DNA 7	Plasmid DNA 8	Plasmid DNA 9	Plasmid DNA 10	Plasmid DNA 11	Plasmid DNA 12
Next relative	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: OTU6	Uncultured bacterium clone MS92 16S ribosomal RNA gene, partial sequence		Uncultured bacterium clone MS92 16S ribosomal RNA gene, partial sequence	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: TS10	Uncultured Acidobacteria bacterium clone HEW_08_383 16S ribosomal RNA gene, partial sequence
Accession number	AB576900.1	JN869173.1		JN869173.1	AB378586.1	HQ598570.1
Max. identity (%)	98	99		99	94	99
Taxonomy						
Next related cultured species	*Hyphomicrobium* sp. KC-IT-W2 16S ribosomal RNA gene, partial sequence	*Pseudomonas* sp. R8 partial 16S rRNA gene, isolate R8	*Azohydromonas lata* strain IAM 12599 16S ribosomal RNA, partial sequence	*Pseudomonas* sp. R8 partial 16S rRNA gene, isolate R8	*Rhodospirillaceae* bacterium enrichment culture clone hao20 16S ribosomal RNA gene, partial sequence	Bacterium Ellin5257 16S ribosomal RNA gene, partial sequence
Accession number	FJ711209.1	AM076674.1	NR_041244.1	AM076674.1	FJ386547.1	AY234608.1
Max. identity (%)	98	99	99	99	91	98
Taxonomy: phylum	Alpha-*Proteobacteria*	Gamma-*Proteobacteria*	Beta-*Proteobacteria*	Gamma-*Proteobacteria*	Alpha-*Proteobacteria*	*Acidobacteria*
Genus	*Hyphomicrobium*	*Pseudomonas*	*Azohydromonas*	*Pseudomonas*	*Rhodospirillaceae*	*Acidobacteriaceae*
Number of sequenced base pairs	787	671	775	748	763	771

Continuation of Table A1: Identified clone sequences of Plasmid DNA obtained from IMMC.

	Sequenced clones of Plasmid DNA						
	Plasmid DNA 13	Plasmid DNA 14	Plasmid DNA 15	Plasmid DNA 16	Plasmid DNA 17	Plasmid DNA 18	Plasmid DNA 19
Next relative	Uncultured bacterium clone 2N1-105 16S ribosomal RNA gene, partial sequence	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: OTU6	Uncultured bacterium clone 11MIC054 16S ribosomal RNA gene, partial sequence		Uncultured bacterium partial 16S rRNA gene, clone FB04F08	Uncultured bacterium clone MS92 16S ribosomal RNA gene, partial sequence	
Accession number	EU160027.1	AB576900.1	JF340946.1		FM872921.1	JN869173.1	
Max. identity (%)	99	99	100		98	99	
Taxonomy							
Next related cultured species	Bacterium Ellin5257 16S ribosomal RNA gene, partial sequence	*Hyphomicrobium vulgare* gene for 16S ribosomal RNA, partial sequence	*Pseudomonas* sp. R8 partial 16S rRNA gene, isolate R8	*Azospirillumzeae* partial 16S rRNA gene, strain Gr35	*Variovorax* sp. S23408 gene for 16S ribosomal RNA, partial sequence	*Pseudomonas* sp. R8 partial 16S rRNA gene, isolate R8	*Alcaligenes* sp. 3013 partial 16S rRNA gene
Accession number	AY234608.1	AB543807.2	AM076674.1	FR667885.1	D84617.2	AM076674.1	AM110970.1
Max. identity (%)	95	99	100	99	97	99	99
Taxonomy: phylum	*Acidobacteria*	Alpha-*Proteobacteria*	Gamma-*Proteobacteria*	Alpha-*Proteobacteria*	Beta-*Proteobacteria*	Gamma-*Proteobacteria*	Beta-*Proteobacteria*
Genus	*Acidobacteriaceae*	*Hyphomicrobium*	*Pseudomonas*	*Azospirillum*	*Variovorax*	*Pseudomonas*	*Alcaligenes*
Number of sequenced base pairs	654	737	667	821	783	652	713

Continuation of Table A1: Identified clone sequences of Plasmid DNA obtained from IMMC.

