

Technische Universität München

Lehrstuhl für Bodenökologie

Bioaugmentation towards isoproturon degradation in agricultural soils: Implications
for biological remediation of soils contaminated with organics

Renyi Li

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: Prof. Dr. Rainer Matyssek

Prüfer der Dissertation:

1. Prof. Dr. Dr. Jean Charles Munch
2. Prof. Dr. Siegfried Scherer
3. Prof. Dr. Jan Roelof van der Meer
(Universität de Lausanne, Schweiz)

Die Dissertation wurde am 20.07.2016 bei der Technischen Universität München eingereicht und durch
die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am
07.09.2016 angenommen.

To my wife Lifang Tang and my son Mu Li.

致我最爱的妻子唐丽芳和儿子李慕。

Abstract

Pesticides remaining in agricultural soil exert threats to the natural ecosystem and human health. Bioaugmentation is regarded as the most promising remedy for soil pollution with organics, without causing additional damages for the soils to be remediated. In this research, isoproturon (IPU), a worldwide extensively used phenylurea herbicide, was used as a model substance to investigate the bioaugmentation strategies to enhance degradation of organic pollutants in agricultural soils. This whole study consists of two parts. In one part, four bioaugmentation approaches to accelerate IPU degradation in soil were investigated to identify the most promising approach (of the tested) for clean-up of IPU in agricultural soil. The other addressed the efficacy, functional sustainability and applicability of this propitious approach in IPU decontamination in different agricultural soils. The primary findings are summarized as below.

I. Isoproturon (IPU) degradation in an agricultural soil inoculated with an **i**solated IPU-degrader **s**train (*Sphingomonas* sp. strain AK1, **IS**) or a **m**icrobial **c**onsortium (**MC**) harboring this strain, with or without carrier material, were investigated in soil microcosm experiments during 46 days. Effect of the carrier material and inoculation size on IPU-degradation efficacy of the inoculants were studied. Mineralization, extractable residues and non-extractable residues of ^{14}C -labeled IPU were analyzed. The low IPU mineralization in untreated soil (7.0% of initially applied IPU) was enhanced to different extents by inoculation of IS (17.4%–46.0%) or MC (58.9%–67.5%). Concentrations of IPU residues in soils amended with MC ($0.002\text{--}0.095\ \mu\text{g g dry soil}^{-1}$) were significantly lower than in soils amended with IS ($0.02\text{--}0.67\ \mu\text{g g dry soil}^{-1}$) and approximately 10 times lower than in the uninoculated soil ($0.06\text{--}0.80\ \mu\text{g g dry soil}^{-1}$). Less extractable residues and non-extractable residues were detected in soil with higher IPU mineralization. Inoculation size (as indicated by the volume of liquid cultures or by the number of carrier particles) determined the IPU-removal efficacy of IS in soil, but this effect was less pronounced for MC. The low sorption of IPU to soil and the decreasing IPU-mineralizing rates suggested incapability of IS to establish the IPU-mineralizing function in the soil. The thorough removal of IPU and persistent IPU-mineralizing activity of soil inoculated with MC indicated a high persistence

of IPU-metabolic trait. Our results showed that microbial consortia might be more efficient than single degrader strains to enhance clean-up of organic chemicals in soil.

II. Soil pH is a limiting factor for biodegradation of IPU in the field. IPU dissipation is hampered in soil with low pH. Efficacy of two IPU-mineralizing microbial communities in IPU dissipation was investigated during 189 d in microcosms of three agricultural soils with contrasting pH values (Marsdorf, pH 3.8; Neumarkt, pH 5.8 and Dürneck, pH 7.3). The microbial communities enriched from an acidic soil (MC-AS) and a neutral soil (MC-NS), respectively, were established on carrier material, namely expanded clay particles, and introduced to the tested soils at a carrier-soil ratio of 1%. IPU was applied to the soils twice, on day 0 and day 133 respectively. The effect of inoculation size, IPU sorption-desorption and pH on biodegradation of IPU were studied. IPU mineralization, extractable residues and non-extractable residues were analyzed with uniformly ring-labeled [¹⁴C]-isoproturon. Both microbial communities resulted in significantly enhanced IPU mineralization (52%–60%) and low concentrations of IPU residues in soil Dürneck (pH 7.3). The acquired IPU-mineralizing activity was persistent in the soil for at least 133 d. Initially a 9-fold higher IPU mineralization rate was attained in soil Neumarkt (pH 5.8) by inoculating MC-AS. However, no difference between treated and untreated soils was detected after IPU re-application. Both communities had negligible effect on the fate of IPU in soil Marsdorf (pH 3.8), where biodegradation of IPU was inhibited, with ≈ 2% IPU mineralized over 189 d. Lowering the carrier-soil ratio to 0.1% sharply reduced the IPU-mineralizing capacity of MC-AS in soil Neumarkt, however, this effect of inoculation size was less pronounced for both microbial communities in soil Dürneck. The low K_d of IPU (1.3–2.0 mL g⁻¹) indicate that IPU bioavailability is not a limiting factor of IPU degradation in the soils. Relationships between pH and maximal IPU mineralization rendered by MC-AS or MC-NS in soil closely approximate those observed in mineral salts liquid medium, suggesting that pH is an important factor influencing biodegradation of IPU by the exogenous microbial communities. It is proposed that MC-AS, which can tolerate a broad range of pH, is a promising candidate for enhancing IPU degradation in acidic soils. The effect of inoculant density on IPU degradation is microbial community–soil specific. Using degrader microorganisms according to

their physiological requirements and properties of the targeted soils may maximize the effectiveness of bioaugmentation.

Zusammenfassung

Pestizide in landwirtschaftlichen Böden können ein Risiko für die Gesundheit von Mensch und Umwelt darstellen. Bioaugmentation wird als die vielversprechendste Methode angesehen zur Sanierung von Böden, die mit organischen Chemikalien verunreinigt sind, und dies ohne weitere Bodenbeschädigung. In der vorliegenden Arbeit wurde das weit verbreitete Phenylharnstoff-Herbizid Isoproturon (IPU) als Modellsubstanz verwendet, um Bioaugmentations-Strategien zum beschleunigten Abbau von organischen Belastungen in Böden zu untersuchen. Die Arbeit besteht aus zwei Teilen. Zum einen wurden verschiedene Bioaugmentations-Ansätze zum beschleunigten IPU-Abbau im Boden untersucht, um darunter einen vielversprechendsten Ansatz zur Minimierung von IPU-Rückständen in landwirtschaftlichen Böden abzuleiten. Zum anderen wurde dieser Ansatz auf seine Wirksamkeit, seine Nachhaltigkeit und seine Anwendbarkeit in Bezug auf IPU-Abbau in mehreren landwirtschaftlichen Böden untersucht. Die Ergebnisse sind im Folgenden zusammengefasst.

I. Landwirtschaftliche Böden wurden mit einem IPU-abbauenden Einzelstamm (*Sphingomonas* sp. strain AK1) oder einer bodenbürtigen mikrobiellen Gemeinschaft, die diesen Einzelstamm enthielt, inokuliert. In Boden-Mikrokosmen wurde der IPU-Abbau während 46 Tagen untersucht. Der Effekt eines Trägermaterials (Blähtonpartikel) sowie der Inokulations-Menge der mikrobiellen Abbauer (variierte Mengen des flüssigen Inokulats oder Anzahl Trägerpartikeln) und auf den IPU-Abbau wurden ebenfalls untersucht. Mineralisierung, extrahierbare Rückstände und nicht-extrahierbare Rückstände des eingesetzten (gleichmäßig ringmarkiertes) ^{14}C -IPU wurden erfasst. Die ursprünglich geringe IPU-Mineralisierung im unbehandelten Boden (7.0% der Dotierung) konnte in unterschiedlichem Ausmaß beschleunigt werden, je nachdem, ob mit dem IPU-abbauenden Einzelstamm AK1 (+ 17.4% bzw. 46.0%) oder der IPU-abbauenden mikrobiellen Gemeinschaft (+ 58.9% bzw. 67.5%) inokuliert wurde. Die IPU-Rückstände waren im Boden, dem die mikrobielle Gemeinschaft zugesetzt wurde, signifikant geringer ($0.002\text{--}0.095\ \mu\text{g g trocken Boden}^{-1}$) als im Boden, der den Einzelstamm enthielt ($0.02\text{--}0.67\ \mu\text{g g trocken Boden}^{-1}$) und ungefähr 10-mal geringer als im nicht-inokulierten Kontrollboden. In Böden mit höherer IPU-Mineralisierung konnten geringere Mengen extrahierbarer und nicht-extrahierbarer Rückstände

nachgewiesen werden. Die IPU-Abbau Effizienz des Einzelstammes wurde von der Inokulations-Menge beeinflusst, während bei der mikrobiellen Gemeinschaft der Einfluss der Inokulations-Menge nicht so ausgeprägt war. In Anbetracht der geringen IPU-Sorption und der abnehmenden IPU-Mineralisierungsraten ist der Einsatz des IPU-abbauenden Einzelstammes nicht so gut geeignet für eine effektive und nachhaltige Reduktion der IPU-Rückstände im Boden. Die Inokulation mit der mikrobiellen Gemeinschaft jedoch resultierte in einem effektiven Rückgang der IPU-Rückstände und in stabilen IPU-Mineralisierungsraten. Diese Ergebnisse lassen darauf schließen, dass der Einsatz von mikrobiellen Gemeinschaften besser für den beschleunigten Abbau von organischen Chemikalien in Böden geeignet sein könnte als der Einsatz von Einzelstämmen.

II. Der pH-Wert des Bodens ist ein limitierender Faktor für den Abbau von Isoproturon im Feld. In Böden mit niedrigem pH-Wert ist der IPU-Abbau eingeschränkt. In Mikrokosmen-Versuchen mit drei landwirtschaftlichen Böden mit unterschiedlichen pH-Werten (Boden Marsdorf, pH 3.8; Boden Neumarkt, pH 5.8 und Boden Dürneck, pH 7.3) wurde die Effektivität von zwei verschiedenen IPU-mineralisierenden mikrobiellen Gemeinschaften während 189 Tagen untersucht. Bei den mikrobiellen Gemeinschaften handelte es sich zum einen um eine mikrobielle Gemeinschaft, die aus einem **sauren** Boden (MC-AS) angereichert wurde und zum anderen um eine mikrobielle Gemeinschaft, die aus einem **neutralen** Boden (MC-NS) angereichert wurde. Beide Gemeinschaften wurden jeweils auf einem Trägermaterial (Blähtonpartikel) etabliert und in die Böden in einem Trägermaterial-Boden Verhältnis von 1/100 inokuliert. IPU (ebenfalls gleichmäßig ringmarkiertes ^{14}C -IPU) wurde zweimal auf den Boden appliziert, am Tag 0 und am Tag 133. Die Bedeutung der Inokulations-Menge, der IPU-Sorption-Desorption und des Boden-pH für den Abbau von Isoproturon wurden erfasst. Mineralisierung, extrahierbare Rückstände und nicht-extrahierbare Rückstände des eingesetzten ^{14}C -IPU wurden quantifiziert. Im Boden Dürneck (pH 7.3) beschleunigten beide mikrobiellen Gemeinschaften die IPU-Mineralisierung signifikant (+ 52% bzw. 60%) und führten zu niedrigen IPU-Rückständen. Die erworbene IPU-Mineralisierungs-Aktivität blieb in diesem Boden für mindestens 133 Tage bestehen. Im Boden Neumarkt (pH 5.8) konnte durch Inokulation mit der mikrobiellen Gemeinschaft MC-AS ursprünglich eine 9-fach höhere IPU-Mineralisierung erzielt werden. Nach der zweiten IPU-Applikation

nach 133 Tagen konnte jedoch keine beschleunigte IPU-Mineralisierung mehr festgestellt werden. Im Boden Marsdorf (pH 3.8) hatten beide mikrobiellen Gemeinschaften einen vernachlässigbar geringen Effekt auf den Abbau von IPU. In diesem Boden war der Abbau von IPU generell eingeschränkt; nach 189 Tagen waren nur 2% des eingesetzten IPU mineralisiert. Das Herabsetzen des Verhältnisses Trägermaterial-Boden auf 1/1000 führte im Boden Neumarkt zu einer stark reduzierten IPU-Mineralisierungskapazität von MC-AS. Dieser Effekt der Inokulations-Größe war jedoch für beide mikrobiellen Gemeinschaften im Boden Dürneck weniger stark ausgeprägt. Da in allen drei Böden niedrige K_d Werte für IPU (1.3–2.0 mL g⁻¹) gemessen wurden, ist davon auszugehen, dass die Bioverfügbarkeit von IPU kein limitierender Faktor für den IPU-Abbau in diesen Böden ist. Die Beziehungen zwischen dem pH-Wert und der maximalen IPU-Mineralisierung, die für MC-AS oder MC-NS im Boden festgestellt wurden, ähneln sehr stark denen im Mineralsalz-Medium; dies zeigt, dass der pH-Wert eine wichtige Einflussgröße für den IPU-Abbau dieser in die Böden eingeführten exogenen mikrobiellen Gemeinschaften ist. Aus den Ergebnissen kann man schließen, dass die mikrobielle Gemeinschaft MC-AS eine breite pH-Toleranz aufweist und daher ein vielversprechender Kandidat für den beschleunigten IPU-Abbau in sauren Böden ist. Der Effekt der Inokulations-Größe auf den IPU-Abbau ist spezifisch für die Kombination „mikrobielle Gemeinschaft - Boden“. Wenn man bei der Auswahl der Bioaugmentations-Techniken sowohl die physiologischen Anforderungen der abbauenden Mikroorganismen als auch die Eigenschaften der Böden, in die sie inokuliert werden, berücksichtigt, dann kann dies zu einem maximalen IPU-Abbau führen.

Table of Contents

Abstract.....	I
Zusammenfassung	IV
Table of Contents.....	VII
List of Tables.....	X
List of Figures	XI
List of Abbreviations	XIII
1 Introduction	1
1.1 Pesticides as a source of environmental pollution and remediation	1
1.2 IPU as a model substance	4
1.3 Factors affecting biodegradation of IPU in soil	6
1.3.1 IPU-mineralizing microorganisms	6
1.3.2 Soil pH.....	7
1.3.3 Soil moisture	9
1.4 Strategies to enhance efficacy of bioaugmentation	10
1.4.1 Utilizing carrier material	11
1.4.2 Using microbial consortia instead of single strains.....	12
1.5 Objectives and Hypotheses	13
1.5.1 A promising approach in soil bioaugmentation	13
1.5.2 Efficacy and sustainability of the microbial-community-carrier-material approach.....	14
2 Materials and Methods	16
2.1 Chemical reagents	16
2.1.1 Uniformly ring-labeled [¹⁴ C]-isoproturon.....	16
2.1.2 Other chemicals.....	16
2.2 IPU-mineralizing microorganisms	17
2.2.1 <i>Sphingomonas</i> sp. strain AK1	17
2.2.2 IPU-mineralizing microbial community enriched from an acidic soil (MC-AS).....	17

2.2.3 IPU-mineralizing microbial community enriched from a neutral soil (MC-NS)	18
2.3 Agar medium and mineral salts media.....	18
2.4 Incubation systems	19
2.4.1 Apparatus for incubating and aerating liquid culture	19
2.4.2 Soil microcosm system.....	21
2.5 Carrier material	22
2.6 Soils	22
2.7 OECD 106 batch experiment	23
2.8 Liquid culture incubation experiment I.....	24
2.8.1 Preparation of inoculants	24
2.8.2 Microbial diversity of MC-AS and MC-NS	25
2.9 Liquid culture incubation experiment II: Effect of pH.....	25
2.10 Soil microcosm incubation experiment I: A promising approach	26
2.11 Soil microcosm incubation experiment II: Efficacy and sustainability	28
2.11.1 Initial IPU application and inoculation.....	28
2.11.2 Re-application of IPU.....	28
2.12 Soil microcosm incubation experiment III: Effect of inoculation size	29
2.13 Analysis of ¹⁴ C-IPU mineralization.....	30
2.14 Quantification of ¹⁴ C-extractable residues	31
2.15 Composition analysis of ¹⁴ C-extractable residues	31
2.16 Quantification of ¹⁴ C-non-extractable residues	32
2.17 Figures, tables and statistical analysis	32
3 Results and Discussion.....	33
3.1 Publication I.....	33
3.2 Publication II.....	34
Publication I: Enhanced degradation of isoproturon in an agricultural soil by a <i>Sphingomonas</i> sp. strain and a microbial consortium	35

Supplementary information of Publication I	44
Publication II: Biodegradation of isoproturon in agricultural soils with contrasting pH by exogenous soil microbial communities.....	51
Supplementary information of Publication II.....	63
Appendix to § 3 Results and Discussion.....	74
Appendix 1: Purification of ¹⁴ C-IPU	75
Appendix 2: IPU sorption to carrier particles	76
Appendix 3: IPU sorption to Teflon vials (<i>Section 2.7</i>).....	77
Appendix 4: Microbial diversity of MC-AS and MC-NS	78
Appendix 5: ¹⁴ C-mass balance of Liquid culture incubation experiment II: Effect of pH (<i>Section 2.9</i>)	80
Appendix 6: Functional sustainability of MC-NS in soil “Konjišče”	82
4 General Discussion and Conclusion	85
4.1 Implications for bioaugmentation of soil pollution with organic chemicals.....	85
4.2 Pros and cons of the microbial-community-carrier-particle approach.....	86
4.3 Future research topics	88
4.4 My opinion about environmental pollution resulting from agrochemicals.....	89
References	91
Acknowledgement	101
Curriculum Vitae	103

List of Tables

Table 1. Comparison between biological remediation and abiotic soil remediation technologies.	3
Table 2. Basic information of isoproturon (UK IPU report, 1995; EU EFSA, 2015).....	5
Table 3. IPU-degrading bacteria isolated during 1999–2014.	8
Table 4. ¹⁴ C-IPU standards used in this thesis.	17
Table 5. Properties of soils originally harboring MC-AS (soil “Cunnersdorf”) and MC-NS (soil “Feldkirchen”) (Folberth et al., 2009).....	18
Table 6. MS-media used to cultivate the IPU-mineralizing microorganisms.	19
Table 7. Physico-chemical properties of soils used for soil incubation experiments.	23
Table 8. Experimental design of Soil microcosm incubation experiment I.	27
Table 9. Experimental design of Soil microcosm incubation experiment II.	29
Table 10. Experimental design of Soil microcosm incubation experiment III.	30
Table 11. HPLC gradient program for detecting IPU and IPU-metabolites.	32