	Sequenced clones of Plasmid DNA					
	Plasmid DNA 20	Plasmid DNA 21	Plasmid DNA 22	Plasmid DNA 23	Plasmid DNA 24	Plasmid DNA 25
Next relative	Uncultured organism clone ELU0037-T187-S-NIPCRAMgANb_000536 small subunit ribosomal RNA gene, partial sequence	Uncultured proteobacterium clone Elev_16S_1389 16S ribosomal RNA			Uncultured bacterium clone C-FCF-16 16S ribosomal RNA gene, partial sequence	Uncultured bacterium clone CN1-05 16S ribosomal RNA gene, partial sequence
Accession number	HQ755796.1	EF020000.1			AF443570.1	HQ218663.1
Max. identity (%)	99	93			95	99
Taxonomy						
Next related cultured species	*Sediminibacterium* sp. HME7863 16S ribosomal RNA gene, partial sequence	*Sphingomonas* sp. AHC-F 16S ribosomal RNA gene, partial sequence	*Azohydromonas lata* gene for 16S rRNA, partial sequence, strain: NBRC 102462	*Azohydromonas lata* gene for 16S rRNA, partial sequence, strain: NBRC 102462	*Azohydromonas lata* gene for 16S rRNA, partial sequence, strain: NBRC 102462	*Mesorhizobium albiziae* strain CCBAU 61158 16S ribosomal RNA, partial sequence
Accession number	JN674641.1	JQ418291.1	AB681804.1	AB681804.1	AB681804.1	NR_043549.1
Max. identity (%)	99	91	96	98	94	98
Taxonomy: phylum	*Bacteroidetes*	Alpha-*Proteobacteria*	Beta-*Proteobacteria*	Beta-*Proteobacteria*	Beta-*Proteobacteria*	Alpha-*Proteobacteria*
Genus	*Sediminibacterium*	*Sphingomonas*	*Azohydromonas*	*Azohydromonas*	*Azohydromonas*	*Mesorhizobium*
Number of sequenced base pairs	839	1007	1005	1002	1004	1006

A1.2 Pre-test for the inoculation procedure: pelletizing of cells of *Sphingomonas* sp. AK1

Aim of the pre-test was to measure to what extent cells of *Sphingomonas* sp. AK1 in liquid are generating a pellet of cells during centrifugation and thus, if cells that were grown on plates prior to inoculation could be washed before using them as inoculum. Details for the procedure are given in chapter 2.5.1.1.

Table A2 gives the number of cells of *Sphingomonas* sp. AK1 which were enumerated by microscoping before and after centrifugation and washing of cells. While before this treatment, 30.6 * 10^7 cells were present, just 14.9 % of applied cells were recovered afterwards. Thus, the majority of free floating cells in liquid got lost during the centrifugation and washing steps. To avoid a high loss of cells during washing, cells of *Sphingomonas* sp. AK1 that were grown on plate were thus not washed after their removal from plate.

Table A1: Number of cells from Sphingomonas sp. AK1 enumerated by microscoping before and after centrifugation and washing of cells.

	total number of cells (*10^7)		
sample	before centrifugation and washing	after centrifugation and washing	loss of cells (% of applied)
1	31.2	4.3	86.1
2	31.2	4.3	86.1
3	33.4	4.7	85.9
4	26.7	4.7	82.3
mean	30.6	4.5	85.1

A.1.3 Curves for mineralization rates as fitted by the nonlinear Gompertz function

A1.3.1 Sustainability of the enhanced IPU mineralization function induced by inoculation of IMMC into soil Scheyern

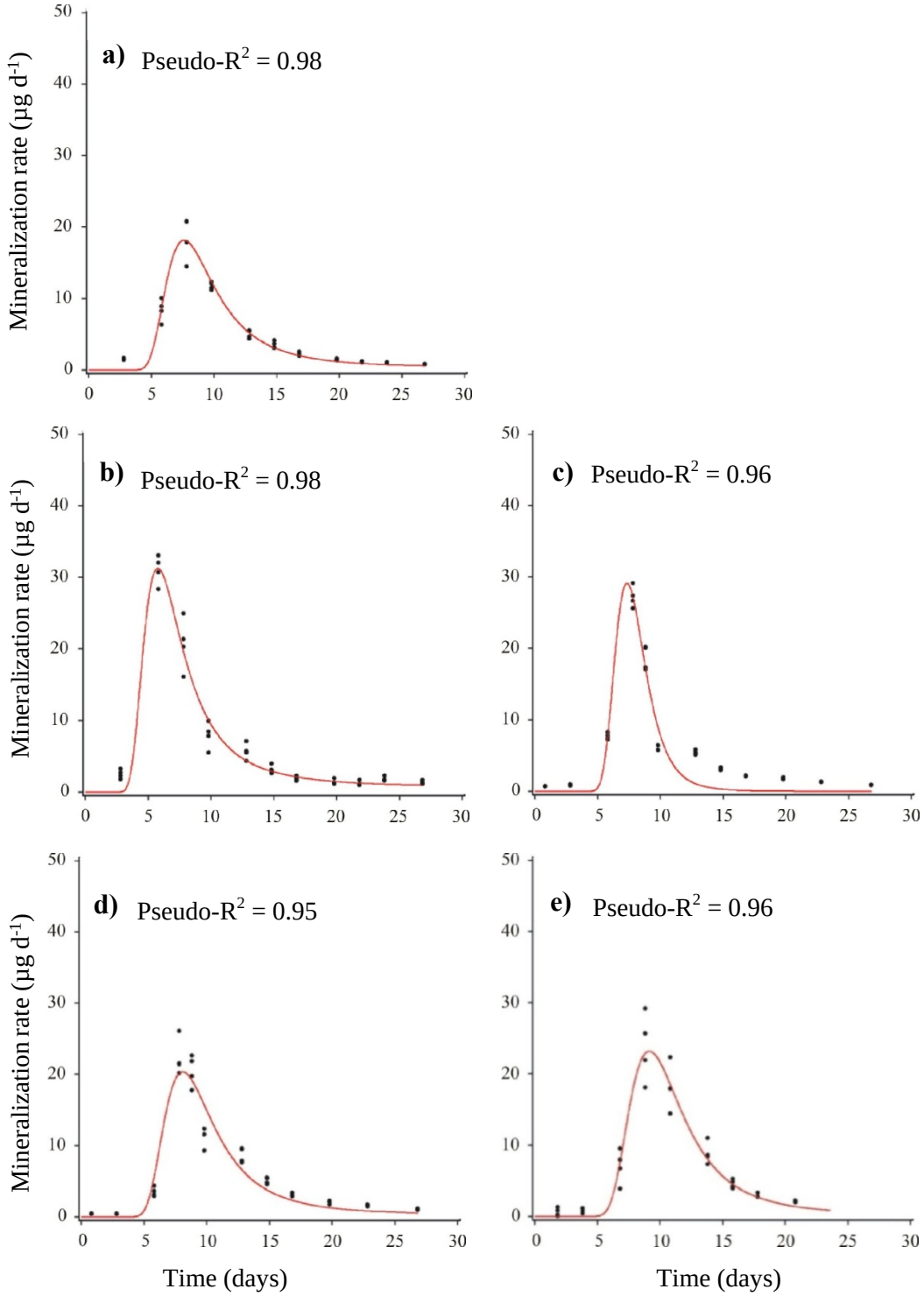

Figure A1: Development of IPU mineralization rates in soil Scheyern, which was inoculated with IMMC, for IPU applications 1 and 2, fitted by the Gompertz function: (a) application 1, (b-e) applications 2a to d (1.5, 6 and 7.5 months after application 1 as well as after simulation of winter). Y-axis: mineralization rate (µg d^{-1}). The red line indicates the fitted curve while measured values of replicates are depicted as black dots. As a measure for the quality of fit, the Pseudo-R2 is given.

A1.3.2 Adaptation of microbes in soils to IPU mineralization by repeated IPU applications: soil Feldkirchen