List of Figures

Fig. 1. A conceptual mode of soil microbial activity influenced by soil moisture (Skopp et al., 1990).	10
Fig. 2. Schematic of the incubating and aerating system for liquid cultures (modified from Kiesel, 2014).	20
Fig. 3. Schematic of the soil microcosm for bioaugmentation experiments (Kiesel, 2014).	21
Fig. A1. Radiochemical purity of ^{14}C -IPU before (a, radiochemical purity 81.6%) and after HPLC purification (b, radiochemical purity 98.1%).	75
Fig. A2. Adsorption of IPU to carrier particles in sterile Milli-Q [®] water. Error bars indicate standard deviation of three replicates.	76
Fig. A3. ^{14}C -IPU recovery in Teflon vials without soil. Error bars represent standard deviations of three replicates.	77
Fig. A4. ^{14}C -IPU mineralization by MC-AS and MC-NS in MS media used as references for the IPU-mineralizing function of MC-AS and MC-NS cultivated in MS-media with non-labeled IPU for DNA extraction (illumina [®] sequencing).....	78
Fig. A5. Taxonomic compositions of MC-AS and MC-NS on family level determined by illumina [®] sequencing of 16S rRNA genes. The group “others” in the figure represents the OTUs with a relative abundance of < 1% and OTUs that are not annotated up to family level.	79
Fig. A6. Fate of ^{14}C -IPU in liquid cultures of MC-AS (a) and MC-NS (b) after incubation for 31 d. Error bars indicate standard deviations of three replicates.	81
Fig. A7. ^{14}C -IPU mineralization rate during 189 d in soil Konjšiče inoculated with MC-NS at a carrier-soil ratio of 0.1% (2 carrier particles in 50 g dw soil). ^{14}C -IPU was applied to the soil on day 0 and day 133. Error bars indicate standard deviations of four replicates.	83
Fig. A8. ^{14}C -IPU mineralization during 57 d after initial application (on day 0, open triangles) and re-application (on day 133, closed triangles) of ^{14}C -IPU in soil Konjšiče inoculated with MC-NS at a carrier-soil ratio of 0.1%. Error bars indicate standard deviations of four replicates.	83

Fig. A9. Fate of ^{14}C -IPU after 189 d in soil Konjišće inoculated with MC-NS at a carrier-soil ratio of 0.1%. ^{14}C -IPU was applied to the soil twice, on day 0 and day 133. Error bars indicate standard deviations of four replicates.84

List of Abbreviations

1,2,4-TCB	1,2,4-trichlorobenzene
2-OH-MDIPU	3-[4-(2-hydroxyisopropylphenyl)]-1-methylurea
4-IA	4-isopropylaniline
AFFS	Agricultural, Forestry and Fishery Statistics (European Union)
ASE	Accelerated Solvent Extractor
CAS	Chemical Abstracts Service
CLU-IN	EPA Contaminated Site Clean-Up Information
d	day(s)
DDIPU	didemethylated IPU [3-(4-isopropylphenyl)-urea]
DNA	deoxyribonucleic acid
dw	dry weight
EFSA	European Food Safety Authority (European Union)
EPA	United States Environmental Protection Agency
ER	extractable residues
EU	European Union
FISH	Fluorescence <i>in situ</i> Hybridization
h	hour(s)
HPLC	High-performance Liquid Chromatography
IPU	isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea]
IPU-MS medium	mineral salts medium with IPU as the sole C source
IS	<u>i</u> solated isoproturon-degrader <u>s</u> train <i>Sphingomonas</i> sp. strain AK1
IS-CP	<u>i</u> solated <u>s</u> train established on <u>c</u> arrier <u>p</u> articles
IS-LM	<u>i</u> solated <u>s</u> train in <u>l</u> iquid <u>m</u> edium
IUPAC	International Union of Pure and Applied Chemistry
K_d	partition ratio (concentration in solid phase vs. concentration in liquid)
MC	<u>m</u> icrobial <u>c</u> ommunity (<u>c</u> onsortium)

MC-AS	IPU-mineralizing <u>m</u> icrobial <u>c</u> ommunity enriched from an <u>a</u> cidic <u>s</u> oil
MC-CP	<u>m</u> icrobial <u>c</u> ommunity established on <u>c</u> arrier <u>p</u> articles
MC-LM	<u>m</u> icrobial <u>c</u> ommunity in <u>l</u> iquid <u>m</u> edium
MC-NS	IPU-mineralizing <u>m</u> icrobial <u>c</u> ommunity enriched from a <u>n</u> eutral <u>s</u> oil
MDIPU	mono-demethylated IPU [3-(4-isopropylphenyl)-1-methylurea]
min	minute(s)
MS-medium	mineral salts medium
NER	non-extractable residues
OECD	Organisation for Economic Co-operation and Development
OTU	operational taxonomic unit
rRNA	ribosomal ribonucleic acid
sec	second(s)
SOM	soil organic matter
SPE	solid phase extraction
TOC	total organic carbon

1 Introduction

1.1 Pesticides as a source of environmental pollution and remediation

Extensively used pesticides in agriculture is a major source of environmental pollution (Enserink et al., 2013). An estimated 2.4 billion kg pesticide active ingredients were used annually in the year 2006 and 2007 globally (EPA, 2011). In Europe the consumption of pesticides amounted to \approx 360 million kg in 2013 (EU AFFS, 2015). While pesticides are required to secure agricultural yield, the active chemicals remaining in soil exert serious threats to the ecosystem and human health. Many pesticides (hereinafter, pesticides refer to active compounds rather than the intact formulations) are identified as carcinogens and endocrine-disruptors (Kavlock et al., 1996). Meanwhile prohibition of approved pesticides is not stopping as toxicity evaluation of the pesticides proceed further. For instance, isoproturon (IPU), a phenylurea herbicide which has been extensively used in twenty-two European countries in the past thirteen years, was recently banned by EU because of new evidences of causing cancer and damaging fertility systems in mammals (EU EFSA, 2015; Commission Implementing Regulation (EU) No 2016/872).

Pesticides introduced into the environment undergo various physical, chemical and biological processes resulting in dispersion (e.g., via surface run-off or leaching), transformation and/or dissipation of the synthetic active substances (Fenner et al., 2013). However, natural attenuation is usually unable to eliminate these chemicals thoroughly from the environment and pesticides come up frequently as priority contaminants in surface- and groundwater exceeding the threshold concentration of pesticides ($0.1 \mu\text{g L}^{-1}$) in drinking water (EU Directive 2013/39/EU, 2013; Fenner et al., 2013). Moreover, excessive pesticides residues in soil may be absorbed and accumulated in plant tissues (Paterson et al., 1990; Yu et al., 2009; Grundmann et al., 2011; Qiu et al., 2016). Therefore, strategies are required to

accelerate dissipation of pesticides in agricultural soils to reduce the amount of pesticides entering water systems or food web.

Current treatment technologies involving abatement or elimination of environmental pollution are categorized into eleven major groups according to the United States Environmental Protection Agency (EPA, 2013). The most frequently adopted soil remedies include physical treatment, chemical treatment, biological treatment and thermal treatment (**Table 1**). During the years 1982–2011, bioremediation amounted to over 10% of 1266 (frequency) selected remediation technologies for source pollution in USA; it was among the top 3 *in situ* treatment technologies and the 5th most-used for *ex situ* remediation (EPA, 2013). Notably, bioremediation topped the list of techniques for groundwater decontamination, representing 37% selected techniques by frequency (EPA, 2013). Despite the limitations of bioremediation (**Table 1**), it is frequently regarded as the most promising strategy for environmental decontamination because of its cost-effectiveness and environment-friendliness (Megharaj et al., 2011).

Definitions of bioremediation and bioaugmentation (EPA, 2013):

“Bioremediation uses microorganisms to degrade organic contaminants in soil, groundwater, sludge, and solids. The microorganisms break down contaminants by using them as an energy source or co-metabolizing them with an energy source. More specifically, bioremediation involves the production of energy in a redox reaction within microbial cells. These reactions include respiration and other biological functions needed for cell maintenance and reproduction. A delivery system that provides one or more of the following is generally required: an energy source (electron donor), an electron acceptor, and nutrients.”

“Bioaugmentation is the addition of microbes to the subsurface where organisms able to degrade specific contaminants are deficient. Microbes may be ‘seeded’ from populations already present at a site and grown in aboveground reactors or from specially cultivated strains of bacteria having known capabilities to degrade specific contaminants.”

Table 1. Comparison between biological remediation and abiotic soil remediation technologies.

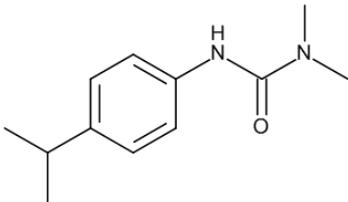
Treatment Technologies*	Typical types	Principal mechanisms	Disadvantages	Advantages		
Physical Treatment	Soil Vapor Extraction	Abiotic treatments	Mobilization, Sorption, Desorption, Separation, Extraction, Oxidation, Reduction, Solubilization etc.	a) Expensive (energy and labor input) b) Damaging soil function c) Restricted application in diffuse pollution	a. Efficient b. Controllable b) Applicable to multiple contamination	
	Physical Separation					
	Soil Washing					
	Solidification and Stabilization					
Chemical Treatment	Chemical Oxidation	Abiotic treatments	Mobilization, Sorption, Desorption, Separation, Extraction, Oxidation, Reduction, Solubilization etc.	a) Expensive (energy and labor input) b) Damaging soil function c) Restricted application in diffuse pollution	a. Efficient b. Controllable b) Applicable to multiple contamination	
	Chemical Reduction					
	Neutralization					
Thermal Treatment	Heating (≥ 100 °C)	Abiotic treatments	Mobilization, Sorption, Desorption, Separation, Extraction, Oxidation, Reduction, Solubilization etc.	a) Expensive (energy and labor input) b) Damaging soil function c) Restricted application in diffuse pollution	a. Efficient b. Controllable b) Applicable to multiple contamination	
	Incineration (648 °C–1648 °C)					
Biological Treatment*	Natural Attenuation	Microorganisms	Biodegradation: biotransformation and mineralization, Bioaccumulation, Biosorption, Bioprecipitation etc.	a) Unguaranteed availability of bioagents (e.g., specific microbial degrader strains) b) Uncontrollable (especially in field-scale) c) Pollutant-specific (e.g., special bacterial degraders for specific compound)	a) Inexpensive (function of microorganisms or plants) b) Relatively efficient c) Conserving soil function d) Applicable to both point source and diffusion pollution	
	Biostimulation					
	Bioaugmentation*					
	Phytoremediation	Plants	Microorganisms and plants	Biodegradation: biotransformation and mineralization, Bioaccumulation, Biosorption, Bioprecipitation etc.	a) Unguaranteed availability of bioagents (e.g., specific microbial degrader strains) b) Uncontrollable (especially in field-scale) c) Pollutant-specific (e.g., special bacterial degraders for specific compound)	a) Inexpensive (function of microorganisms or plants) b) Relatively efficient c) Conserving soil function d) Applicable to both point source and diffusion pollution
	Constructed Treatment Wetlands					

* Treatment technologies are according to EPA (2013).

1.2 IPU as a model substance

This thesis is focusing on bioremediation of soil contaminated with organic pollutants. IPU (**Table 2**) was selected as a model substance for this study. The major reasons for selecting IPU are: (1) Usage and pollution. IPU is one of the most-used pesticides globally and a frequently detected contaminants in drinking water sources (e.g., groundwater) in many countries (Fenner et al., 2013; Hussain et al., 2015). (2) Hazards to environment and human. Toxicity studies revealed that IPU has a high potential of causing cancer and impairing fertility systems in mammals (EU EFSA, 2015). Moreover, IPU is very toxic to aquatic organisms, birds and wild animals, posing long-term threats on natural ecosystems. (3) Physico-chemical properties. The volatility of IPU and sorption capacity of IPU to soil are of major consideration. The involatility of IPU (at room temperature, atmospheric pressure) facilitates an accurate ^{14}C -mass recovery for fate analyses of IPU in soil. IPU generally has a low solid-liquid partition ratio (K_d) in soil, which is primarily determined by soil organic matter (SOM) and by pH, clay content and calcite content to a certain extent (Spark and Swift, 2002; Boivin et al., 2005; El Arfaoui et al., 2012; Jarvis, 2016). The low K_d of IPU may suggest a high bioavailability of IPU in soil, which guarantees that the effect of bioremediation can be easily detected. The appropriateness of comparing the efficacy of bioaugmentation in different soils is also enhanced when IPU bioavailability does not differ substantially in the tested soils.

Table 2. Basic information of isoproturon (UK IPU report, 1995; EU EFSA, 2015).

Physico-chemical properties	
IUPAC name	3-(4-isopropylphenyl)-1,1-dimethylurea
CAS number	34123-59-6
Molecular formula	C ₁₂ H ₁₈ N ₂ O
Molecular weight	206.3
Molecular structure	
Melting point	157–159 °C
Decomposition temperature	239 °C
Vapor pressure	3.19–3.33×10 ⁻⁶ Pa (20 °C) 1.18×10 ⁻⁴ Pa (40 °C) 2.85×10 ⁻³ Pa (60 °C)
Solubility in water	60–70 mg L ⁻¹ (20 °C, pH independence)
Solubility in methanol	65–75 g L ⁻¹ (20 °C)
Partition ratio (log K _{ow})	2.48 (25°C)
As active substance in herbicides	
Weeds control	pre- and post-emergence control of annual grass and broadleaved weeds in cereals
Approval in EU*	01.01.2003–30.06.2016
Application rate	1.5 kg ha ⁻¹
Application frequency	1–2, in autumn and/or spring
Concentration in soil at 1.5 kg ha ⁻¹	4.6 µg g ⁻¹ (soil depth 1.2 cm, soil density 1.3 g cm ⁻³)

* EU Pesticides database <http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.detail&language=EN&selectedID=1495>

1.3 Factors affecting biodegradation of IPU in soil

A variety of biotic and abiotic factors together shape the IPU degradation dynamics in soil (Hussain et al., 2015), among which soil indigenous microbial community, soil pH and soil moisture are the major contributors. Other soil parameters and anthropogenic soil management such as temperature (Alletto et al., 2006; Sebaï et al., 2011), organic amendments (Vieubl e Gonod et al., 2009; 2016) and soil depth (Larsen et al., 2000; Sonia Rodr iguez-Cruz et al., 2006) also play a certain role, however, they are not in the research scope of this thesis.

1.3.1 IPU-mineralizing microorganisms

Biodegradation of IPU in the environment is predominantly mediated by microbial activity under aerobic conditions (Hussain et al., 2015). Soil microorganisms which have acclimatized to metabolize IPU are the intrinsic and determinant driving-force of IPU degradation. Since 1999, eleven IPU-mineralizing bacteria have been isolated and characterized (**Table 3**). However, genes responsible for metabolism of IPU largely remain unknown (Hussain et al., 2015). Gu et al. (2013) identified a demethylase gene *pdmAB* in an IPU-mineralizing bacterium *Sphingobium* sp. strain YBL2, which is responsible for initial *N*-demethylation of IPU and consequent transformation to mono-demethylated IPU (MDIPU). However, initial *N*-demethylation is unlikely a rate-limiting step of IPU degradation in soil. MDIPU was frequently detected as the most abundant IPU-metabolite in soil with low IPU-mineralizing potential (Schroll and K uhn, 2004; Suhadolc et al., 2004; Alletto et al., 2006; Grundmann et al., 2011), which suggest that IPU could easily be metabolized to MDIPU by the soil microbes. Genes responsible for the initial *N*-demethylation of IPU are likely ubiquitous in soil. Therefore, it may not be used as specific probes for detecting growth and activity of IPU-degraders introduced to soil. Up to date, the molecular basis of the aromatic-ring cleavage of IPU is still missing. The unavailability of specific IPU-mineralizing genes makes cumbersome the measurement of proliferation and activity of IPU-degrader microorganisms in complex environment (e.g., soil).

1.3.2 Soil pH

pH is one of the most important factors controlling microbial degradation of IPU in soil. Not only the in-field spatial distribution pattern of IPU-degrading activity in soil depends heavily on pH (Walker et al., 2001; Bending et al., 2003; El Sebai et al., 2007; Hussain et al., 2013), but also IPU mineralization by the IPU-degraders in mineral salts media (Bending et al., 2003; Sun et al., 2009; Hussain et al., 2011). Generally high IPU-degradative activity occurs in soil with higher pH ($\text{pH} \geq 7$). Shi and Bending (2007) discovered that the pH-dependence of IPU degradation in an agricultural field was a consequence of the spatial distribution of the IPU-metabolizing *Sphingomonas* sp. community as determined by soil pH. Furthermore, all of the reported IPU-degrader bacteria up to date were exclusively isolated from weakly alkaline soils (pH 7.1–8.1) and showed a narrow optimal pH range (7.0–7.5) for IPU degradation (**Table 3**). Attempts to accelerate IPU dissipation in acidic soils using the IPU-degrading strains or enriched IPU-mineralizing microbial communities were hampered because of the inhibited activity of the degraders at low pH (Bending et al., 2003; Grundmann et al., 2007; Sun et al., 2009). Elevating soil pH to the optimal pH range of the IPU-degraders might be possible to help overcome the effect of low pH, however, such method is limited to pH increase in a small range. Besides, increasing soil pH considerably may exert detrimental effect on native soil microorganisms that have adapted to acidic environment. IPU-degrading microbes originated from acidic soils may provide a better solution to clean up IPU in acidic soils.

Table 3. IPU-degrading bacteria isolated during 1999–2014.

Strain	Origin	Soil history	Soil pH	pH of IPU-MS media	pH tested in MS media	pH tested in soil	Optimal pH in MS media	Optimal pH in soil	Reference
<i>Arthrobacter globiformis</i> strain D47	UK	cereals, isoproturon, ≥ 3 yr	7.8	6.9	–	–	–	–	Cullington and Walker (1999)
<i>Sphingomonas</i> sp. strain SRS2	UK	winter barley, isoproturon, 20 yr	7.19	7.2	6.5, 7.0, 7.5, 8.0 (< 7.0, > 7.5, inhibited)	pH 6.26, inhibited; pH 6.5, inhibited; pH 7.5, enhanced	7.5	7.5	Sørensen et al., (2001)
<i>Arthrobacter</i> sp. N2	France	garden path soil, diuron, 3 yr	–	6.5, 6.6	–	–	–	–	Tixier et al. (2002); Widehem et al. (2002)
<i>Sphingomonas</i> sp. strain F35	UK	winter barley, isoproturon, 20 yr	7.1–7.5	–	6.5, 7.0, 7.5, 8.0 (< 7.0, > 7.5, inhibited)	–	7.5	–	Bending et al. (2003)
<i>Methylopila</i> sp. TES	France	winter wheat, isoproturon, 10 yr	7.4	–	–	–	–	–	El Sebai et al. (2004)
<i>Sphingobium</i> sp. strain YBL1	China	herbicide plant field, phenylurea herbicides, 10 yr	–	–	–	4.6, inhibited; 6.7, 8.3, enhanced	–	6.7	Sun et al. (2009)
<i>Sphingobium</i> sp. strain YBL2	China	herbicide plant field, phenylurea herbicides, 10 yr	–	–	3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 (≤ 6.0 , ≥ 8.0 , inhibited)	See above	7.0	6.7	Sun et al. (2009)
<i>Sphingobium</i> sp. strain YBL3	China	herbicide plant field, phenylurea herbicides, 10 yr	–	–	–	See above	–	6.7	Sun et al. (2009)
<i>Pseudomonas aeruginosa</i> strain JS-11	–	wheat, isoproturon	–	7.2 \pm 0.2	–	–	–	–	Dwivedi et al. (2011)
<i>Sphingomonas</i> sp. strain SH	France	rape seed/winter wheat/barley, isoproturon, 10 yr	7.66–7.83	6.6	5.5, 6.5, 7.5, 8.5 (≤ 6.5 , ≥ 8.5 , inhibited)	–	7.5	–	Hussain et al. (2011)
<i>Sphingomonas</i> sp. strain AK1	Germany	–	7.2 (pH _{CaCl₂})	7.2	–	–	–	–	Kiesel (2014)

– not available/not investigated. Soil pH in the table were measured with 1:1 soil-water ratio, unless specified.