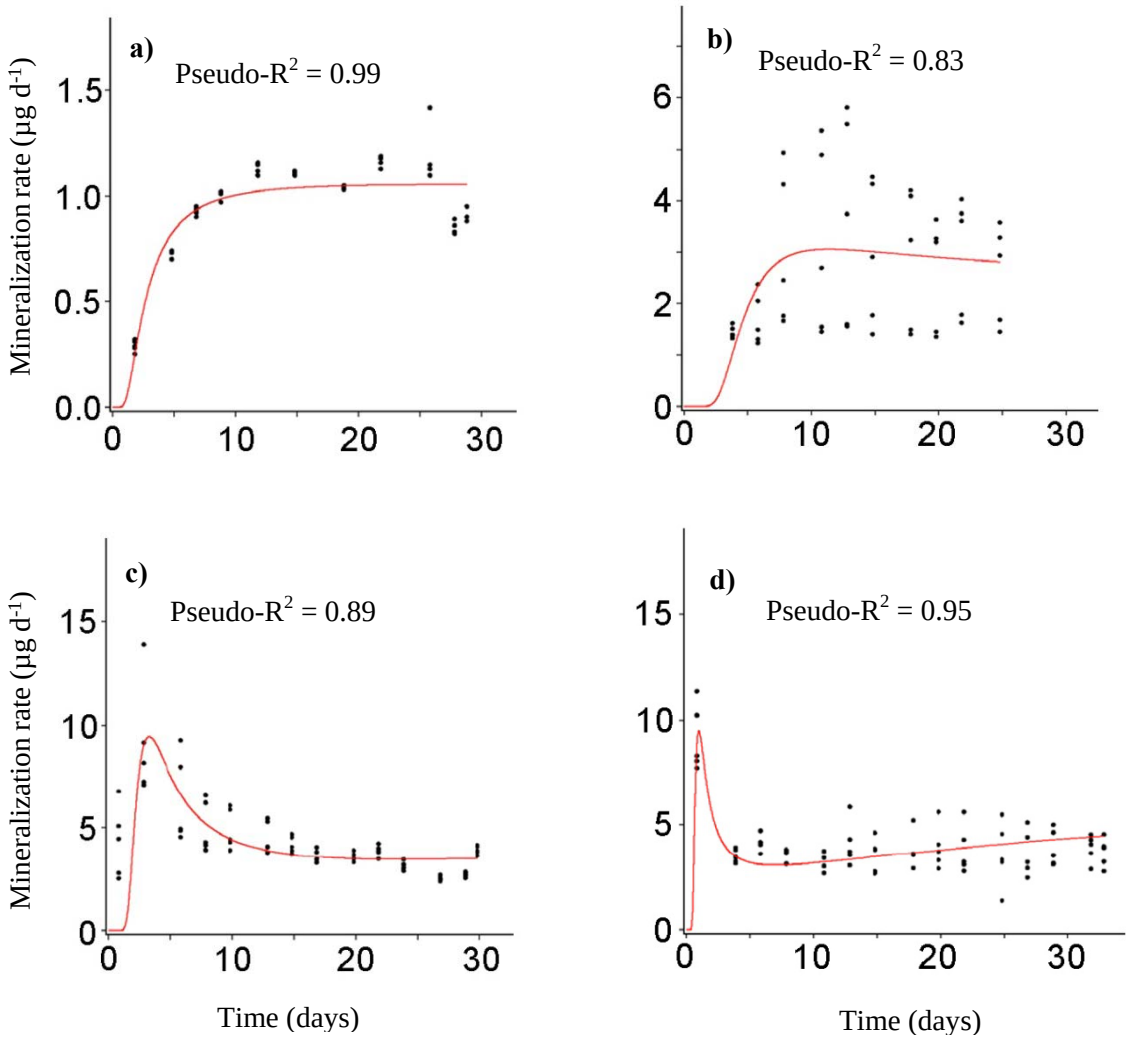

Figure A2: Development of daily IPU mineralization rates for native soil Feldkirchen for IPU applications 1-4 fitted by the Gompertz function: (a) application 1, (b) application 2, (c) application 3 and (d) application 4. Y-axis: mineralization rate (µg d^{-1}). The red line indicates the fitted curve while measured values of replicates are depicted as black dots. As replicate F1 showed a completely different mineralization dynamic than other replicates, it was excluded from fitting. As a measure for the quality of fit, the Pseudo-R^2 is given.

A1.3.3 Adaptation of microbes in soils to IPU mineralization by repeated IPU applications: soils Scheyern, Konjišče and Kelheim

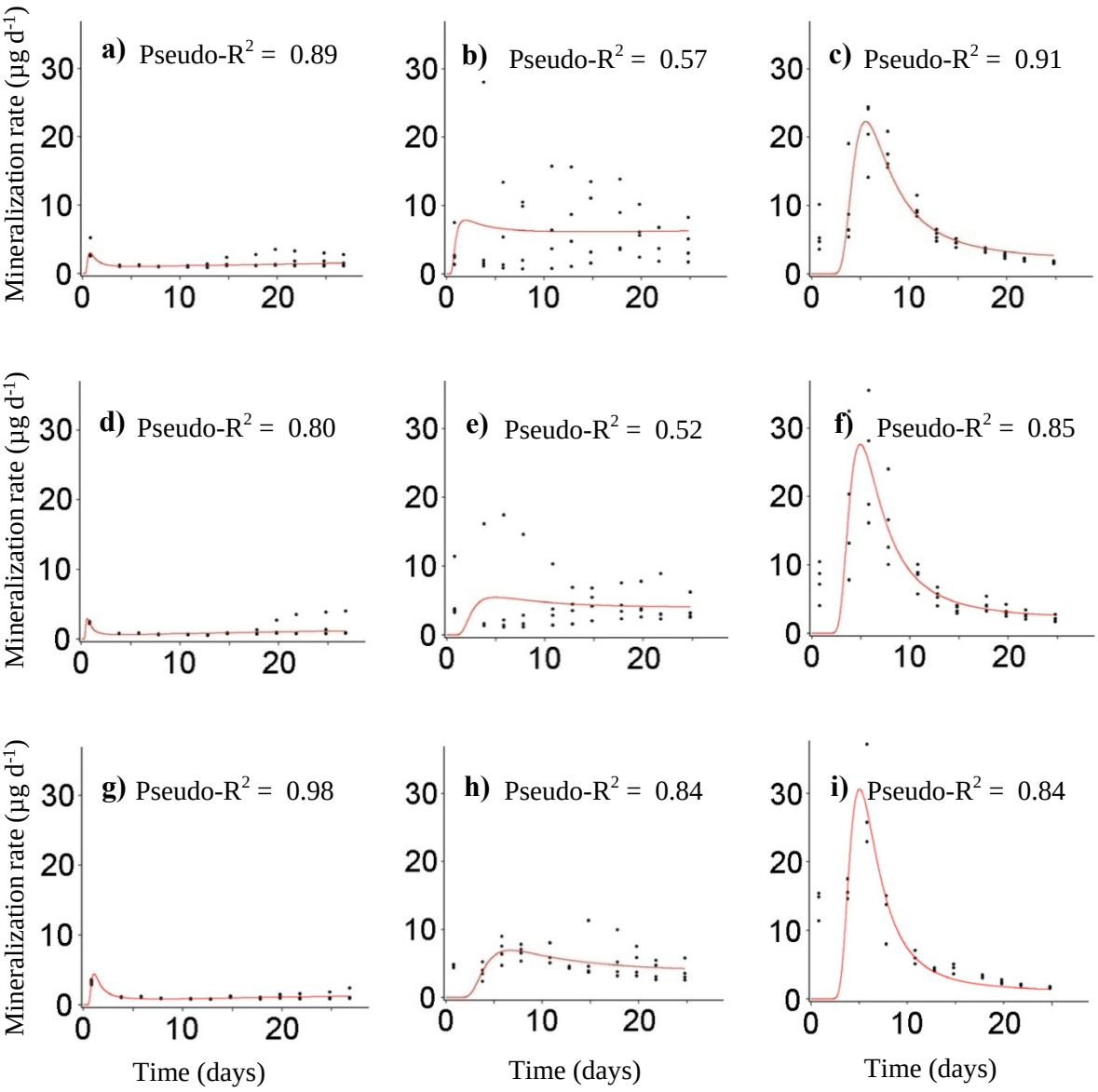

Figure A3: Development of IPU mineralization rates in soils Scheyern (a-c), Konjišče (d-f) and Kelheim (g-i) for IPU applications 1, 2 and 3 fitted by the Gompertz function: (a, d, g) application 1, (b, e, h) application 2 and (c, f, i) application 3. Y-axis: mineralization rate (µg d^{-1}). The red line indicates the fitted curve while measured values of replicates are depicted as black dots. As replicate KE1 shows a completely different mineralization dynamics in phase 3 as other replicates of soil Kelheim and also other soils, it was excluded for fitting. As a measure for the quality of fit, the Pseudo-R^2 is given.

A1.4 Determination of bioavailable IPU at application time of ^{14}C-IPU for soil Scheyern

In chapters 2.8, 3.4 and 4.3, it was investigated how long and to what degree the increased IPU mineralization, which was induced by inoculation of IMMC, was preserved in soil when IPU was applied only once and was no more applied for several months. Soil Scheyern was selected as this soil was shown to degrade IPU cometabolically after a single application of IPU (Grundmann et al., 2007; Folberth et al., 2009).

To determine the supply of microbes with IPU, IPU residues dissolved in pore water were measured at several points in time during phases 1 and 2 (chapter 2.8.6.1). The respective results are shown in chapter 3.4.2.1, Figure 24. To picture the full dynamic of bioavailable IPU in soil Scheyern, it was also necessary to examine the amount of bioavailable IPU at time of IPU application 1 (= day 0). This was done in two successive experiments. The results of experiments 1 and 2 are presented in the following.

When IPU is applied to soil, it disperses and a portion is adsorbed to soil particles, while another portion remains dissolved. However, IPU was reported to plays a major role in the degradation of IPU (Hussain et al., 2009). To prevent the immediate beginning of IPU degradation as soon as IPU was applied and thus falsified results, it was necessary to inhibit microbial activity.

Aim of experiment 1 was to determine the concentration of sodium azide (NaN_3) sufficient to inhibit microbial activity to a sufficient extent. In experiment 2, soil samples were prepared with a NaN_3-concentration as defined adequate in experiment 1. By means of them, it was determined after which period of time the sorption equilibrium of IPU is achieved. At that point of time, the maximum amount of IPU that is bioavailable for microbes could be measured.

A1.4.1 Experiment 1: Determination of optimum NaN_3-concentration for inhibition of microbial activity in soil Scheyern

For evaluation of NaN_3-incubation effectiveness, the following parameters were used:

(1) mineralization of IPU,
(2) the number of living cells as an indicator for microbial growth and
(3) the formation of metabolites illustrating microbial activity and degradation.

The aim was to reduce mineralization and thus microbial activity to a mineralization level < 1 % of applied ^{14}C-IPU.

Mineralization at 1, 10, 100 and 1000 ppm NaN_3

Figure A4 presents the daily mineralization rates and the cumulative mineralization for replicates that were supplemented with NaN_3 in concentrations of 1, 10, 100 or 1000 µg g^{-1} dry soil. Curves show constant IPU mineralization rates at low level for all variants (Fig. A4a). Furthermore, mineralization seemed to be increasingly inhibited by a rising amount of NaN_3 applied to soil, with

a low deviation only in the variant that received 1000 µg NaN_3 g^{-1} dry soil (Fig. A4a,b). However, no statistically significant differences were detected for the IPU amounts mineralized at the end of the experiment by post-hoc tests (Tukey HSD) (Fig. A4b).

In variants supplemented with 1, 10, 100 or 1000 µg NaN_3 g^{-1} dry soil 2.0 ± 2.1, 1.6 ± 1.6, 1.0 ± 1.3 respectively 0.2 ± 0.4 % of applied ^{14}C were mineralized until the end of experiment.