1.3.3 Soil moisture

Water is an elemental constituent of soil. Soil without water is not livable. Soil water and soil air share the fixed soil pore volume formed by the solid soil matrix. In the case of aerobic biodegradation, soil water content could determine soil microbial activity by affecting substrate availability (e.g., solubility and diffusion) and oxygen diffusion concomitantly (**Fig. 1**). Many studies have reported the effect of soil moisture on a broad range of soil functions mediated by soil microbes such as soil general respiration, nitrification and pollutant degradation (Orchard and Cook, 1983; Stark and Firestone, 1995; Schroll et al., 2006). A positive correlation between soil moisture and soil respiration or soil nitrification was observed within a soil water potential ranging from -8.5 MPa to -0.01 MPa (Orchard and Cook, 1983; Stark and Firestone, 1995). Alletto et al. (2006) discovered that degradation of IPU in a soil was 10–15 folds faster when the soil water content increased from 50% water holding capacity (WHC) to 90% WHC. The effect of soil moisture on soil microbial activity can be soil-specific as influenced by soil physical properties such as clay content, total organic carbon (TOC) and bulk density (Moyano et al., 2012). According to the well-established conceptual model (**Fig. 1**), an optimal soil moisture for maximal aerobic microbial activity is expected when the limitation of soil water content on substrate diffusion and oxygen diffusion are equal. By investigating mineralization of three ^{14}C -labeled pesticides in ten different soils, Schroll et al. (2006) found that within the range of soil water potential from -20 MPa to -0.015 MPa, increasing mineralization of pesticides were linearly correlated with higher soil moisture; the optimal soil moisture for biodegradation of organic pollutants was -0.015 MPa. Similar optimal soil water potentials for global soil respiration and nitrification was reported by Schjøning et al. (2003) and Moyano et al. (2012). In this thesis, all soil microcosm experiments were conducted with a soil water potential of -0.015 MPa to ensure the comparability of IPU-degradation in different soils.

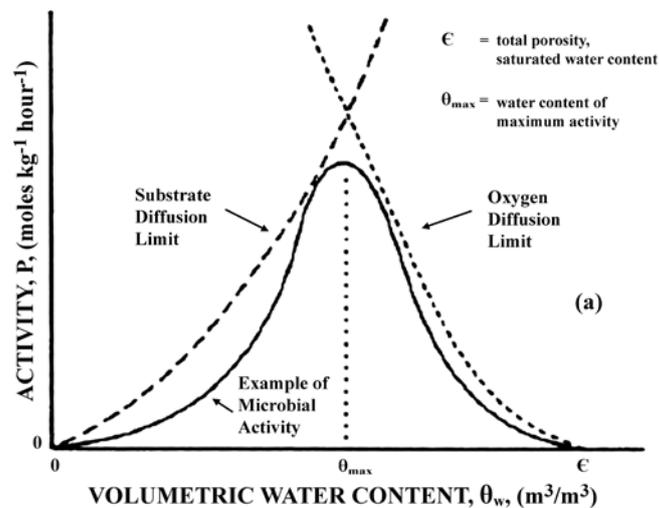


Fig. 1. A conceptual mode of soil microbial activity influenced by soil moisture (Skopp et al., 1990).

1.4 Strategies to enhance efficacy of bioaugmentation

Growth and activity of pollutant-degrading microbes in soil determine the success of bioremediation. However, soil generally represent a hostile environment for exogenous microorganisms; decrease in cell numbers of inoculant microbes frequently occurred shortly after introduction into soil (van Veen et al., 1997; Gentry et al., 2004). The death of inoculated cells can be attributed to various physico-chemical and biological soil factors such as pH, nutrient availability, redox potential, existence of other toxic pollutants, predation by soil protozoa, competition with indigenous soil microbiome, diversity of endogenous soil microbial community etc. (Tyagi et al., 2011; van Elsas et al., 2012; Husson, 2013; Ma et al., 2013). To circumvent these challenges, strategies were developed to alleviate the adverse effect of unfavorable properties of recipient soil on inoculated microorganisms or to improve the competence of inocula to increase the survival chance. These strategies include improving soil properties (e.g., increasing soil pH with limestone), supplementing soil with extra nutrients, adding specific electron acceptors, soil ventilation, pre-adaptation of inocula to contaminated soil conditions, utilizing carrier materials for inoculation, using degrader strain together with its supporting microbes or using co-culture of complementary partial degraders (e.g., EPA CLU-IN Website; Van Dyke and Prosser, 2000;

Owsianiak et al., 2010; De Roy et al., 2014). In the following sections, state of the art of two strategies that directly related to this study are presented.

1.4.1 Utilizing carrier material

Carrier material are viewed as a desirable tool to promote establishment of introduced microorganisms in new soil environment (Gentry et al., 2004). A variety of organic and inorganic material such as peat, alginate, agro-biowaste, biochar, clay minerals, expanded clay particles etc., were used to deliver microbes into soil for various purposes (Gentry et al., 2004; Grundmann et al., 2007; Albareda et al., 2008; Sun et al., 2016). Sterile soil or non-sterilized soil (as a natural source of degrader microorganisms) were also used as carrier material in many research (Van Dyke and Prosser, 2000; Grundmann et al., 2007; Jablonowski et al., 2013). Studies (e.g., as reviewed by Owsianiak et al., 2010) found that the shelf life of inoculated microbial cells in soil were promoted when the microbes were inoculated via carriers, compared with inoculation of free cells. Several mechanisms were considered to be responsible for the beneficial effect of carriers on survival of inoculant cells in soil (van Veen et al., 1997; Gentry et al. 2004). Basically, carriers serve as protective sites and newly-created niches for the inoculant microorganisms in the “new” soil. Some carrier material (e.g., peat and biochar) can also supply nutrients for the proliferation of the inoculant microbes shortly after inoculation. Moreover, when the inoculant microbes are cultivated with carrier material, the cells are allowed to form biofilm on the surface or inside the porous structure of carriers, therefore, hold many advantages that suspended microbial cells may not have, such as high resistance to fluctuating conditions, facilitated microbial communication and nutrient cross-feeding (Sørensen et al., 2002; Wang et al., 2013). An issue for consideration is that utilization of carriers in bioremediation should be adjusted to the abundance, availability and cost of carrier material when formulating bioaugmentation strategies based on carriers (Albareda et al., 2008).

1.4.2 Using microbial consortia instead of single strains

Usually laboratory soil bioaugmentation experiments were conducted with monocultures of isolated xenobiotic-degrader strains. In this case the inoculum potential solely depends on the growth and functioning of the single strain after introduction into soil. However, increasing attention and interest have been paid to co-cultures of different bacterial strains (i.e., synthetic microbial community), which exhibit promising potential of application in various fields, including environmental decontamination (Brenner et al., 2008; De Roy et al., 2014). Synthetic microbial ecosystems are based on communication between constituent microbial groups (e.g., quorum sensing, nutrient cross-feeding) and division of functions in specific sub-populations; these two features enable co-cultured microbial communities to undertake complicated tasks that cannot be realized by pure cultures and to withstand environmental fluctuations such as nutrient limitation or invasion by other microbes (Brenner et al., 2008).

However, more effort was given to isolation of the key degraders, while accompanying microorganisms of these key degraders usually receive less attention. Interaction between degrader microorganisms and co-existing microbes, which may play an indispensable role in the functioning of the key degraders, are frequently underestimated or neglected during the dilution-plating isolation process with artificial agar media. Nevertheless, several studies demonstrated the advantages of co-cultures of bacterial strains in degradation of phenylurea herbicides. Sørensen et al. (2002) found that the ultimate IPU-degrading strain *Sphingomonas* sp. strain SRS2 could only mineralize IPU intensively in mineral salts medium with IPU as the sole C-source in the presence of certain non-IPU-degrader bacteria that supply strain SRS2 with methionine. Devers-Lamrani et al. (2014) reported synergistic degradation of diuron by cooperation between two partial diuron-degraders, *Arthrobacter* sp. BS2 and *Achromobacter* sp. SP1, in which strain BS2 transformed the parent compound to 3,4-dichloroaniline and strain SP1 mineralized the intermediate to CO₂ ultimately. Similar findings were reported in the biodegradation of linuron (Dejonghe et al., 2003).

In addition to synthetic bacterial consortia, fungi could also be employed in alliance with bacteria to construct artificial microbial communities to augment degradation of unwanted chemicals in soil. Apart

from the diverse contaminant-metabolizing potential of fungi (Harms et al., 2011), the extensive fungal mycelial network can function as “fungal highway” to disperse pollutant-degrading bacteria (Kohlmeier et al., 2005; Wick et al., 2007) or as “fungal pipeline” to translocate pollutant molecules in soil (Furuno et al., 2012; Schamfuß et al., 2013). By increasing mobility of degrader microorganisms and bioaccessibility of pollutants in soil, degradation of xenobiotics can be enhanced. In summary, using microbial communities instead of isolated strains could help overcome hurdles in survival and activity of inoculant microbes in soil bioaugmentation.

1.5 Objectives and Hypotheses

Based on *Introduction*, two principal research topics were identified and investigated in this thesis. It is expected that the findings from this study could enrich our knowledge on bioremediation strategies to clean up agricultural soils contaminated with IPU and contribute insights to designing microbial inocula for other purposes.

1.5.1 A promising approach in soil bioaugmentation

According to *Section 1.4*, low survival rate and activity of inoculant microorganisms in contaminated soil is a common obstacle in soil bioaugmentation aiming at accelerated organic pollutant degradation. Delivering degrader microorganisms using carrier material or including microbes beneficial to the key degrader in the inocula were found able to promote the efficacy of bioaugmentation. However, little has been done to systematically investigate the efficiency of inoculating approaches considering both carrier material *vs.* suspended cells and isolated strains *vs.* microbial consortia. In a previous study of our lab (Lab Schroll, Institute of Soil Ecology, Helmholtz Zentrum München), Wang et al. (2010) showed that a microbial community established on carrier particles was more efficient than the community without carrier material or a single degrader strain with or without carriers to degrade 1,2,4-trichlorobenzene (1,2,4-TCB) in soil. However, further studies are required to corroborate the finding of Wang et al. (2010) to generalize about the promising approach, microbial community established on carrier particles,

for soil bioaugmentation. In the first part of this study, biodegradation of IPU in an agricultural soil by an exogenous IPU-degrader bacterium *Sphingomonas* sp. strain AK1 (Kiesel, 2014) and the enriched soil microbial community (Grundmann et al., 2007) originally harboring the degrader strain were investigated with soil microcosm experiments under specific soil conditions. The isolated IPU-mineralizing strain and the microbial consortium were inoculated to soil via carrier material or liquid medium.

The general objective of this experiment is to identify a highly promising bioaugmentation approach to enhance biodegradation of IPU in soil. Specifically, the aims are (i) to compare the capability of an isolated degrader strain (*Sphingomonas* sp. strain AK1) and a microbial consortium to degrade IPU in soil; (ii) to investigate the effect of carrier material on IPU-degrading activity of the inoculant microorganisms, and (3) to examine the functional stability (in terms of IPU biodegradation) of the most promising approach in soil.

It is hypothesized that efficacy of the inoculants in IPU degradation is as follows: microbial community > single strain, inoculants using carrier material > inoculants without carrier material; the microbial community established on carrier material is the most effective in biodegradation of IPU in soil.

1.5.2 Efficacy and sustainability of the microbial-community-carrier-material approach

One major challenge in bioaugmentation to accelerate degradation of IPU in soil is that the activity of IPU-degrading microorganisms are limited by a narrow pH range (pH 7.0–7.5) (*Section 1.3*). IPU degradation in soils with pH beyond this range was restricted. While elevating soil pH (e.g., by addition of limestone) to a significantly higher level (e.g., from pH 5 to pH 7) to fulfil the physiological requirement of IPU-degraders is practically or economically infeasible, IPU-degrading microorganisms derived from acidic soil might be a solution to overcome the detrimental effect of low pH on IPU biodegradation in soil. Previously, two IPU-mineralizing microbial community were enriched by our lab from an acidic agricultural soil (pH_{CaCl₂} 5.8) and a weakly alkaline agricultural soil (pH_{CaCl₂} 7.2), respectively. In this experiment, bioaugmentation of IPU in soils with contrasting pH_{CaCl₂} (3.8–7.3)

using the two microbial consortia were investigated; the factors influencing IPU degradation by the inocula were also studied.

The objectives of this experiment is to investigate the versatility and applicability of the two IPU-mineralizing microbial communities in bioaugmentation of IPU-contaminated soils. The specific goals are (i) to examine the efficiency and functional stability of the two soil-borne IPU-mineralizing microbial consortia in different soils with disparate pH; (ii) to look into the effect of pH, inoculation size, IPU sorption on IPU degradation by the two microbial communities.

The hypotheses are (i) IPU-mineralizing microbial communities originated from the acidic soil and the neutral soil have different optimal pH for IPU degradation; (ii) IPU degradation is enhanced by the microbial communities in soil with pH close to the pH of soils harboring the microbial communities; (iii) IPU bioavailability is not a limiting factor for IPU degradation in the soils.

2 Materials and Methods

2.1 Chemical reagents

2.1.1 Uniformly ring-labeled [^{14}C]-isoproturon

Uniformly ring-labeled [^{14}C]-isoproturon (^{14}C -IPU, specific radioactivity $9.96 \text{ kBq } \mu\text{g}^{-1}$; GE Healthcare, Amersham Place Little Chalfont, UK) was used to study the fate of IPU during biodegradation in soil. ^{14}C -IPU used for this research was purified with HPLC to reach a radiochemical purity of 95.1%–98.1% (**Fig. A1, Appendix**). To reduce the risks and costs of using highly radioactive pure ^{14}C -IPU, non-labeled-IPU (purity 99.0%; LGC Standards, Teddington, UK) was mixed proportionally with ^{14}C -IPU in methanol (HPLC grade) to prepare the ^{14}C -IPU standards for specific experiments according to the requirements of radioactivity and IPU concentrations (**Table 4**).

2.1.2 Other chemicals

3-(4-isopropylphenyl)-1-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU), 3-[4-(2-hydroxyisopropylphenyl)]-1-methylurea (2-OH-MDIPU) and 4-isopropylaniline (4-IA) were obtained from LGC Standards. Scintillation cocktails (Ultima GoldTM XR, Ultima-FloTM AF and Permafluor[®] E+) and Carbo-Sorb[®] E were purchased from PerkinElmer (Waltham, USA). Acetonitrile (ROTISOLV[®] HPLC) was purchased from Carl Roth (Karlsruhe, Germany) and Casamino Acids from United States Biological (Salem, USA). Methanol (LiChrosolv[®]), HPLC water (LiChrosolv[®]), 0.1 M NaOH solution (Titripur[®]), ammonium acetate and chemicals for mineral salts liquid media (MS-media) were obtained from Merck (Darmstadt, Germany) unless specified.

Table 4. ^{14}C -IPU standards used in this thesis.

^{14}C -IPU standard	Specific radioactivity (Bq μg^{-1})	Experiment	Final IPU concentration
A	18	OECD 106 batch experiment	5 $\mu\text{g g}^{-1}$
B	6.7	Liquid culture experiment I	25 $\mu\text{g mL}^{-1}$
C	26	Liquid culture experiment II	5 $\mu\text{g mL}^{-1}$
D	736	Soil microcosm experiment I	5 $\mu\text{g g}^{-1}$
E	723	Soil microcosm experiment II: 1 st application	5 $\mu\text{g g}^{-1}$
F	677	Soil microcosm experiment II: 2 nd application	5 $\mu\text{g g}^{-1}$
G	326	Soil microcosm experiment III	5 $\mu\text{g g}^{-1}$

2.2 IPU-mineralizing microorganisms

2.2.1 *Sphingomonas* sp. strain AK1

The IPU-mineralizing bacterial strain *Sphingomonas* sp. strain AK1 was isolated by Kiesel (2014) from an agricultural soil “Feldkirchen” (**Table 5**). Strain AK1 is able to mineralize IPU and its metabolites (MDIPU, DDIPU and 4-IA) in MS-medium supplemented with casamino acids (Kiesel, 2014).

2.2.2 IPU-mineralizing microbial community enriched from an acidic soil (MC-AS)

MC-AS was enriched by Dr. Ulrike Dörfler (Group Schroll, Institute of Soil Ecology, Helmholtz Zentrum München, data not published) from a moderately acidic soil “Cunnersdorf” (pH 5.8, **Table 5**) with a high intrinsic IPU-mineralizing capacity (Folberth et al., 2009). The community was cultivated in MS-medium with IPU as the sole C source and grown onto carrier particles as described by Grundmann et al. (2007). The IPU-mineralizing capability of MC-AS established on carrier particles (“active particles”) was monitored with ^{14}C -IPU in MS-medium over two years. Portions of MC-AS “active” particles were conserved in glycerol:1×PBS solution (1:4) at $-80\text{ }^{\circ}\text{C}$.

2.2.3 IPU-mineralizing microbial community enriched from a neutral soil (MC-NS)

MC-NS was enriched from a weakly alkaline soil “Feldkirchen” (pH 7.2, **Table 5**) according to the same procedures as described above. The microbial consortium was able to mineralize IPU, MDIPU, DDIPU and 4-IA in MS-medium. *Sphingomonas* sp. strain AK1 was isolated from this enriched microbial community. Further information about the isolated degrader strain and the community are available in the thesis of Kiesel (2014).

Table 5. Properties of soils originally harboring MC-AS (soil “Cunnersdorf”) and MC-NS (soil “Feldkirchen”) (Folberth et al., 2009).

Soil	pH _{CaCl₂}	Clay < 2 μm %	Silt 2–63 μm %	Sand 63–2000 μm %	TOC %	Total N %	CaCO ₃ %	Water content at –15 kPa*
Cunnersdorf	5.8	8	12	80	2.3	0.2	< 0.1	16.0
Feldkirchen	7.2	33	34	33	2.7	0.3	5.1	32.8

* soil moisture optimal for biodegradation of organic pollutants (Schroll et al., 2006).