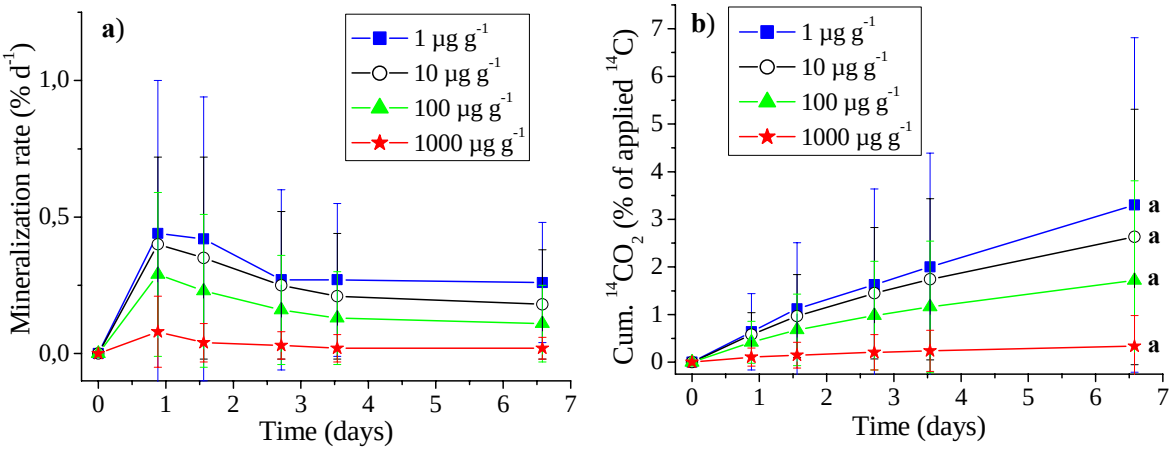

Figure A4: Development of IPU mineralization in soil Scheyern supplemented with 1, 10, 100 respectively 1000 µg NaN_3 g^{-1} dry soil: (a) daily mineralization rates, (b) cumulative mineralization. Bars indicate standard deviation. Values with the same letter assigned are not significantly different (α = 0.05) according to One-way ANOVA and Tukey HSD test.

Number of living cells at 1, 10, 100 and 1000 ppm NaN_3

Bacteria

Figure A5 presents the number of living bacterial cells at days 0 (= application of NaN_3 to soil), 3, 7 and 10. Compared to untreated soil at day 0, the number of cells reduced significantly by the application of NaN_3. This reduction was independent from the applied concentration of NaN_3.

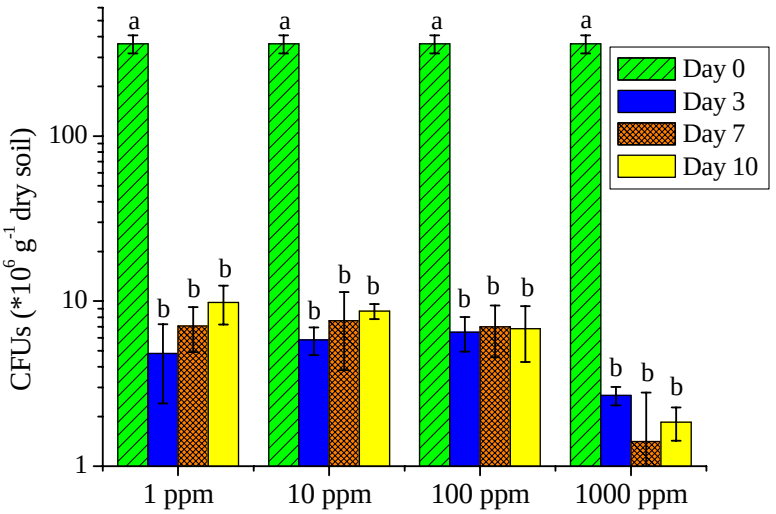

Figure A5: Numbers of Colony Forming Units for bacteria in soil Scheyern treated with 1, 10, 100 respectively 1000 µg NaN_3 g^{-1} dry soil as well as in untreated soil (= day 0). Bars indicate standard deviation. Values with the same letter assigned are not significantly different (α = 0.05) according to One-way ANOVA and Tukey HSD test.

Fungi

The number of fungi present in untreated soil was considerable lower than those for bacteria (Figure A6). As seen for bacteria, the application of NaN_3 resulted in a significant reduction of fungal cells compared to untreated soil, independent from the used concentration.

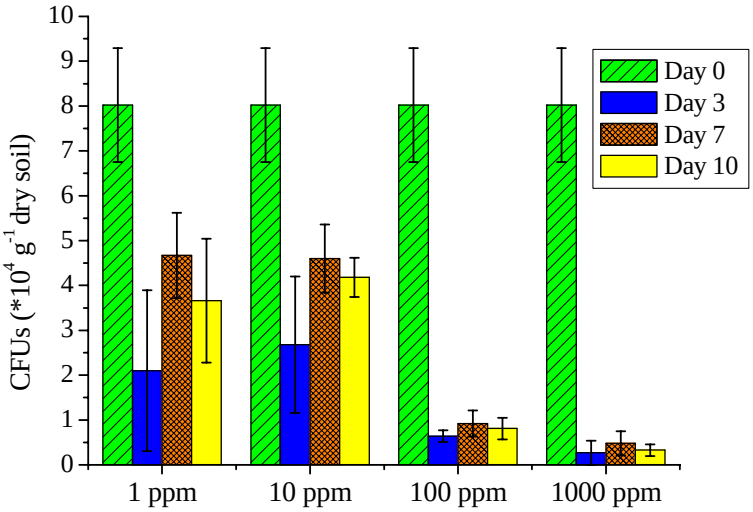

Figure A6: Numbers of Colony Forming Units for fungi in soil Scheyern treated with 1, 10, 100 respectively 1000 µg NaN_3 g^{-1} dry soil as well as in untreated soil (= day 0). Bars indicate standard deviation.

Significant differences in fungal cell numbers were detected between different NaN_3 concentrations and incubation times. For a better overview, significances for cell counts are given in Table A3.

The impact of NaN_3 – concentration on survival of fungal cells persisted for different periods of time. At 1 ppm, significant more cells were measured between day 3 and 7, indicating that for this

NaN_3-concentration, just short term duration of fungal suppression could be attained. In contrast, no significant changes for the investigated period of 10 days could be detected in cell numbers for concentrations of 10, 100 respectively 1000 µg g^{-1} dry soil.

Table A2: Test for homogeneity of subsets for fungal Colony Forming Units detected in soil Scheyern treated with 1, 10, 100 respectively 1000 µg NaN_3 g^{-1} dry soil. Stars indicate significant differences according to One-way ANOVA (α = 0.05) and Tukey HSD test.

	NaN_3-concentration (µg g^{-1} dry soil)	0	1	10	100	1000	1	10	100	1000	1	10	100	1000
Day 0	0													
Day 3	1	*												
	10	*												
	100	*												
	1000	*		*										
Day 7	1	*	*		*	*								
	10	*	*		*	*								
	100	*					*	*						
	1000	*					*	*						
Day 10	1	*			*	*			*	*				
	10	*			*	*			*	*				
	100	*					*	*			*	*		
	1000	*		*			*	*			*	*		

NaN_3-concentration (µg g^{-1} dry soil)

Day 0	Day 3	Day 7	Day 10

Statistical differences were also seen concerning the intensity of NaN_3-concentration on fungal survival. At day 3, cell numbers at 10 ppm were significantly higher than those at 1000 ppm. At days 7 and 10, significantly more CFUs were detected at 1 and 10 ppm than at 100 and 1000 ppm. Thus, the inhibiting impact of NaN_3 on fungi was in general stronger at 100 and 1000 ppm.

Formation of metabolites at 1, 10, 100 and 1000 ppm NaN_3

Table A4 lists the portion of metabolites extracted by ASE. As pore water was not extracted before, values include also the radioactivity dissolved in pore water. All in all, five metabolites were detected, three of them could be identified by HPLC. There are clear trends noticeable concerning the variants with different concentrations of NaN_3. The higher the amount of added NaN_3, the fewer metabolites were detected in the SPE eluates, indicating a higher level of cometabolic degradation at low NaN_3-concentrations This is in accordance with mineralization results (Figure A4). Besides this, the mass of extracted compounds was increasing with increasing NaN_3 concentration. On average, 74.4, 94.8, 105.1 respectively 109.9 % of applied ^{14}C were extractable

by ASE in variants treated with 1, 10, 100 respectively 1000 µg NaN_3 g^{-1} dry soil. In all variants, IPU was the compound with highest share in the mass of all detected pesticide residues.

Table A3: Composition of IPU residues in pore water plus MeOH-extracts in soil Scheyern treated with 1, 10, 100 or 1000 µg NaN_3 g^{-1} dry soil. Quantities of unknown metabolites are given in µg IPU equivalents. Metabolites Unknown 1 and 2 could not be identified by HPLC as standards were not available.

	Retention time	Portion of peak area in the eluate of SPE and quantity of respective metabolites							
		Amount of added NaN_3 (µg g^{-1} dry soil)							
		1		10		100		1000	
	(min)	%	µg	%	µg	%	µg	%	µg
Unknown 1	12.99 ± 0.04	3.7	5.0	2.7	4.7	1.0	1.9	0.8	1.5
Unknown 2	13.80 ± 0.03	0.8	1.1						
DD-IPU	17.58 ± 0.15	0.2	0.3	0.1	0.2				
MD-IPU	18.62 ± 0.07	11.0	15.1	8.1	14.0	1.3	2.2	5.0	12.4
IPU	19.78 ± 0.05	84.3	114.8	89.1	155.1	97.7	188.5	98.7	187.5
Sum		100.0	136.3	100.0	173.8	100.0	192.6	104.5	201.4
Number of metabolites		5		4		3		3	

Mass balance

107.1 to 114.0 % of applied ^{14}C were recovered in this experiment (Table A5). In all variants, the extractable fraction (MER + PW) accounted by far for the largest fraction while cumulative mineralization formed the smallest portion. The formation of NER was decreasing with increasing concentration of NaN_3, with a range of 22.3 (variant 1 µg g^{-1}) to 1.6 % of applied ^{14}C (variant 1000 µg g^{-1}).

Table A4: Distribution and recovery of applied ^{14}C for soil Scheyern treated with 1, 10, 100 or 1000 µg NaN_3 g^{-1} dry soil. Min = cumulative mineralization, MER = Methanol-extractable residues, PW = pore water, NER = non-extractable residues.