2.3 Agar medium and mineral salts media

Sphingomonas sp. strain AK1 was exclusively grown on R2A agar (Merck, Darmstadt, Germany) and cultivate at 20 °C for 4–6 weeks until colonies were visible (Kiesel, 2014). MS-media used to cultivate *Sphingomonas* sp. strain AK1, MC-AS and MC-NS were prepared according to Sørensen et al. (2001) with modifications (**Table 6**). All media and glassware were autoclaved (121 °C, 15 min) using a Systec V-65 autoclave (Systec, Linden, Germany) before use.

Table 6. MS-media used to cultivate the IPU-mineralizing microorganisms.

Mineral salt	Concentration (per L)
KH_2PO_4	1.36 g
$\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$	1.78 g
$\text{MgSO}_4 \times 7 \text{H}_2\text{O}$	0.05 g
CaCl_2	0.01 g
H_3BO_3	2.86 mg
$\text{MnSO}_4 \times \text{H}_2\text{O}$	1.54 mg
$\text{CuSO}_4 \times 5 \text{H}_2\text{O}$	0.04 mg
ZnCl_2	0.021 mg
$\text{CoCl}_2 \times 6 \text{H}_2\text{O}$	0.041 mg
$\text{Na}_2\text{MoO}_4 \times 2 \text{H}_2\text{O}$	0.025 mg
$\text{FeCl}_3 \times 6 \text{H}_2\text{O}$	0.00514 mg
Casamino acids*	0.1 g
pH	5.8† or 7.2‡

* for *Sphingomonas* sp. strain AK1.

† for MC-AS.

‡ for *Sphingomonas* sp. strain AK1 and MC-NS.

2.4 Incubation systems

2.4.1 Apparatus for incubating and aerating liquid culture

The IPU-degrading microorganisms were cultivated in MS-media with IPU as the sole C source (IPU-MS media) in 100 mL Erlenmeyer flasks (DURAN, Mainz, Germany). At the air inlet and outlet of the incubators sterile filters with a pore size of 0.2 μm (Midisart[®] 2000, Sartorius, Göttingen, Germany) were installed to avoid contamination of the liquid culture by microorganisms from the air (**Fig. 2**). The flasks were shaken at 75 rpm on a two-story orbital shaker (Edmund Bühler, Hechingen, Germany) in a

20 °C incubation chamber in the dark. Three times per week the incubators were connected to a trapping system (**Fig. 2**) and aerated at an air flow of 1.8 L h^{-1} for 2 h. The aerating system consists of a washing bottle and a pump sucking air through the filters into the incubators. NaOH solution (10 mL, 0.1 M) was filled in the flushing bottle to trap $^{14}\text{C-CO}_2$ evolved from mineralization of $^{14}\text{C-IPU}$.

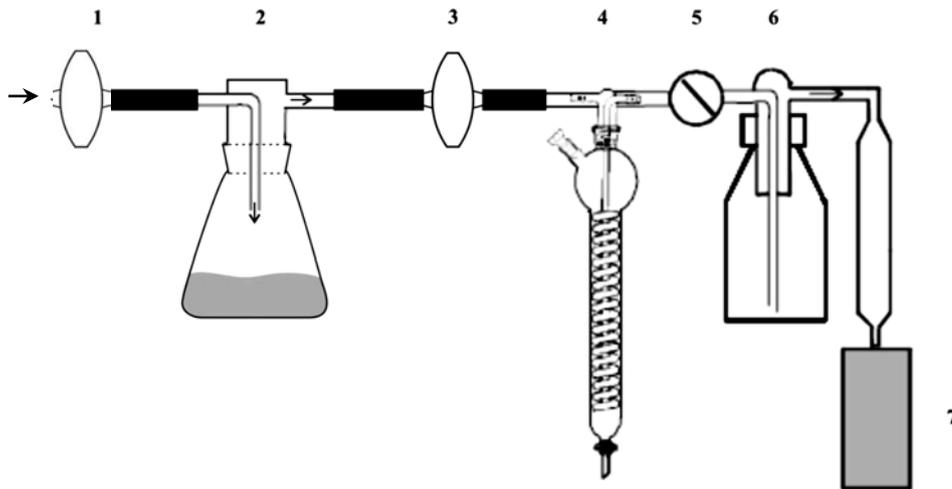


Fig. 2. Schematic of the incubating and aerating system for liquid cultures (modified from Kiesel, 2014).

→: direction of air flow

1: sterile filter with a pore size of $0.2 \mu\text{m}$, for filtering microorganisms in air

2: 100 mL Erlenmeyer flask containing 20 mL IPU-MS medium

3: sterile filter with a porosity of $0.2 \mu\text{m}$, for filtering microbes in air and connection to the flushing bottle. The Erlenmeyer flask was closed with two clamps between 1–2 and 2–3 when the liquid culture was incubated on a shaker.

4: flushing bottle filled with 10 mL 0.1 M NaOH for trapping $^{14}\text{C-CO}_2$ from $^{14}\text{C-IPU}$ mineralization

5: precise regulation valve

6: Woulff bottle

7: pump

2.4.2 Soil microcosm system

The soil microcosm is as shown in **Fig. 3**. The facility comprises a 250-mL amber glass bottle (250 mL; diameter, 63 mm; body height, 90 mm) for soil incubation and a trapping system for absorbing ^{14}C - CO_2 . Inside the bottle a 25-mL plastic beaker was fixed to the bottom side of the rubber cap and filled with 10 mL 0.1 M NaOH. The incubator was connected to the atmosphere via a hollow steel needle penetrating through the rubber cap to guarantee O_2 -supply to the soil inside. Outside the incubation flask the needle was connected to a syringe reservoir filled with sodalime pellets (Merck, Darmstadt, Germany) to filter CO_2 from air to avoid saturation of NaOH solution in the plastic beaker by atmospheric CO_2 . The soil was incubated at 20 ± 1 °C in the dark. The NaOH solution was exchanged regularly to measure ^{14}C -IPU mineralization. The incubators were weighed weekly to monitor the soil water content. The respective amount of water was added to maintain the soil water potential at -15 kPa (the corresponding gravimetric water contents were given in **Table 7**).

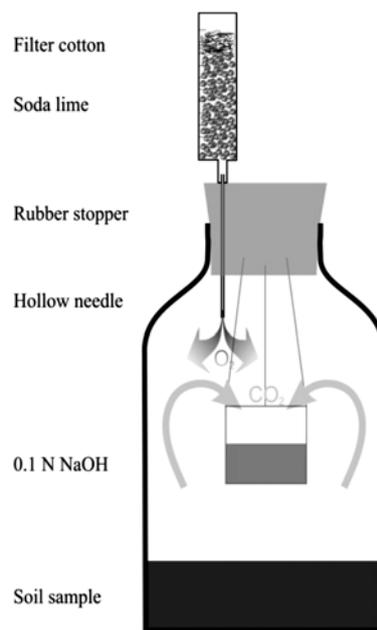


Fig. 3. Schematic of the soil microcosm for bioaugmentation experiments (Kiesel, 2014).

2.5 Carrier material

Expanded clay particles (Seramis, Mogendorf, Germany) were used as carrier material to inoculate the IPU-mineralizing microorganisms into soil. The particles are mainly made of kaolinite, illite and quartz. Properties of the carrier material are as follows (data from manufacturer): diameter, ≈ 4 mm; pore volume, $> 80\%$; $\text{pH}_{\text{H}_2\text{O}}$, ≈ 7 ; total N, $3\text{--}8 \text{ mg L}^{-1}$; P_2O_5 , $5\text{--}10 \text{ mg L}^{-1}$ and K_2O , $100\text{--}120 \text{ mg L}^{-1}$. The dry weight of 20 carrier particles is 0.52 ± 0.06 g. The carrier material showed a negligible IPU sorption potential and had no effect on IPU degradation (**Fig. A2, Appendix**). This carrier material has been used previously to inoculate 1,2,4-TCB degrading microorganisms and showed a beneficial effect on the functioning of the degraders in soil (Wang et al., 2010).

2.6 Soils

Four agricultural soils (**Table 7**) were used in incubation experiments to study IPU biodegradation. These soils were selected because they all showed a low IPU-mineralizing capacity and represent diverse soil properties such as pH and SOM content. After sampling, the soil material was air-dried, sieved to ≤ 2 mm and stored at -20 °C. Before start of the incubation experiments, the soils were defrosted at 4 °C for one week, wetted close to the water potential of -15 kPa (see corresponding gravimetric water contents in **Table 7**) and equilibrated for another week at 20 ± 1 °C. All soil incubation experiments were conducted using the soil microcosm system (*Section 2.4.2*) with 50 g (dry weight, dw) soil at a soil density of 1.3 g cm^{-3} and a soil water potential of -15 kPa (Schroll et al., 2006). The nominal IPU concentration in soil was fixed at $5 \text{ } \mu\text{g g}^{-1}$, the approximate concentration of IPU in topsoil at an IPU application rate of 0.75 kg ha^{-1} .

Table 7. Physico-chemical properties of soils used for soil incubation experiments.

Soil	pH _{CaCl₂}	Clay < 2 µm %	Silt 2–63 µm %	Sand 63–2000 µm %	TOC %	Total N %	CaCO ₃ %	Water content at –15 kPa† %
Konjšče*	7.1	7	60	33	1.9	0.2	nd.	35.7
Marsdorf*	3.8	9	16	75	1.4	0.1	0.1	13.3
Neumarkt*	5.8	4	8	88	1.0	0.1	2.7	10.1
Dürneck	7.3	7.6	63.9	28.5	4.0	0.27	60.6	35.7

* according to Folberth et al. (2009).

† soil moisture optimal for biodegradation of organic pollutants (Schroll et al., 2006).

nd.: not determined.

2.7 OECD 106 batch experiment

Sorption and desorption of IPU in the four soils (**Table 7**) were investigated according to the OECD guideline 106 (OECD, 2000) at a soil-solution ratio of 1:5 in parallel batch experiments. ¹⁴C-IPU standard A (**Table 4**) was dissolved in 0.01 M CaCl₂ to prepare ¹⁴C-IPU working solution with an IPU concentration of 1 µg mL⁻¹. Four g soil (dw) was weighed into a 30-mL Nalgene™ Teflon tube (Thermo Fisher Scientific, Sunnyvale, USA) and added with 20 mL ¹⁴C-IPU working solution. The samples were incubated on a GFL 3025 over-head shaker (GFL, Burgwedel, Germany) at 60 rpm min⁻¹, 20 ± 1 °C. In total, 21 replicates were prepared for each soil. At time points 1 h, 2 h, 4 h, 21 h, 24 h, 48 h and 72 h after the start of incubation, three replicates were centrifuged at 4500×g, 20 °C for 20 min using a Sorvall RC6 Plus centrifuge (Thermo Fisher Scientific, Sunnyvale, USA). Two 1-mL aliquots of the supernatant from each vial were mixed with 4 mL Ultima Gold™ XR and measured in a liquid scintillation counter (Tri-Carb® 1900TR, PerkinElmer, Waltham, USA) to determine the radioactivity in the aqueous phase. Three vials with 20 mL ¹⁴C-IPU working solution but without soil were subjected to the same treatment to measure the adsorption of IPU to the testing tubes. IPU sorption to the test vials were negligible (**Fig.**

A3, Appendix). IPU adsorption was calculated as the difference between the radioactivity in the liquid before and after incubation with soil.

The partition ratio K_d of IPU was calculated according to the formula below.

$$K_d = \frac{C_S}{C_A}$$

K_d : solid–liquid partition ratio of IPU in soil (mL g^{-1})

C_S : concentration of IPU in soil ($\mu\text{g g}^{-1}$)

C_A : concentration of IPU ($\mu\text{g mL}^{-1}$) in aqueous phase (supernatant)

Desorption of IPU was conducted with the soil samples centrifuged after shaking for 72 h in the sorption experiment. Twenty mL IPU-free 0.01 M CaCl_2 was added to the tube after removing the supernatant. The soil was re-suspended in the solution and incubated on the overhead shaker at 60 rpm min^{-1} , $20 \pm 1 \text{ }^\circ\text{C}$ for another 144 h. After 24 h, 48 h, 72 h and 144 h the samples were centrifuged and the supernatant was replaced by 20 mL CaCl_2 solution. ^{14}C in the supernatant was measured in the same way as in the sorption experiment.

2.8 Liquid culture incubation experiment I

2.8.1 Preparation of inoculants

To reactivate and multiply inocula, 200 μL cryoculture of *Sphingomonas* strain AK1 or 20 “active” carrier particles pre-colonized by MC-AS or MC-NS, respectively, were inoculated to 20 mL IPU-MS medium (^{14}C -IPU Standard B, **Table 4**). Approximately 80 sterilized carrier particles were added to the liquid culture to allow the microbes to settle down and grow on the carriers. Incubation and aeration of liquid cultures were as described in *Section 2.4.1*. After aeration, an aliquot (2 mL) of the trapping solution was mixed with 3 ml Ultima-Flo™ AF to measure the radioactivity in the liquid scintillation counter. When the liquid cultures showed a cumulative IPU mineralization of $> 40\%$ and a daily

mineralization rate of 4%–5% d⁻¹ (liquid cultures of *Sphingomonas* strain AK1 and MC-NS, for *Section 2.10 Soil microcosm experiment I*) or 1% d⁻¹ (liquid cultures MC-AS and MC-NS, for *Section 2.11 Soil microcosms experiment II* and *Section 2.12 Soil microcosm experiment III*), the respective microbes were inoculated into soil. The microorganisms were inoculated with similar IPU-mineralizing activity to ensure the comparability of the different inoculants.

2.8.2 Microbial diversity of MC-AS and MC-NS

In order to analyze the composition of the two IPU-mineralizing microbial consortia, five “active” particles of MC-AS or MC-NS (*Section 2.8.1*), respectively, were inoculated to 20 mL IPU-MS media with non-labeled IPU (25 µg mL⁻¹). Each liquid culture was added with 35 sterile carrier particles to multiply the microbial community on carriers. The liquid cultures were incubated and aerated as described in *Section 2.4.1* for 28 d. In a parallel experiment, the microbial consortia were cultivated in IPU-MS media with ¹⁴C-IPU (¹⁴C-IPU standard B, **Table 4**) to serve as references for the IPU-mineralizing function of MC-AS and MC-NS cultivated with non-labeled IPU. After the incubation was ended, carrier particles from the liquid culture with non-labeled IPU were frozen at –80 °C for DNA extraction. DNA was extracted from 20 “active” particles of MC-AS or MC-NS using the FastDNA[®] SPIN Kit for Soil and the FastPrep[®] Instrument (MP Biomedicals, Santa Ana, USA) according to manufacturer’s instructions. Microbial 16S rRNA gene sequencing was conducted according to the Illumina[®] standard protocol (Illumina, San Diego, USA) using paired-end sequencing. The primers are: S-D-Bact-0008-a-S-16 (forward primer 5’→3’) AGA GTT TGA TCM TGG C and S-D-Bact-0343-a-A-15 (reverse primer 5’→3’) CTG CTG CCT YCC GTA. Details about the oligonucleotide probes are available at probeBase (<http://probebase.csb.univie.ac.at/>).

2.9 Liquid culture incubation experiment II: Effect of pH

The effect of pH on IPU mineralization by MC-AS and MC-NS was studied. Briefly, four “active” carrier particles of MC-AS or MC-NS (*Section 2.8.1*), respectively, were inoculated to 20 mL IPU-MS

media (^{14}C -IPU standard C, **Table 4**) with pH 3.8, 5.8, 7.2 or 8.0 in a 100 mL Erlenmeyer flask. For each microbial consortium–pH combination, three replicates were prepared. The liquid cultures were incubated and aerated over 31 d as previously described (*Section 2.4.1*). ^{14}C -IPU mineralization was measured as previously reported. After incubation was terminated, carrier particles were separated from liquid medium and combusted using a Sample Oxidizer Model 307 (PerkinElmer, Waltham, USA) to measure the radioactivity. Eight mL of the liquid culture was filtered with a Millex-GP Syringe Filter Unit, 0.22 μm (Merck, Darmstadt, Germany) to separate suspended microbial cells from liquid medium. Radioactivity of the liquid medium before and after filtration was determined by mixing 1 mL medium with 4 mL Ultima GoldTM XR and measuring in the liquid scintillation counter. The radioactivity detected in ^{14}C -CO₂, suspended solids (difference between filtered and unfiltered medium), filtered liquid medium and carrier particles were used to calculate the ^{14}C mass balance. Composition of ^{14}C -residues in the filtered medium was determined using ^{14}C -HPLC.

2.10 Soil microcosm incubation experiment I: A promising approach

Soil microcosm incubation experiment I was conducted with soil “Konjščee” (**Table 7**) to identify a highly promising bioaugmentation approach for enhancing IPU biodegradation in soil. The experiment was conducted with the soil microcosm system described in *Section 2.4.2*. ^{14}C -IPU standard D (240 μL , **Table 4**) was applied via a 250- μL Hamilton[®] MicroliterTM syringe (Hamilton Company, Bonaduz, Switzerland) to 3.5 g pulverized oven-dry (105 °C) soil aliquot in a small glass beaker and carefully mixed with the soil material until methanol evaporated. This spiked soil was mixed homogeneously with 46.5 g (dw) equilibrated soil in a 250-mL amber glass bottle to achieve a nominal IPU concentration of 5 $\mu\text{g g}^{-1}$. The soil was then inoculated with *Sphingomonas* sp. strain AK1 or MC-NS with or without carrier particles from the above mentioned liquid cultures (*Section 2.8.1*). In approaches with the liquid culture, soil material was condensed to 1.3 g cm^{-3} by pressing the soil with an end-bended steel spatula to a defined line on the incubator which marked the volume corresponding to a soil density of 1.3 g cm^{-3} . After compacting the soil, 0.5 mL or 1.5 mL liquid culture of either *Sphingomonas* sp. strain AK1 or MC-NS was spread evenly on the soil surface using an Eppendorf pipette. In the approaches using carrier

particles, the soil was mixed with 20 or 60 carrier particles from the respective liquid culture before condensing to 1.3 g cm^{-3} . IPU carry-over from the inocula to soil was negligible (results not presented). After inoculation, the soil was added with distilled water to a soil water content of 35.7% (corresponding to -15 kPa water potential). Soil subjected to the same treatment, but without inoculation of degraders served as control. All soil treatments were performed with four replicates. The soil was incubated for 46 d as described in *Section 2.4.2*. NaOH in the plastic beakers was exchanged thrice per week. To measure ^{14}C -IPU mineralization, 2 mL NaOH was mixed with 3 mL Ultima-Flo™ AF and measured by liquid scintillation counting. At the end of incubation, the soil material was conserved at $-20 \text{ }^\circ\text{C}$ until analysis of ^{14}C -extractable residues (^{14}C -ER) and ^{14}C -non extractable residues (^{14}C -NER). A summary of the experimental design was given in **Table 8**.

Table 8. Experimental design of Soil microcosm incubation experiment I.