Concentration of NaN_3 (µg g^{-1} dry soil)	Recovery of applied ^{14}C in %			
	Min	MER + PW	NER	Sum
1	1.8	89.8	22.3	114.0
10	1.4	96.6	9.0	107.1
100	0.9	105.5	1.9	108.3
1000	0.2	103.5	1.6	105.3

Optimum NaN_3-concentration for inhibition of microbial activity

Given the impact of NaN_3-concentration on duration and strength for the survival of fungal cells in soil as well as on the quantity and quality of extracted metabolites, an addition of 100 and 1000 µg NaN3 g^{-1} dry soil resulted in better results than when applying 1 or 10 ppm to soil Scheyern. As a reliable inhibition of microbial degradation activity below a cumulative mineralization < 1 % of applied radioactivity seems to be most probable for the variant of 1000 µg NaN3 g^{-1} dry soil, the following experiment was conducted at this concentration level for NaN_3.

A1.4.2 Experiment 2: Determination of the time period after which the sorption equilibrium for IPU is attained

After a chemical enters the soil matrix, it disperses by diffusion within the pore water and penetrates also into small soil pores. As diffusion in soil is known to be a time–dependent process (Beulke et al., 2004), the sorption equilibrium is achieved also after a certain time period.

The aim of experiment 2 was to determine the time period after which the sorption equilibrium between soil matrix and soil water was achieved for ^{14}C-IPU in soil Scheyern. At that time, the maximum amount of IPU dissolved in pore water could be reliably measured. This amount indicates the maximum amount of IPU that is bioavailable at time of IPU application 1, when no degradation processes occurred yet.

Achievement of the sorption equilibrium

Figure A7 shows the amount of radioactivity measured in the pore water at the day of ^{14}C-IPU application (= day 0) as well as 1, 2, 3, 4 and 7 days after application.

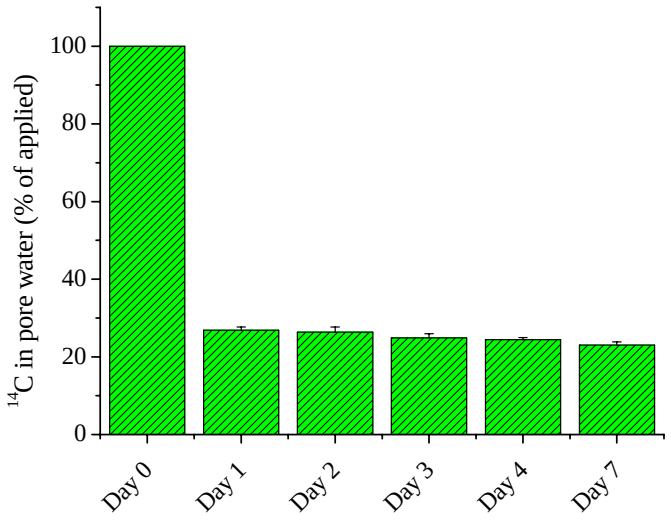

Figure A7: Recovery of applied ^{14}C in pore water of soil Scheyern incubated with 1000 µg NaN_3 g^{-1} dry soil. ^{14}C-IPU was applied at day 0 and recovery of ^{14}C measured in pore water 1, 2, 3, 4 and 7 days after application. Bars indicate standard deviation.

Already one day after application, just 26.9 % of applied ^{14}C-IPU was detected in the pore water. As it was shown that mineralization and the formation of NER played a negligible role at a concentration of 1000 µg g^{-1} NaN_3 in soil Scheyern (experiment 1, chapter A1.4.1, Fig. A4 and Table A5), it was assumed that the residual ^{14}C would be present in soil as methanol-extractable residues. During the following days, the quantity of ^{14}C in pore water decreased just slowly, with 23.1 % of applied ^{14}C measured in pore water at day 7. Thus, the main sorption process occurred clearly within one day after application of ^{14}C-IPU to soil.

On average, a negligible percentage of 5.22 ± 2.49 % of the applied ^{14}C was lost during the filtration process.

Mass balance

Table A5: Distribution and recovery of applied ^{14}C 1, 2, 3, 4 and 7 days after application of ^{14}C-IPU to soil Scheyern incubated with 1000 µg NaN_3 g^{-1} dry soil. Min = cumulative mineralization, MER = Methanol-extractable residues, PW = pore water, NER = non-extractable residues.

Days after application of ^{14}C-IPU	Recovery of applied ^{14}C in %			
	Min	PW	MER + NER	Sum
1	0.02	23.7	81.4	105.0
2	0.01	23.2	84.8	108.0
3	0.07	21.9	86.0	107.9
4	0.02	21.5	86.3	107.9
7	0.03	20.3	85.2	105.5

Table A5 shows the mass balances. 105.0 to 108.8 % of applied ^{14}C were recovered at the respective time points, indicating plausibility of results.

A1.4.3 Amount of ^{14}C-IPU dissolved in pore water of soil Scheyern at time of IPU application 1

It was shown that, when microbial activity is inhibited in soil Scheyern, 26.9 % of applied ^{14}C-IPU was detected in the pore water under equilibrium conditions (chapter A1.4.2).

Therefore, when applying 250 µg of ^{14}C-IPU to soil Scheyern, a maximum amount of 67.3 µg IPU (= 26.9 % of 250 µg IPU) were dissolved in the pore water and thus bioavailable to microbes. The residual 182.8 µg (= 73.1 % of 250 µg IPU) were adsorbed to soil particles and might be delivered subsequently from the Methanol extractable fraction.