Soil treatments	Inoculation size	Type of inoculation	Inoculation ratio carrier to soil (w/w)
IS-LM 0.5	0.5 mL	liquid culture of strain AK1	–
IS-LM 1.5	1.5 mL	liquid culture of strain AK1	–
IS-CP 20	0.5 g (20 CP)	carrier particles with strain AK1	1%
IS-CP 60	1.5 g (60 CP)	carrier particles with strain AK1	3%
MC-LM 0.5	0.5 mL	liquid culture of MC-NS	–
MC-LM 1.5	1.5 mL	liquid culture of MC-NS	–
MC-CP 20	0.5 g (20 CP)	carrier particles with MC-NS	1%
MC-CP 60	1.5 g (60 CP)	carrier particles with MC-NS	3%
Control	no inoculation	–	–

2.11 Soil microcosm incubation experiment II: Efficacy and sustainability

2.11.1 Initial IPU application and inoculation

Soil microcosm incubation experiment II was performed with three agricultural soils, “Marsdorf”, “Neumarkt” and “Dürneck” (**Table 7**), to study the efficacy and sustainability of the promising approach to degrade IPU in soils with contrasting physico-chemical properties, especially pH. Briefly, 150 μL ^{14}C -IPU standard E (**Table 4**) was applied to 50 g (dw) soil to result in 5 $\mu\text{g g}^{-1}$ IPU in the soil as described in *Section 2.10*. The dosed soil was inoculated and mixed with 20 “active” particles of MC-AS or MC-NS (prepared according to *Section 2.8.1*) before being condensed to 1.3 g cm^{-3} . After inoculation, the soil was moistened with distilled water to a soil water potential of -15 kPa (see **Table 7** for the corresponding gravimetric water contents). Soil subjected to the same treatment, but without inoculation of microbes served as control. Each soil treatment was conducted with four replicates. The soil samples were incubated for 133 days at 20 ± 1 °C. The soils were wetted weekly to maintain the soil water potential at -15 kPa. NaOH was exchanged three times per week from day 0 to day 57 and twice per week from day 57 to day 133. ^{14}C -IPU mineralization was measured as reported above. An overview of the experimental set-up is given in **Table 9**.

2.11.2 Re-application of IPU

After 133 d, 150 μL ^{14}C -IPU standard F (**Table 4**) was applied to each of the soil samples, corresponding to 5 $\mu\text{g g}^{-1}$ IPU concentration in soil. An aliquot of 3.5 g (dw) soil from each incubator was dried and used for IPU application. The procedures were as described above. The soils were incubated for 56 d under the same conditions. ^{14}C -IPU mineralization was measured thrice per week during this period. On day 189, the soil was sampled and conserved in a -20 °C freezer until ^{14}C -residues analyses.

Table 9. Experimental design of Soil microcosm incubation experiment II.

Treatments	Type of inoculants	Quantity of inocula	carrier to soil ratio (w/w)	No. of IPU application
Soil Marsdorf (pH 3.8)				
Control	uninoculated	–	–	2
MC-AS	carrier particles with MC-AS	0.5 g	1%	2
MC-NS	carrier particles with MC-NS	0.5 g	1%	2
Soil Neumarkt (pH 5.8)				
Control	uninoculated	–	–	2
MC-AS	carrier particles with MC-AS	0.5 g	1%	2
MC-NS	carrier particles with MC-NS	0.5 g	1%	2
Soil Dürneck (pH 7.3)				
Control	uninoculated	–	–	2
MC-AS	carrier particles with MC-AS	0.5 g	1%	2
MC-NS	carrier particles with MC-NS	0.5 g	1%	2

2.12 Soil microcosm incubation experiment III: Effect of inoculation size

In Soil microcosm incubation experiment III, soil “Neumarkt” and soil “Dürneck” (**Table 7**) were inoculated with two “active” carrier particles (carrier-soil ratio w/w, 0.1%) of MC-AS or MC-NS to examine the effect of inoculation size on IPU dissipation in soil. The experimental design is shown in **Table 10**. ^{14}C -IPU application (^{14}C -IPU standard G, **Table 4**), inoculation of microbial degraders, soil incubation and measurement of ^{14}C -IPU mineralization were performed as previously described. The soils were incubated for 55 d; afterwards the soil was frozen at $-20\text{ }^{\circ}\text{C}$ for analyses of ^{14}C -residues.

Table 10. Experimental design of Soil microcosm incubation experiment III.

Treatments	Soil	Type of inoculation	Quantity of inocula	carrier to soil (w/w)
N-MC-AS-2	Neumarkt	carrier particles with MC-AS	0.05 g	0.1%
D-MC-AS-2	Dürneck	carrier particles with MC-AS	0.05 g	0.1%
D-MC-NS-2	Dürneck	carrier particles with MC-NS	0.05 g	0.1%

2.13 Analysis of ^{14}C -IPU mineralization

Mineralization of ^{14}C -IPU was determined by measuring the radioactivity of ^{14}C - CO_2 trapped by NaOH solution in the incubation systems. Briefly, 2 mL trapping solution was mixed with 3 mL Ultima-Flo™ AF and measured in the liquid scintillation counter. The degree of IPU mineralization (cumulative IPU mineralization) was calculated as the radioactivity of ^{14}C - CO_2 in % of initially added ^{14}C . IPU mineralization rates were calculated as μg IPU-mass equivalents d^{-1} g dry soil $^{-1}$ according to the formula below.

$$\text{MR}_{\text{IPU}} = \frac{\text{CM}_t - \text{CM}_{t-1}}{\text{R}_{\text{spec.IPU}} * t * m}$$

MR_{IPU} : mineralization rate of IPU (μg d^{-1} g dry soil $^{-1}$)

CM_t : cumulative IPU mineralization (kBq) as ^{14}C - CO_2 at sampling time t (d)

CM_{t-1} : cumulative IPU mineralization (kBq) as ^{14}C - CO_2 at sampling time $t-1$ (d), the time point previous to time t

$\text{R}_{\text{spec.IPU}}$: specific radioactivity of the ^{14}C -IPU standard (kBq μg^{-1})

t : time (d) between two adjacent sampling times ($t-1$ and t)

m : dry mass of soil (g)

2.14 Quantification of ¹⁴C-extractable residues

After incubation the soil was extracted using an Accelerated Solvent Extractor ASE[®] 200 (Thermo Fisher Scientific, Sunnyvale, USA) equipped with 33-mL steel extraction cells (Schroll and Kühn 2004). For each soil replicate, 25 g soil (dw) was mixed with 1–2 g diatomaceous earth (Sigma-Aldrich, St. Louis, USA) and extracted with methanol at 90 °C, 10 MPa. The ASE[®] extraction program was as follows: Preheat, 1 min; Heat and pressurize, 5 min; Static, 1 min; Flush, 80% of the cell volume; Cycles, 3 (from static to flush); Purge, 300 sec; End relief, 1 min. Each soil sample was extracted three times to ensure exhaustive extraction of ¹⁴C-ER. Two aliquots (1 mL each) of crude extracts were mixed with 4 mL Ultima Gold[™] XR and measured in the liquid scintillation counter to determine the radioactivity. The quantity of ¹⁴C-ER was calculated as the radioactivity of crude extract in % of applied ¹⁴C.

2.15 Composition analysis of ¹⁴C-extractable residues

The crude ¹⁴C-ER were further processed to identify the composition of ¹⁴C-ER. The extracts were evaporated at 50–55 °C, 15–20 kPa using a rotary evaporator (Rotavapor R-114, BÜCHI, Flawil, Switzerland) to about 3–8 mL depending on the water contents of soil samples. The liquid was then diluted with Milli-Q[®] water to 250 mL and homogenized in a 250-mL measuring cylinder. Two 1-mL aliquots of the diluted extract were mixed with 4 mL Ultima Gold[™] XR and measured by liquid scintillation counting. The extract was then cleaned with solid phase extraction (SPE; Bond Elut ENV, 200 mg, 3 mL, Agilent Technologies, Santa Clara, USA) at a flow rate of $\approx 9 \text{ mL min}^{-1}$. The SPE columns were dried with nitrogen and stored at –20 °C. For further analysis, the cartridges were eluted with 10 mL methanol. The radioactivity of the eluates was determined by measuring two aliquots (100 μL each) with 5 mL Ultima Gold[™] XR in the liquid scintillation counter. The eluates were evaporated with the rotary evaporator (50–55 °C, 15–20 kPa) to dryness and re-dissolved in an appropriate volume of methanol to achieve a concentration of 42–167 Bq μL^{-1} in the samples. Twenty μL of each sample was injected into a ¹⁴C-HPLC system described by Schroll and Kühn (2004). The HPLC gradient to

identify IPU and its metabolites is presented in **Table 11**. Peaks in the HPLC chromatograms below the quantification limit of the ^{14}C -HPLC (20 Bq per 20 μL) were not integrated.

Table 11. HPLC gradient program for detecting IPU and IPU-metabolites.

Time (min)	Mobile phases	
	Acetonitrile (%)	10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ (%)
0	5	95
15	60	40
20	60	40
25	5	95
35	5	95

2.16 Quantification of ^{14}C -non-extractable residues

The ASE[®]-extracted soil was ground intensively until it was homogeneous (looks like flour powder). For each soil sample, three aliquots (300–400 mg per aliquot) of pulverized soil material were mixed with 10 drops of saturated sugar solution, dried at 70 °C for 5 h and combusted in a Model 307 Sample Oxidizer (PerkinElmer, Waltham, USA). ^{14}C -CO₂ released from the combustion was absorbed by 8 mL Carbo-Sorb[®] E which was mixed with 12 mL Permafluor[®] E+ and measured in the liquid scintillation counter to determine the radioactivity. Quantity of ^{14}C -NER was the amount of soil radioactivity after extraction in % of added ^{14}C .

2.17 Figures, tables and statistical analysis

Figures in this thesis were created using OriginPro 2015 (OriginLab Corporation, MA, USA). Tables were created using Microsoft Office Word 2013 (Microsoft Corporation, Washington, USA). Statistical analysis (Duncan test, $p < 0.05$) is performed using IBM[®] SPSS[®] Statistics Version 20 (IBM Corporation, New York, USA).

3 Results and Discussion

This chapter comprises two papers published in two peer-reviewed journals.

3.1 Publication I

Li, R., Dörfler, U., Munch, J.C., Schroll, R., 2017. Enhanced degradation of isoproturon in an agricultural soil by a *Sphingomonas* sp. strain and a microbial consortium. *Chemosphere* 168, 1169–1176.

This paper corresponds to *Section 1.5.1*. In this paper, effectiveness of four bioaugmentation approaches to promote degradation of IPU in soil was investigated. An IPU-mineralizing bacterium (*Sphingomonas* sp. strain AK1) and the microbial community naturally harboring this strain were inoculated to an agricultural soil as suspended cells or as cells established on carrier material. IPU mineralization, transformation, formation of extractable residues and non-extractable residues were analyzed in the treated and untreated soil using ¹⁴C-IPU. Functional sustainability of the microbial–community–carrier–particle inoculant in soil was evaluated. IPU sorption–desorption in soil was determined.

This paper is based on the following experiments (see *Chapter 2*):

- *Section 2.7 OECD 106 batch experiment* (results about soil “Konjšiče”)
- *Section 2.8 Liquid culture experiment I*
- *Section 2.10 Soil microcosm incubation experiment I: A promising approach*
- *Section 2.11 Soil microcosm incubation experiment II: Efficacy and sustainability* (results about soil “Konjšiče”)

I designed the experiments with R. Schroll, U. Dörfler and J.C. Munch. I conducted the experiments and analyzed the data. I wrote the manuscript and act as corresponding author of the paper.

3.2 Publication II

Li, R., Dörfler, U., Schroll, R., Munch, J.C., 2016. Biodegradation of isoproturon in agricultural soils with contrasting pH by exogenous soil microbial communities. *Soil Biology and Biochemistry* 103, 149–159.

This research corresponds to *Section 1.5.2*. In this paper, efficacy and functional sustainability of two IPU-mineralizing microbial communities to enhance IPU degradation were investigated in three different agricultural soils with contrasting pH. IPU mineralization, transformation, formation of extractable residues and non-extractable residues in soils were analyzed using ^{14}C -IPU. Effect of inoculation size, IPU sorption and pH on IPU degradation in the soils by the inoculant microbial communities were studied.

Publication II is based on the following experiments (see *Chapter 2*):

- *Section 2.7 OECD 106 batch experiment* (results about soil “Marsdorf, Neumarkt and Dürneck”)
- *Section 2.8 Liquid culture experiment I*
- *Section 2.9 Liquid culture experiment II: Effect of pH*
- *Section 2.11 Soil microcosm incubation experiment II: Efficacy and sustainability* (results about soil “Marsdorf, Neumarkt and Dürneck”)
- *Section 2.12 Soil microcosm incubation experiment III: Effect of inoculation size*

I designed the experiments with R. Schroll, U. Dörfler and J.C. Munch. I conducted the experiments and analyzed the data. I wrote the manuscript and act as corresponding author of the paper.

Publication I: Enhanced degradation of isoproturon in an agricultural soil by a *Sphingomonas* sp. strain and a microbial consortium

Li et al., 2017. *Chemosphere* 168, 1169–1176.



Enhanced degradation of isoproturon in an agricultural soil by a *Sphingomonas* sp. strain and a microbial consortium



Renyi Li ^{a,*}, Ulrike Dörfler ^a, Jean Charles Munch ^b, Reiner Schroll ^{a,**}

^a Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany

^b Lehrstuhl für Grünlandlehre, Technische Universität München, 85764 Neuherberg, Germany

HIGHLIGHTS

- Isoproturon (IPU) degradation by a microbial strain and a consortium was compared.
- Both the single strain and microbial consortium enhanced IPU degradation in soil.
- Maximal IPU degradation in soil was achieved by the microbial community.
- Carrier material had positive effect on IPU degradation by the single strain.
- Effect of inoculation size was less notable on the community than on single strain.

ARTICLE INFO

Article history:

Received 31 March 2016
Received in revised form
5 October 2016
Accepted 22 October 2016
Available online 3 November 2016

Handling Editor: Chang-Ping Yu

Keywords:

¹⁴C-label
Pesticide
Microbial consortium
Mineralization
Metabolites
Non-extractable residues

ABSTRACT

Isoproturon (IPU) degradation in an agricultural soil inoculated with an isolated IPU-degrader strain (*Sphingomonas* sp. strain AK1, **IS**) or a microbial consortium (**MC**) harboring this strain, with or without carrier material, were investigated in soil microcosm experiments during 46 days. Effect of the carrier material and inoculation size on IPU-degradation efficacy of the inoculants were studied. Mineralization, extractable residues and non-extractable residues of ¹⁴C-labeled IPU were analyzed. The low IPU mineralization in untreated soil (7.0%) was enhanced to different extents by inoculation of IS (17.4%–46.0%) or MC (58.9%–67.5%). Concentrations of IPU residues in soils amended with MC (0.002–0.095 μg g dry soil⁻¹) were significantly lower than in soils amended with IS (0.02–0.67 μg g dry soil⁻¹) and approximately 10 times lower than in the uninoculated soil (0.06–0.80 μg g dry soil⁻¹). Less extractable residues and non-extractable residues were detected in soil with higher IPU mineralization. Inoculation size (as indicated by the volume of liquid cultures or by the number of carrier particles) determined the IPU-removal efficacy of IS in soil, but this effect was less pronounced for MC. The low sorption of IPU to soil and the decreasing IPU-mineralizing rates suggested incapability of IS to establish the IPU-mineralizing function in the soil. The thorough removal of IPU and persistent IPU-mineralizing activity of soil inoculated with MC indicated a high persistence of IPU-metabolic trait. Our results showed that microbial consortia might be more efficient than single degrader strains to enhance clean-up of organic chemicals in soil.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Isoproturon (IPU), 3-(4-isopropylphenyl)-1,1-dimethylurea, is one of the most-used herbicides worldwide for pre- and post-

emergence control of annual grasses and broadleaved weeds in cereal crops (Fenner et al., 2013). Due to its extensive use and high mobility in soil, the active substance has been widely detected in groundwater and surface water exceeding the maximum allowable limit of pesticides in drinking water in European Union (EU; 0.1 μg L⁻¹) (Fenner et al., 2013; Hussain et al., 2015). IPU is potentially carcinogenic and highly toxic to aquatic organisms. In addition, a recent report from EFSA (2015) classified IPU as an endocrine-disruptor because of new proofs of damaging fertility

* Corresponding author.

** Corresponding author.

E-mail addresses: renyili2009@gmail.com (R. Li), schroll@helmholtz-muenchen.de (R. Schroll).

system of mammals. Because of the high risks of IPU to human health and the environment, IPU was prohibited by EU in July 2016 (Commission Implementing Regulation (EU) No 2016/872, 2016).

Biodegradation of IPU in soil under field conditions varies considerably as influenced by soil parameters such as pH, soil indigenous microbiome, temperature, water content, amendment of sorbent (e.g., biochar) and distribution of soil organic matter etc. (Bending et al., 2003; Alletto et al., 2006; Schroll et al., 2006; Eibisch et al., 2015; Vieubl e Gonod et al., 2016). Natural adaptation of microorganisms to IPU degradation has occurred in soils that were frequently exposed to IPU, usually via regular pesticide application in agriculture. Since 2001 eleven IPU-degrading microorganisms have been isolated from different soils, seven of these strains belonging to the *Sphingomonas* genus (Li et al., 2016). Pesticides may persist for a long time in soil without adapted degrader microorganisms, exerting toxicity to non-targeted organisms and risks of contaminating water resources. To accelerate dissipation of xenobiotics in soil, microorganisms carrying the metabolic traits of the chemicals have been introduced into contaminated soils without self-cleaning capabilities. The potential of microbial inoculants to enhance the removal of organic chemicals in soils has been investigated in many studies with specific degrading strains or microbial consortia, such as reviewed by Dejonghe et al. (2001) and Owsianiak et al. (2010). However, as affected by abiotic and biotic soil factors, such as pH, nutrient status, availability of pollutants, competition with native microorganisms etc., microbial cells introduced into soil often have a limited survival time and activity (van Veen et al., 1997; Dejonghe et al., 2001). Studies e.g., as reviewed by Owsianiak et al. (2010) found that the persistence of inoculant microbial cells in soil was enhanced when the microorganisms were inoculated via carriers, compared with free cells. Several mechanisms were considered to be responsible for the carrier-promoting effect on inoculant cell survival in soil (Gentry et al., 2004). Briefly, carrier materials offer protective sites and newly-created niches for the inoculant microorganisms in inoculated soil. Some carrier materials (e.g., peat, biochar) can supply nutrients for the proliferation of the inoculant cells (Sun et al., 2016). Moreover, the inoculant microbial cells could form biofilm on the surface or inside the pore structure of carrier particles, therefore, having many biological advantages that free cells may not have (Wang et al., 2010, 2013).

Both single strains and microbial communities have shown their capabilities to enhance biodegradation of organic pollutants in soil bioaugmentation. However, little has been done to compare the efficacy of a single degrader and a microbial consortium to degrade the same substance in soil. A previous report of Wang et al. (2010) showed that the microbial community had many advantages over the single strain (*Bordetella* sp. strain F2) in degradation of 1,2,4-trichlorobenzene (1,2,4-TCB) in soil. Nevertheless, further studies are required to corroborate this finding to help identify a potentially promising approach, presumably “microbial community established on carrier particles”, for inoculation of beneficial microorganisms to enhance degradation of organic contaminants in soil. IPU is selected as a model substance for bioaugmentation experiments in this research. The concept of considering a microbial consortium as a whole, contrary to the conventional bottom-up approach starting with isolating single degraders, may offer a different view on designing strategies for enhancing the persistence and activity of microorganisms introduced into soil. The objectives of this study were therefore (i) to evaluate how the IPU-degradation potential of a pure culture versus a consortium containing the degraders can be established in a non-degrading soil, (ii) to investigate the effects of a carrier material (expanded clay particles) on IPU-degrading activity of the inoculant microorganisms, and (iii) to study the effect of inoculation size on dissipation of

IPU in soil.

2. Materials and methods

2.1. Chemicals

[Uniformly-ring-¹⁴C]-labeled IPU (¹⁴C-IPU, radiochemical purity > 98%, specific radioactivity 9.96 kBq μg⁻¹) was purchased from GE Healthcare (Amersham Place Little Chalfont, UK). ¹⁴C-IPU and non-labeled IPU (purity 99.0%; Dr. Ehrenstorfer, Augsburg, Germany) were dissolved in methanol and mixed proportionally to prepare ¹⁴C-IPU standards for liquid culture experiments (Standard A, specific radioactivity 6.7 Bq μg⁻¹), soil experiments (Standard B, specific radioactivity 736 Bq μg⁻¹) and batch experiments (Standard C, specific radioactivity, 17.8 Bq μg⁻¹). IPU degradation products 3-(4-isopropylphenyl)-1-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU), 3-[4-(2-hydroxyisopropylphenyl)]-1-methylurea (2-OH-MDIPU) and 4-isopropylaniline (4-IA) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Scintillation cocktails (Ultima-Flo™ AF, Ultima Gold™ XR and Permafluor® E⁺) and Carbo-Sorb® E were purchased from PerkinElmer (Waltham, USA). Methanol (LiChrosolv®), HPLC water (LiChrosolv®), 0.1 M NaOH solution (Titripur®), ammonium acetate and mineral salts for the liquid media were obtained from Merck (Darmstadt, Germany) unless specified. Acetonitrile (ROTISOLV® HPLC) was purchased from Carl Roth (Karlsruhe, Germany). Casamino acids were bought from United States Biological (Salem, USA).

2.2. Soil and carrier material

The studied soil (Fluvisol) was collected from Ah horizon (0–20 cm) of an agricultural field in the north-east of Slovenia (latitude 46.710°, longitude 15.821°, altitude 230 m). The soil material was sieved to ≤ 2 mm and stored at -20 ± 1 °C. This soil was selected because it showed a low IPU-mineralization potential and has a neutral pH (Folberth et al., 2009). The main physico-chemical properties of this soil were as follows: clay (< 2 μm), 7%; silt (2–63 μm), 60%; sand (63–2000 μm), 33%; total organic carbon, 1.9%; total nitrogen, 0.2%, pH (CaCl₂), 7.1, and gravimetric water content at -15 kPa water potential, 35.7% (Folberth et al., 2009). Two weeks before start of the soil incubation experiments, the soil was defrosted at 4 °C for 7 days, followed by wetting the soil close to a water potential of -15 kPa (Schroll et al., 2006) and equilibration at 20 ± 1 °C for another week. Properties of the carrier material (Seramis® expanded clay particles, diameter ≈ 4 mm) was described by Li et al. (2016). This carrier material had been utilized to inoculate 1,2,4-TCB degrading microorganisms to soil and showed a potential to enhance the survival of the degraders in soil (Wang et al., 2010).

2.3. Microbial inocula

A bacterium (designated as *Sphingomonas* sp. strain AK1) and a microbial consortium that are capable to mineralize IPU and its metabolites (MDIPU, DDIPU and 4-IA), were used as inocula for the soil inoculation experiment. The microbial consortium was enriched from an agricultural soil with a high IPU-degradation potential (Grundmann et al., 2007). *Sphingomonas* sp. strain AK1 was isolated from this soil microbial community (Kiesel, 2014). The auxotrophy of *Sphingomonas* sp. strain AK1 was identified when the strain was unable to degrade IPU when casamino acids were excluded from the mineral salts media (Kiesel, 2014). The IPU-mineralizing capacity and characteristics of *Sphingomonas* sp. strain AK1 and the community were described in detail by Kiesel

(2014). To prepare the inocula, 200 μL cryoculture of strain AK1 or 20 “active” carrier particles pre-colonized by the microbial consortium, respectively, were inoculated to 20 mL IPU-mineral salt medium (IPU concentration 25 $\mu\text{g mL}^{-1}$, ^{14}C -IPU Standard A). Composition of the media was described in Table S1, **Supporting Information**. Approximately 80 sterilized carrier particles were then added to each of the incubators to allow the microbes to settle down and grow on the carriers. For each liquid culture, six replicates were prepared. The flasks were shaken at 75 rpm on a GFL 3005 orbital shaker (GFL, Burgwedel, Germany) at 20 °C in darkness. Three times per week they were connected to a trapping system and aerated. The flasks and trapping systems are as described by Kiesel (2014). Each incubator was aerated for 2 h with an air flow of 1.8 L h⁻¹. $^{14}\text{CO}_2$ evolved from mineralization of ^{14}C -IPU was trapped with 10 mL 0.1 M NaOH solution in the washing bottles. A 2-ml aliquot of the trapping solution was mixed with 3 mL Ultima-Flo™ AF and the radioactivity was measured in a liquid scintillation counter (Tri-Carb® 1900TR, PerkinElmer, Waltham, USA). When the liquid cultures showed a cumulative mineralization of > 40% and an IPU-mineralization rate of 4%–5% d⁻¹, the respective microbes were inoculated into soil. The microorganisms were inoculated with similar IPU-mineralizing activity to ensure the comparability of the inocula.

2.4. IPU application and inoculation

^{14}C -IPU standard B (240 μL) was applied via a 250- μL Hamilton® Microliter™ syringe (Hamilton Company, Bonaduz, Switzerland) to 3.5 g milled oven-dry (105 °C) soil aliquot in a small glass beaker and carefully mixed with the soil material until methanol evaporated. This soil aliquot was then mixed homogeneously with 46.5 g (dry weight, dw) equilibrated soil in a 250-mL amber bottle to achieve a nominal IPU concentration of 5 $\mu\text{g g}^{-1}$ (3.75 kBq g⁻¹). The actually measured IPU concentration in soil was 5.1–5.4 $\mu\text{g g}^{-1}$. The spiked soil was then inoculated with the single strain (*Sphingomonas* sp. strain AK1) or the microbial consortium from the above mentioned liquid culture, with or without carrier particles at different inoculation sizes. An overview of the experimental set-up is given in Table S2, **Supporting Information**. In the treatments with the liquid culture, the spiked soil was compacted to 1.3 g cm⁻³ by pressing the soil with an end-bended steel spatula to a defined line of the incubator which marked the volume corresponding to a soil density of 1.3 g cm⁻³. After compacting the soil, 0.5 mL and 1.5 mL, respectively, liquid culture of either strain AK1 or the microbial consortium was spread evenly on the soil surface using an Eppendorf pipette. In the treatments using carrier particles, the soil was mixed with 20 and 60 carrier particles, respectively, from the liquid culture of the isolated strain or the microbial consortium before being compacted to 1.3 g cm⁻³. IPU carry-over from the inocula to soil was negligible (results not presented). After inoculation, distilled water was added to soil giving a soil water potential of –15 kPa (i.e., 35.7% gravimetric water content). Soil subjected to the same treatment, but without inoculation of degraders served as control. All the experiments were performed with four replicates.

2.5. Soil incubation and measurement of ^{14}C -IPU mineralization

The soil was incubated using the incubation system depicted by Kiesel (2014). The soil was incubated at 20 ± 1 °C for 46 days. $^{14}\text{CO}_2$ evolved from mineralization of ^{14}C -IPU was absorbed by 10 mL 0.1 M NaOH in the plastic beaker inside the incubator; the NaOH was replaced with fresh NaOH three times per week. The incubators were weighed weekly and the lost water was added to the soil to maintain the soil water content at 35.7%. The evolved $^{14}\text{CO}_2$ was quantified by mixing 2 mL NaOH from the plastic beakers with

3 mL Ultima-Flo™ AF and measured by liquid scintillation counting. Degree of IPU mineralization was calculated as the amount of $^{14}\text{CO}_2$ in % of initially applied ^{14}C . The IPU-mineralization rate was calculated as $^{14}\text{CO}_2$ as $\mu\text{g IPU-mass equivalents d}^{-1} \text{ g dry soil}^{-1}$. The formula used to calculate the IPU-mineralization rate in soil is as below:

$$\text{MR}_{\text{IPU}} = \frac{\text{CM}_t - \text{CM}_{t-1}}{\text{R}_{\text{spec.IPU}} * t * m}$$

MR_{IPU}: mineralization rate of IPU ($\mu\text{g d}^{-1} \text{ g dry soil}^{-1}$)
 CM_t: cumulative IPU mineralization (kBq) as $^{14}\text{CO}_2$ at sampling time t (d)
 CM_{t-1}: cumulative IPU mineralization (kBq) as $^{14}\text{CO}_2$ at sampling time $t-1$ (d), the time point previous to time t
 R_{spec.IPU}: specific radioactivity of the ^{14}C -IPU standard
 t: time (d) between two adjacent sampling times ($t-1$ and t)
 m: dry mass of soil (g)

IPU-mineralization rate, rather than cell number of microorganisms in the inocula, was used to indicate the IPU-metabolic activity of the various inoculants. The main reasons are as below. (1) IPU-catabolic potential of the four types of inocula are not simply comparable based on microbial cell numbers. Cell numbers may be insufficient to represent the IPU-degrading capacity of the various inoculants, because the physiology and metabolism of microbial cells in a pure culture or within a consortium (approaches with the single strain or the consortium), as well as microbial cells in the form of suspended cells or biofilm (approaches using liquid culture or carrier particles) can vary dramatically (Stewart and Franklin, 2008; Mitri and Foster, 2013). (2) The IPU-mineralizing microbial consortium used in this study comprises various different microbial strains, making cell counting of all the microorganisms cumbersome. In this study ^{14}C was labeled on the aromatic ring of IPU, where the mineralized $^{14}\text{CO}_2$ originated. Transcriptomics of an IPU-ring-cleavage gene would be ideal to follow in soil the IPU-degrader activity related to the IPU-mineralizing dynamics, however, such genes have not been identified up to now.

2.6. Analysis of ^{14}C -extractable residues (^{14}C -ER)

After incubation for 46 days the soil was extracted using an ASE® 200 Accelerated Solvent Extractor (Thermo Fisher Scientific, Sunnyvale, USA) equipped with 33-mL extraction cells (Li et al., 2016). Briefly, 35 g wet soil was mixed with 1 g diatomaceous earth (Sigma-Aldrich, St. Louis, USA) and extracted with methanol at 90 °C, 10 MPa. Each soil was extracted thrice to ensure exhaustive extraction of ^{14}C -ER. Two aliquots (100 μL each) of crude extracts were taken, mixed with 5 mL Ultima Gold™ XR and measured in a liquid scintillation counter to determine the radioactivity of ^{14}C -ER. To prepare the samples for HPLC, the extracts were evaporated at 50 °C, 20 kPa using a rotary evaporator (Rotavapor R-114, BÜCHI, Flawil, Switzerland) to about 8 mL. The aliquot was then diluted with Milli-Q water to 250 mL and cleaned with solid phase extraction (SPE; Bond Elut ENV, Agilent Technologies, Santa Clara, USA) at a flow rate of $\approx 9 \text{ mL min}^{-1}$. The SPE columns were dried with nitrogen and stored at –20 °C. For further analysis the cartridges were eluted with 10 mL methanol. The radioactivity of the eluates was determined by measuring two aliquots (100 μL each) with 5 mL Ultima Gold™ XR in the liquid scintillation counter. The eluates were evaporated with a rotary evaporator (50 °C, 20 kPa) to dryness and re-dissolved in an appropriate volume of methanol to

achieve a concentration of 42 Bq μL^{-1} in the samples. Twenty μL of each sample was injected into a ^{14}C -HPLC system described by Schroll and Kühn (2004). The HPLC gradient is presented in Table S3, Supporting Information. Peaks in the HPLC chromatograms below the quantification limit of the ^{14}C -HPLC (20 Bq per 20 μL) were not integrated.

2.7. Analysis of ^{14}C -non-extractable residues (^{14}C -NER)

The extracted soil was milled intensively until it was homogeneous. Aliquots of approximately 300 mg soil were mixed with 10 drops of saturated sugar solution, dried at 70 °C for 5 h and combusted in a Model 307 Sample Oxidizer (PerkinElmer, Waltham, USA). $^{14}\text{CO}_2$ released from the combustion was absorbed by 8 mL Carbo-Sorb® E which was mixed with 12 mL Permafluor® E+ and the radioactivity was measured in a liquid scintillation counter. For each soil replicate, three aliquots were combusted. Radioactivity found in ^{14}C -CO₂, ^{14}C -ER and ^{14}C -NER were used to make the mass balance of initially applied ^{14}C .

2.8. IPU sorption and desorption

Sorption and desorption of IPU in the soil was investigated according to the OECD guideline (OECD, 2000) at a soil-solution ratio of 1:5 and an IPU concentration of 1 $\mu\text{g mL}^{-1}$ (^{14}C -IPU standard C) in parallel batch experiments. Details about the batch experiments were as described by Li et al. (2016).

2.9. Statistics

Statistical analysis (Duncan test, $p < 0.05$) was performed using IBM® SPSS® Statistics Version 20 (IBM Corporation, New York, USA) to determine the significance of differences. All figures used in this study were produced using OriginPro 2015 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. ^{14}C -IPU mineralization

The control soil showed a constantly low IPU mineralization rate during the incubation period of 46 days (Fig. 1a, b). This native IPU-mineralizing activity was considerably enhanced by inoculating either the *Sphingomonas* sp. strain AK1 (IS; Fig. 1a) or the microbial consortium (MC; Fig. 1b). Soils inoculated with 3-fold more liquid culture and “active” carrier particles showed 2.5–3.3 times higher initial IPU mineralization rates (R_i) than the corresponding lower inoculation treatments (i.e., IS-LM 0.5 vs IS-LM 1.5; IS-CP 20 vs IS-CP 60; MC-LM 0.5 vs MC-LM 1.5 and MC-CP 20 vs MC-CP 60, Table 1). The single strain and the community inoculated via the same number of carriers showed similar R_i in the soil (IS-CP 20 vs MC-CP 20 and IS-CP 60 vs MC-CP 60, Table 1). IPU-mineralization rates were maximal on the first day in soil treated with IS and decreased continuously until the end of the incubation (Fig. 1a). In contrast, R_i of soil amended with the community increased and reached their maxima (R_m , 0.32–0.62 $\mu\text{g d}^{-1} \text{g dry soil}^{-1}$) within 4–8 days (Fig. 1b; R_m for each treatment is shown in Table 1). Afterwards IPU-mineralization rates in these soils started to decrease (Fig. 1b). Soils inoculated with 0.5 and 1.5 mL liquid culture of the microbial consortium showed R_i which were over 10-fold lower than the isolated-strain-counterparts, but attained a 3–6 times higher R_m (MC-LM 0.5 vs IS-LM 0.5, MC-LM 1.5 vs IS-LM 1.5, Table 1). From the 32nd day onwards the IPU mineralization rate was 0.01 $\mu\text{g d}^{-1} \text{g dry soil}^{-1}$ in all treatments (Fig. 1a, b). During 46 days, a high cumulative IPU mineralization (58.9%–67.5%) was measured in soils

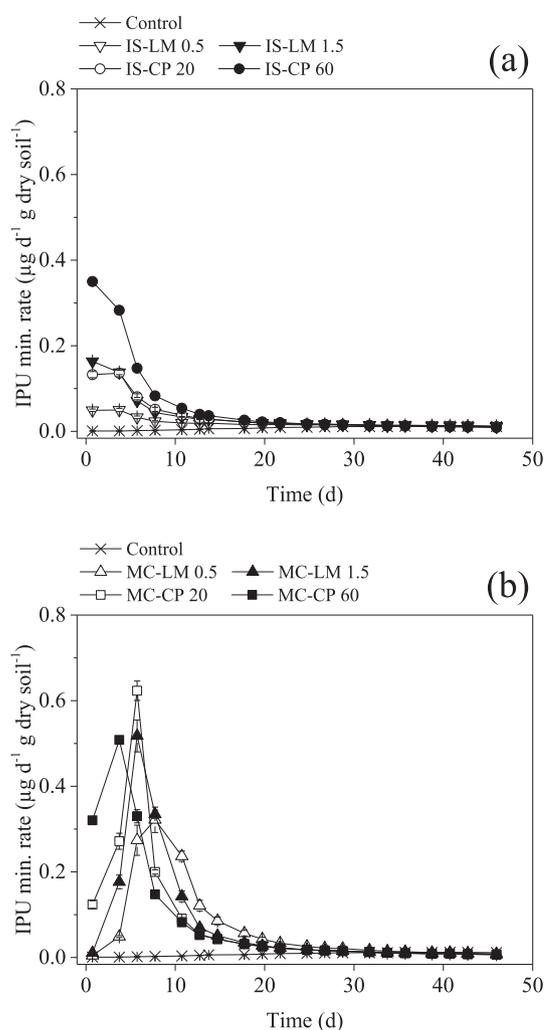


Fig. 1. IPU mineralization rates in soils inoculated with *Sphingomonas* sp. strain AK1 (a) or the microbial consortium (b). Control: uninoculated soil; IS-LM 0.5 and IS-LM 1.5: soil inoculated with the isolated strain (IS, *Sphingomonas* sp. strain AK1) via 0.5 mL and 1.5 mL liquid medium (LM), respectively; IS-CP 20 and IS-CP 60: soil inoculated with the isolated strain (IS) via 20 and 60 carrier particles (CP) as carrier material, respectively; MC-LM 0.5 and MC-LM 1.5: soil inoculated with the microbial consortium (MC) via 0.5 mL and 1.5 mL liquid medium (LM), respectively; MC-CP 20 and MC-CP 60: soil inoculated with the microbial consortium (MC) via 20 and 60 carrier particles (CP), respectively. Error bars indicate standard deviations of four replicates.

treated with MC, regardless of the huge variation in the initial IPU mineralization rates (Table 1 and Fig. S1, Supporting Information). On the contrary, cumulative IPU mineralization of soil treated with IS was all lower and varied considerably (Table 1).

3.2. Quantity of ^{14}C -extractable residues and ^{14}C -non-extractable residues

As shown in Fig. 2, ^{14}C -ER amounted to half of applied radioactivity in the control after 46 days of incubation. In soils treated with IS the portion of ^{14}C -ER was significantly lower than in the control ($p < 0.05$) and ranged from 15.9% to 38.9%, depending on the type and amount of inocula. Inoculation with 60 “active” carrier particles resulted in the smallest amount of ^{14}C -ER in IS-treated soils. The amount of ^{14}C -ER in MC-amended soil were much less than in IS-amended soil ($p < 0.05$), and variations between the inoculation treatments were small (3.0%–4.0%). ^{14}C -NER represented about 40% of applied radioactivity in the control and in soil treated with IS. In soils inoculated with

Table 1

IPU mineralization parameters of inoculated soils. Abbreviations of soil treatments are as described in Fig. 1. Figures after \pm indicate standard deviation of four replicates. Letters after the figures denote the significance of differences between the soil treatments (Duncan test, $p < 0.05$).

Soil treatments	IPU mineralization parameters					
	R_i ($\mu\text{g d}^{-1} \text{g dry soil}^{-1}$)	R_m ($\mu\text{g d}^{-1} \text{g dry soil}^{-1}$)	T_m (d)	M_m (% of applied ^{14}C)	R_f ($\mu\text{g d}^{-1} \text{g dry soil}^{-1}$)	M_f (% of applied ^{14}C)
IS-LM 0.5	0.049 \pm 0.003 e	0.049 \pm 0.003 f	0.7	0.7 \pm 0.04 f	0.012 \pm 0.0005	17.4 \pm 0.60 g
IS-LM 1.5	0.164 \pm 0.005 c	0.164 \pm 0.005 d	0.7	2.3 \pm 0.004 f	0.011 \pm 0.0001	28.7 \pm 0.60 f
IS-CP 20	0.133 \pm 0.007 d	0.133 \pm 0.007 e	0.7	1.9 \pm 0.10 f	0.011 \pm 0.0001	28.7 \pm 1.53 f
IS-CP 60	0.350 \pm 0.008 a	0.350 \pm 0.008 c	0.7	5.0 \pm 0.12 e	0.009 \pm 0.0001	46.0 \pm 0.59 e
MC-LM 0.5	0.004 \pm 0.0005 f	0.322 \pm 0.030 c	7.7	25.0 \pm 2.41 d	0.009 \pm 0.0004	58.9 \pm 0.38 d
MC-LM 1.5	0.011 \pm 0.002 f	0.518 \pm 0.037 b	5.7	29.3 \pm 2.09 c	0.007 \pm 0.0004	63.4 \pm 0.64 c
MC-CP 20	0.124 \pm 0.009 d	0.623 \pm 0.023 a	5.7	40.1 \pm 0.33 a	0.007 \pm 0.0001	64.8 \pm 0.36 b
MC-CP 60	0.320 \pm 0.008 b	0.508 \pm 0.008 b	3.7	32.8 \pm 0.54 b	0.007 \pm 0.0002	67.5 \pm 0.64 a

R_i : initial IPU mineralization rate. R_m : maximal IPU mineralization rate. T_m : time required to achieve the maximal IPU mineralization rate. M_m : degree of IPU mineralization when the maximal mineralization rate was achieved. R_f : final IPU mineralization rate when incubation was terminated on day 46. M_f : final cumulative IPU mineralization when incubation was terminated on day 46.

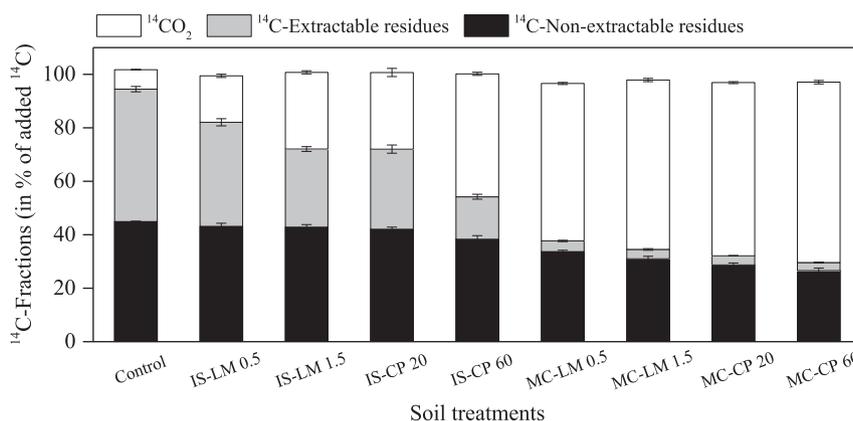


Fig. 2. Distribution of applied ^{14}C in soils after 46 days of incubation. Error bars indicate standard deviations of four replicates. Abbreviations are as described in Fig. 1.

MC, ^{14}C -NER fraction was slightly reduced and ranged from 26.6% to 33.7% with the minimum in the treatment with 60 “active” carrier particles. Generally, the amount of ^{14}C -ER and ^{14}C -NER declined with increasing IPU mineralization. The quantity of ^{14}C -ER was found to be linearly negatively correlated with the cumulative IPU mineralization in the soil ($R^2 = 0.996$; Fig. S2, Supporting Information). ^{14}C -recovery in the soils ranged from 96.6% to 101.8%.

3.3. Concentration and composition of IPU residues in soil

The concentration and relative proportion of IPU residues are shown in Table 2 and Table 3, respectively. With the increase of IPU

mineralization, the concentrations of IPU and IPU-metabolites in soil decreased, while the relative proportion of the parent compound (IPU) in ^{14}C -ER increased. IPU concentrations in soil inoculated with MC were 8–10 times lower than in the control and 3–9-fold lower than in soil inoculated with IS. MDIPU was the major IPU metabolite in all soils, which represented about 30% of ^{14}C -ER in the control and soil treated with IS, and approximately 10% ^{14}C -ER in soil treated with MC.

3.4. IPU sorption and desorption

As shown in Fig. 3, 30% IPU was adsorbed to the soil when the equilibrium was achieved after 3 days ($K_d = 2.1 \text{ mL g}^{-1}$), and 70% of

Table 2

Concentrations of IPU residues in soils at the end of incubation (day 46) as measured by ^{14}C -HPLC. Retention times (min) of the corresponding residue substances are presented below its name. Abbreviations of soil treatments are as described in Fig. 1. Figures after \pm indicate standard deviation of four replicates. Letters after the figures denote the significance of differences between the soil treatments (Duncan test, $p < 0.05$).

Soil treatments	Concentration of IPU residues in soil ($\mu\text{g IPU-mass equivalents/g dry soil}$)					
	2-OH-MDIPU	Unknown 1	Unknown 2	MDIPU	IPU	Unknown 3
	11.98 \pm 0.08	13.01 \pm 0.09	13.99 \pm 0.09	18.76 \pm 0.07	20.01 \pm 0.07	22.08 \pm 0.06
Control	0.11 \pm 0.02 a	0.29 \pm 0.03 a	0.06 \pm 0.01 a	0.69 \pm 0.04 a	0.80 \pm 0.01 a	*
IS-LM 0.5	0.08 \pm 0.01 b	0.23 \pm 0.02 b	0.05 \pm 0.01 b	0.47 \pm 0.02 b	0.67 \pm 0.05 b	*
IS-LM 1.5	0.06 \pm 0.01 c	0.16 \pm 0.03 c	0.04 \pm 0.01 c	0.35 \pm 0.06 c	0.50 \pm 0.08 d	*
IS-CP 20	0.05 \pm 0.01 d	0.18 \pm 0.02 c	0.04 \pm 0.005 c	0.32 \pm 0.03 c	0.58 \pm 0.05 c	*
IS-CP 60	0.04 \pm 0.004 e	0.08 \pm 0.01 d	0.02 \pm 0.003 d	0.17 \pm 0.01 d	0.30 \pm 0.02 e	*
MC-LM 0.5	*	0.015 \pm 0.002 e	0.005 \pm 0.001 e	0.018 \pm 0.002 e	0.095 \pm 0.004 f	0.004 \pm 0.0003 a
MC-LM 1.5	*	0.009 \pm 0.001 e	0.004 \pm 0.001 e	0.010 \pm 0.002 e	0.086 \pm 0.010 f	0.003 \pm 0.001 b
MC-CP 20	*	0.007 \pm 0.001 e	0.004 \pm 0.0004 e	0.011 \pm 0.001 e	0.081 \pm 0.003 f	0.003 \pm 0.001 b
MC-CP 60	*	0.005 \pm 0.001 e	0.003 \pm 0.0004 e	0.009 \pm 0.001 e	0.076 \pm 0.005 f	0.002 \pm 0.0003 b

*: Below quantification limit (20 Bq per 20 μL).

Table 3
Portions of IPU residues in ^{14}C -extractable residues of soils at the end of incubation (day 46) as measured by ^{14}C -HPLC. Retention times (min) of the corresponding residue substances are presented below its name. Abbreviations of soil treatments are as described in Fig. 1. Figures after \pm indicate standard deviation of four replicates. Letters after the figures denote the significance of differences between the soil treatments (Duncan test, $p < 0.05$).

Soil treatments	Portion of IPU residues (in % of ^{14}C -extractable residues)					
	2-OH-MDIPU	Unknown 1	Unknown 2	MDIPU	IPU	Unknown 3
	11.98 \pm 0.08	13.01 \pm 0.09	13.99 \pm 0.09	18.76 \pm 0.07	20.01 \pm 0.07	22.08 \pm 0.06
Control	5.5 \pm 0.7 b	15.1 \pm 1.3 a	3.1 \pm 0.5 ab	35.4 \pm 1.4 a	40.9 \pm 0.5 f	*
IS-LM 0.5	5.5 \pm 0.6 b	15.5 \pm 0.7 a	3.5 \pm 0.6 ab	31.0 \pm 2.0 b	44.6 \pm 2.4 e	*
IS-LM 1.5	5.8 \pm 1.2 ab	14.6 \pm 0.8 a	3.7 \pm 0.5 ab	31.1 \pm 1.9 b	44.9 \pm 2.3 e	*
IS-CP 20	4.5 \pm 0.8 c	15.2 \pm 1.1 a	3.0 \pm 0.4 b	27.7 \pm 2.6 c	49.6 \pm 2.5 d	*
IS-CP 60	6.6 \pm 0.9 a	12.6 \pm 0.5 b	3.5 \pm 0.6 ab	28.1 \pm 0.5 c	49.2 \pm 1.8 d	*
MC-LM 0.5	*	10.8 \pm 0.8 c	4.0 \pm 0.7 a	13.4 \pm 1.0 d	69.2 \pm 1.8 c	2.7 \pm 0.2 a
MC-LM 1.5	*	7.6 \pm 0.4 d	3.4 \pm 0.7 ab	9.3 \pm 0.8 e	77.2 \pm 0.8 ab	2.5 \pm 0.3 a
MC-CP 20	*	7.1 \pm 1.0 d	3.5 \pm 0.4 ab	10.2 \pm 0.6 e	76.5 \pm 1.9 b	2.8 \pm 0.7 a
MC-CP 60	*	5.6 \pm 1.0 e	3.2 \pm 0.3 ab	9.0 \pm 1.2 e	79.6 \pm 1.0 a	2.6 \pm 0.3 a

*: Below quantification limit (20 Bq per 20 μL).

the adsorbed IPU was allowed to be desorbed during 6 days.

4. Discussion

4.1. IPU mineralization

Inoculation of the isolated strain (*Sphingomonas* sp. strain AK1, **IS**) or the microbial consortium (**MC**) enhanced IPU degradation in soil. In general IPU mineralization in soil varied according to the

type of inoculants and inoculation size (as determined by the volume of liquid cultures or by the number of carrier particles). These findings are in agreement with a previous study on bio-augmentation of 1,2,4-TCB in soil using the various inoculating approaches (Wang et al., 2010). However, in this research IPU mineralization was less affected by the inoculant density in soil inoculated with MC than in soil inoculated with IS, and inoculation of MC resulted in more thorough IPU removal in soil. For instance, soil inoculated with MC on carrier particles (i.e., MC-CP 20 and MC-CP 60) mineralized 36.1% and 21.5% more IPU than soil inoculated with IS on 20 and 60 carriers, respectively. Nutrients supply to the IPU degrader *Sphingomonas* sp. strain AK1 from other microbes in MC such as amino acids (Kiesel, 2014), and possible existence of other IPU degraders in MC might have contributed to the greater mineralization of IPU in MC-amended soils. The lower initial IPU mineralization rate in soil inoculated with free cells (i.e., IS-LM and MC-LM treatments) in comparison to soil inoculated with the microbes on carriers (i.e., IS-CP and MC-CP treatments) might be because of the smaller amount of degrader cells in the liquid inoculants. However, despite the lowest initial IPU mineralization rates in soils inoculated with MC via liquid culture (MC-LM 0.5 and MC-LM 1.5), the rates increased and IPU was mineralized exhaustively in these MC-treated soils, resulting in substantial cumulative IPU mineralization and marginal amounts of ^{14}C -ER. These results suggest that MC is more effective than IS to decontaminate IPU in soil. Soil treated with IS-CP 20 showed significantly ($p < 0.05$) lower initial IPU mineralization rate than soil treated with IS-LM 1.5, but in the end IS-CP 20 mineralized as much IPU as IS-LM 1.5 did and resulted in the same amount of ^{14}C -ER and ^{14}C -NER as in the soil amended with IS-LM 1.5. The enhanced performance of IS-CP 20 is likely attributed to the effect of carrier material (e.g., offering protective sites for the inoculated IS cells in the soil and allowing formation of IS-biofilm).

The IPU mineralization rate in soil is determined by both IPU availability and the quantity and activity of the degrader cells. IPU has generally a low sorption capacity in soil that is controlled by several soil characteristics (Li et al., 2016). The low sorption and high desorption of IPU in soil showed that IPU availability is not a limiting factor, thus, the decrease of IPU mineralization rates in IS-inoculated soils is due to decay or reduced activity of degrader cells rather than restricted IPU availability. The increasing IPU mineralization rate in MC-treated soils is a result of the proliferation of the IPU degraders in the microbial consortium. It is shown in Fig. S3, Supporting Information that the IPU-mineralizing potential of MC could remain stable in the soil for at least 189 d, suggesting a long persistence of the introduced MC degraders in the soil. Therefore,

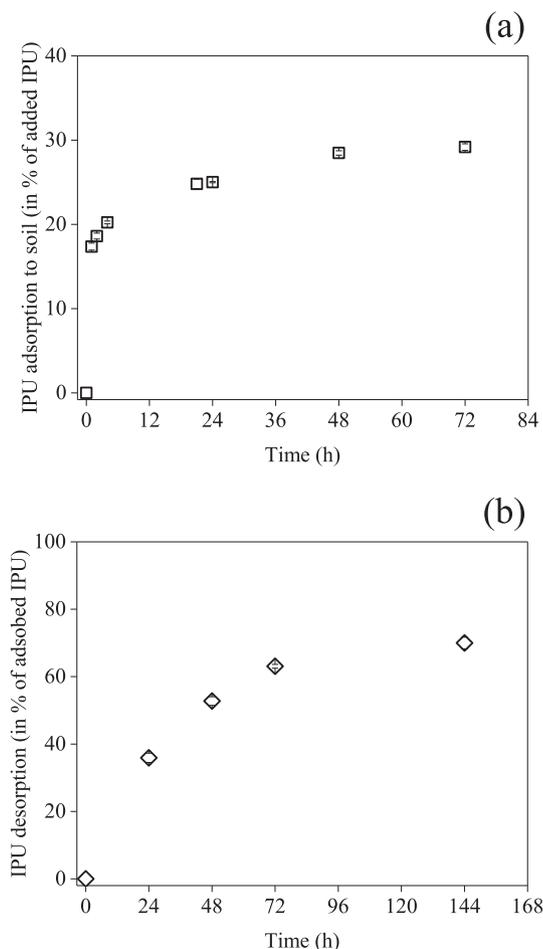


Fig. 3. IPU sorption (a) and desorption (b) in soil as measured by OECD batch experiment. Error bars indicate standard deviation of three replicates.

loss of degrader cells in the soil could unlikely explain the reduced IPU mineralization rates (after the maximal rates) in soils inoculated with MC. On the other hand, when IPU remaining in soil was not sufficient (due to mineralization by MC) to sustain the increasing mineralization rates, the rates started to decrease. Quantification of genes responsible for the IPU-ring C-respiration (mineralization) may enable to trace the proliferation and activity of inoculated IPU degraders in soil (e.g., by qPCR and transcriptomics analysis). Such data could improve interpretation of IPU degradation dynamics in soil from a microbiological aspect.

4.2. IPU residues in soil

Concentrations of IPU residues remaining in soil indicate the thoroughness of IPU dissipation by the various processes. Degradation products of xenobiotics are of environmental concern because metabolites are frequently found more toxic than their parent compounds (Boxall et al., 2004; Escher and Fenner, 2011). For instance, toxicity of the IPU-metabolite, 4-IA, is 600 times higher than IPU as determined by Microtox[®] (Tixier et al., 2002). However, this metabolite was not detected in the control and inoculated soils. The high relative portion and low concentration of IPU in soil inoculated with MC suggested that IPU is degraded exhaustively without accumulation of dead-end products. The trace amount of IPU in these soils might be the portion that was bound to soil particles and/or entrapped in soil matrix, being inaccessible for the degraders but extractable under harsh conditions (e.g., ASE[®]). MDIPU was the major IPU metabolite in all soils and was detected in the control at the highest concentration suggesting that initial N-demethylation of IPU is not a rate-limiting step of IPU degradation by the indigenous soil microbiome.

Non-extractable residues refer to parent molecules and/or metabolites of a chemical in soil, which are unextractable by methods that do not change the properties of the residues (IUPAC definition, Roberts, 1984). These residues can be regarded as a “chemical time-bomb” in soil or environmentally innocuous depending on the quality of the residues and likelihood of their release (Barraclough et al., 2005). Substantial progress has been achieved recently in the nature of non-extractable residues of xenobiotics in soil (Kästner et al., 2014). By using stable isotope labeling, Nowak et al. (2010, 2013) revealed that NER formed in soil by metabolic degradation of a herbicide 2,4-D and a pharmaceutical ibuprofen almost solely comprised biomolecules (e.g., amino acids, phospholipid fatty acids) derived from cell residues of the pollutant-degrading microorganisms. This type of NER that are composed of necromass of degrader cells formed via anabolism of xenobiotic molecules, are defined as bioNER to differentiate from “real” NER as defined by IUPAC (Kästner et al., 2014). Formation of the environmentally irrelevant bioNER can be considered as another desirable fate of xenobiotics in soil in addition to mineralization to CO₂. Poßberg et al. (2016) reported the formation of bioNER in soil with low degradative potential of the organic pollutant, where bioNER constituted only a certain portion of total NER in soil. In this study, the composition of ¹⁴C-NER was not analyzed. However, based on the previous findings and theories, ¹⁴C-NER in soil treated with MC, where IPU was mineralized intensively and utilized as C source by the degrader microorganisms, probably consist of cell residues of IPU degraders. By transforming IPU to biomolecules the risks of IPU is eliminated. In soils with low IPU degradation capacity such as the control and some IS-inoculated soils, IPU and/or its degradation products bound to soil material may account for the major part of NER.

5. Conclusion

Although there may be major differences in the number of active degrading organisms in the MC- and IS-inoculants, the community serves better the nutritional requirements in soil, which is shown by the increase of the mineralization rates (which finally means growth) after a short lag time; whereas the isolated strain inoculation shows an immediate loss of mineralization activity. The microbial consortium is more effective than the single strain to degrade IPU and can maintain the degradative potential in soil over a long period. The carrier particles exert positive effect on IPU degradation by the IS-inoculants. Influence of inoculation size on IPU degradation by the single strain is more notable than by the community. This study suggests that enriched natural microbial communities can be an efficient and cost-effective strategy for solving the pressing soil pollution problems posed by organic chemicals. Employing a microbial community as a whole, rather than using an isolated strain, may offer a more advantageous approach for microbial inoculation in various application fields, such as bioaugmentation, inoculation of beneficial microbes to promote plant growth, or biocontrol of phytopathogen in soil.

Acknowledgements

This work is part of the PhD project of R. Li, which was funded by German Academic Exchange Service (DAAD; Grant No. 57076385). Helmholtz Zentrum München offered the facilities and consumables for conducting the experiments.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2016.10.084>.

References

- Alletto, L., Coquet, Y., Benoit, P., Bergheud, V., 2006. Effects of temperature and water content on degradation of isoproturon in three soil profiles. *Chemosphere* 64, 1053–1061.
- Barraclough, D., Kearney, T., Croxford, A., 2005. Bound residues: environmental solution or future problem? *Environ. Pollut.* 133, 85–90.
- Bending, G.D., Lincoln, S.D., Sørensen, S.R., Morgan, J.A.W., Amand, J., Walker, A., 2003. In-field spatial variability in the degradation of the phenyl-urea herbicide isoproturon is the result of interactions between degradative *Sphingomonas* spp. and soil pH. *Appl. Environ. Microbiol.* 69, 827–834.
- Boxall, A.B.A., Sinclair, C.J., Fenner, K., Kolpin, D., Maund, S.J., 2004. Peer reviewed: when synthetic chemicals degrade in the environment. *Environ. Sci. Technol.* 38, 368A–375A.
- Commission Implementing Regulation (EU) No 2016/872 of 1 June 2016 concerning the non-renewal of approval of the active substance isoproturon, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending Commission Implementing Regulation (EU) No 540/2011. *Off. J. Eur. Union L* 145, 2.6.2016, 7–9.
- Dejonghe, W., Boon, N., Seghers, D., Top, E.M., Verstraete, W., 2001. Bioaugmentation of soils by increasing microbial richness: missing links. *Environ. Microbiol.* 3, 649–657.
- EFSA (European Food Safety Authority), 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance isoproturon. *EFSA J.* 13 (8), 99. <http://dx.doi.org/10.2903/j.efsa.2015.4206>, 4206.
- Eibisch, N., Schroll, R., Fuß, R., 2015. Effect of pyrochar and hydrochar amendments on the mineralization of the herbicide isoproturon in an agricultural soil. *Chemosphere* 134, 528–535.
- Escher, B.I., Fenner, K., 2011. Recent advances in environmental risk assessment of transformation products. *Environ. Sci. Technol.* 45, 3835–3847.
- Fenner, K., Canonica, S., Wackett, L.P., Elsner, M., 2013. Evaluating pesticide degradation in the environment: blind spots and emerging opportunities. *Science* 341, 752–758.
- Folberth, C., Scherb, H., Suhadolc, M., Munch, J.C., Schroll, R., 2009. In situ mass distribution quotient (IMDQ) – a new factor to compare bioavailability of chemicals in soils? *Chemosphere* 75, 707–713.
- Gentry, T., Rensing, C., Pepper, I.A.N., 2004. New approaches for bioaugmentation as a remediation technology. *Crit. Rev. Environ. Sci. Technol.* 34, 447–494.
- Grundmann, S., Fuß, R., Schmid, M., Laschinger, M., Ruth, B., Schulin, R., Munch, J.C.,

- Schroll, R., 2007. Application of microbial hot spots enhances pesticide degradation in soils. *Chemosphere* 68, 511–517.
- Hussain, S., Arshad, M., Springael, D., Sørensen, S.R., Bending, G.D., Devers-Lamrani, M., Maqbool, Z., Martin-Laurent, F., 2015. Abiotic and biotic processes governing the fate of phenylurea herbicides in soils: a review. *Crit. Rev. Environ. Sci. Technol.* 45, 1947–1998.
- Kästner, M., Nowak, K.M., Miltner, A., Trapp, S., Schäffer, A., 2014. Classification and modelling of nonextractable residue (NER) formation of xenobiotics in soil - a synthesis. *Crit. Rev. Environ. Sci. Technol.* 44 (19), 2107–2171.
- Kiesel, C.A., 2014. Enhanced Degradation of Isoproturon in Soils: Sustainability of Inoculated, Microbial Herbicide Degraders, and Adaptation of Native Microbes. Ph.D. Dissertation. Technische Universität München, Munich, Germany. <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:bvb:91-diss-20141120-1188688-0-1>.
- Li, R., Dörfler, U., Schroll, R., Munch, J.C., 2016. Biodegradation of isoproturon in agricultural soils with contrasting pH by exogenous soil microbial communities. *Soil Biol. Biochem.* 103, 149–159.
- Mitri, S., Foster, K.R., 2013. The genotypic view of social interactions in microbial communities. *Annu. Rev. Genet.* 47, 247–273.
- Nowak, K.M., Girardi, C., Miltner, A., Gehre, M., Schäffer, A., Kästner, M., 2013. Contribution of microorganisms to non-extractable residue formation during biodegradation of ibuprofen in soil. *Sci. Total Environ.* 445–446, 377–384.
- Nowak, K.M., Miltner, A., Gehre, M., Schäffer, A., Kästner, M., 2010. Formation and fate of bound residues from microbial biomass during 2,4-D degradation in soil. *Environ. Sci. Technol.* 45, 999–1006.
- OECD, 2000. Adsorption - Desorption Using a Batch Equilibrium Method 106. Guidelines for the Testing of Chemicals. Organisation of Economic Co-operation and Development, Paris, pp. 1–45.
- Owsianiak, M., Dechesne, A., Binning, P.J., Chambon, J.C., Sørensen, S.R., Smets, B.F., 2010. Evaluation of bioaugmentation with entrapped degrading cells as a soil remediation technology. *Environ. Sci. Technol.* 44, 7622–7627.
- Poßberg, C., Schmidt, B., Nowak, K., Telscher, M., Lajojda, A., Schaeffer, A., 2016. Quantitative identification of biogenic nonextractable pesticide residues in soil by 14C-Analysis. *Environ. Sci. Technol.* 50 (12), 6415–6422.
- Roberts, T.R., 1984. Non-extractable residues in soils and plants. *Pure Appl. Chem.* 56 (7), 945–956.
- Schroll, R., Becher, H.H., Dörfler, U., Gayler, S., Grundmann, S., Hartmann, H.P., Ruoss, J., 2006. Quantifying the effect of soil moisture on the aerobic microbial mineralization of selected pesticides in different soils. *Environ. Sci. Technol.* 40, 3305–3312.
- Schroll, R., Kühn, S., 2004. Test system to establish mass balances for 14C-labeled substances in Soil–Plant–Atmosphere systems under field conditions. *Environ. Sci. Technol.* 38, 1537–1544.
- Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* 6, 199–210.
- Sun, D., Hale, L., Crowley, D., 2016. Nutrient supplementation of pinewood biochar for use as a bacterial inoculum carrier. *Biol. Fertil. Soils* 1–8.
- Tixier, C., Sancelme, M., Aït-Aïssa, S., Widehem, P., Bonnemoy, F., Cuer, A., Truffaut, N., Veschambre, H., 2002. Biotransformation of phenylurea herbicides by a soil bacterial strain, *Arthrobacter* sp. N2: structure, ecotoxicity and fate of diuron metabolite with soil fungi. *Chemosphere* 46, 519–526.
- van Veen, J.A., van Overbeek, L.S., van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. *Microbiol. Mol. Biol. Rev.* 61, 121–135.
- Vieublé Gonod, L., El Arfaoui, A., Benoit, P., 2016. Impact of spatial distribution of exogenous organic matter on C mineralization and isoproturon fate in soil. *Soil Biol. Biochem.* 95, 180–188.
- Wang, F., Dörfler, U., Schmid, M., Fischer, D., Kinzel, L., Scherb, H., Munch, J.C., Jiang, X., Schroll, R., 2010. Homogeneous inoculation vs. microbial hot spots of isolated strain and microbial community: what is the most promising approach in remediating 1,2,4-TCB contaminated soils? *Soil Biol. Biochem.* 42, 331–336.
- Wang, F., Fekete, A., Harir, M., Chen, X., Dörfler, U., Rothballer, M., Jiang, X., Schmitt-Kopplin, P., Schroll, R., 2013. Soil remediation with a microbial community established on a carrier: strong hints for microbial communication during 1,2,4-Trichlorobenzene degradation. *Chemosphere* 92, 1403–1409.

Supplementary information of Publication I

Table S1 Composition of the mineral salt media used to cultivate *Sphingomonas* sp. strain AK1 and the IPU-mineralizing microbial consortium (Kiesel 2014).

Ingredients	Concentration (per L)
KH ₂ PO ₄	1.36 g
Na ₂ HPO ₄ * 2 H ₂ O	1.78 g
MgSO ₄ * 7 H ₂ O	0.05 g
CaCl ₂	0.01 g
H ₃ BO ₃	2.86 mg
MnSO ₄ * H ₂ O	1.54 mg
CuSO ₄ * 5 H ₂ O	0.04 mg
ZnCl ₂	0.021 mg
CoCl ₂ * 6 H ₂ O	0.041 mg
Na ₂ MoO ₄ * 2 H ₂ O	0.025 mg
FeCl ₃ * 6H ₂ O	0.00514 mg
Casamino acids	0.1 g
Isoproturon	25 mg
pH	7.2

Table S2 Experimental design of the soil incubation experiment. Weight of 20 carrier particles is ≈ 0.5 g. Abbreviations are as described in Fig. 1 of the manuscript.

Soil treatments	Inoculation size	Type of inoculation	Inoculation ratio carrier to soil (w/w)
IS-LM 0.5	0.5 mL	liquid culture of IS	–
IS-LM 1.5	1.5 mL	liquid culture of IS	–
IS-CP 20	0.5 g	carrier particles with IS	1%
IS-CP 60	1.5 g	carrier particles with IS	3%
MC-LM 0.5	0.5 mL	liquid culture of MC	–
MC-LM 1.5	1.5 mL	liquid culture of MC	–
MC-CP 20	0.5 g	carrier particles with MC	1%
MC-CP 60	1.5 g	carrier particles with MC	3%
Control	no inoculation	–	–

Table S3 HPLC gradient program for detecting IPU and IPU-metabolites.

Time (min)	Mobile phases	
	Acetonitrile (%)	10 mM NH ₄ CH ₃ CO ₂ (%)
0	5	95
15	60	40
20	60	40
25	5	95
35	5	95

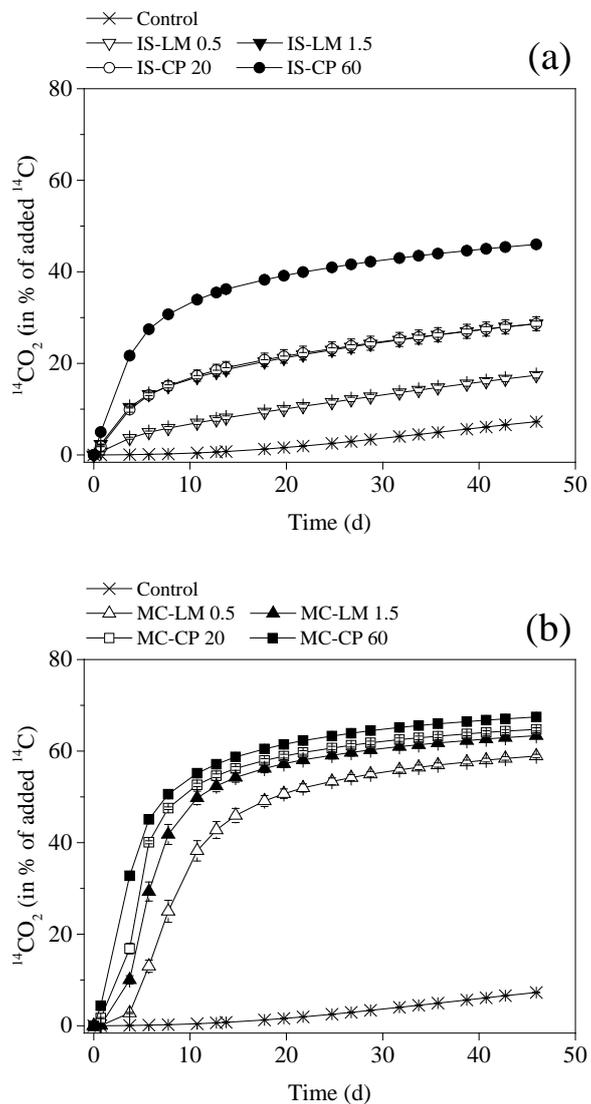


Fig. S1 Cumulative mineralization of ^{14}C -IPU in soils inoculated with *Sphingomonas* sp. strain AK1 (a) or the microbial consortium (b). Abbreviations are as described in Fig. 1 of the manuscript. Error bars indicate standard deviation of four replicates.

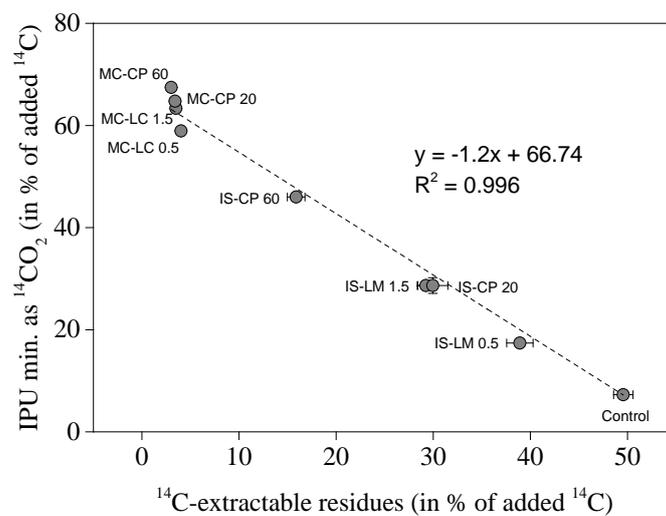


Fig. S2 Correlation between ^{14}C -ER and IPU mineralization in soils. Abbreviations of soil treatments are as described in Fig. 1 in the manuscript. Error bars indicate standard deviation of 4 replicates.

In another experiment, 2 “active” carrier particles with MC were inoculated into 50 g (dry weight) soil and incubated for 189 days as described in “Materials and Methods” in the manuscript. The soil was applied with ^{14}C -IPU standard ($723 \text{ Bq } \mu\text{g}^{-1}$) on day 0 and re-applied with ^{14}C -IPU standard ($677 \text{ Bq } \mu\text{g}^{-1}$) on day 133. Four replicates were performed for this experiment. The IPU mineralization rate after the first and second application was calculated according to the formula in “Materials and Methods”, based on the specific radioactivity of the ^{14}C -IPU standard applied, respectively.

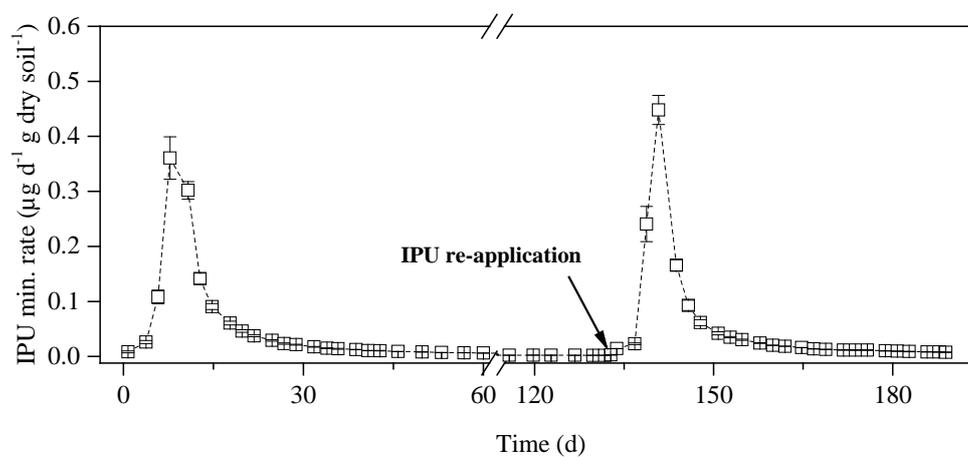
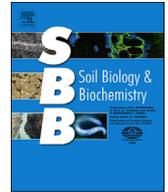


Fig. S3 IPU mineralization rate in soil inoculated with MC via two carrier particles during 189 days. Error bars indicate standard deviation of 4 replicates.

Publication II: Biodegradation of isoproturon in agricultural soils with contrasting pH by exogenous soil microbial communities

Li et al., 2016. *Soil Biology and Biochemistry* 103, 149–159.



Biodegradation of isoproturon in agricultural soils with contrasting pH by exogenous soil microbial communities



Renyi Li ^{a, *}, Ulrike Dörfler ^a, Reiner Schroll ^a, Jean Charles Munch ^b

^a Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany

^b Lehrstuhl für Grünlandlehre, Technische Universität München, 85354 Freising, Germany

ARTICLE INFO

Article history:

Received 19 April 2016

Received in revised form

17 August 2016

Accepted 18 August 2016

Keywords:

Isoproturon-mineralizing microbial communities

Carrier material

Mineralization

Metabolites

Non-extractable residues

pH tolerance

ABSTRACT

Soil pH is a limiting factor for biodegradation of isoproturon (IPU) in the field. IPU dissipation is hampered in soil with low pH. Efficacy of two IPU-mineralizing microbial communities in IPU dissipation was investigated during 189 d in microcosms of three agricultural soils with contrasting pH (Marsdorf, pH 3.8; Neumarkt, pH 5.8 and Dürneck, pH 7.3). The microbial communities enriched from an acidic soil (MC-AS) and a neutral soil (MC-NS), respectively, were established on carrier material, namely expanded clay particles, and introduced to the tested soils at a carrier-soil ratio of 1%. IPU was applied to the soil twice, on day 0 and day 133. The effect of inoculation size, sorption–desorption and pH on biodegradation of IPU were studied. IPU mineralization, extractable residues and non-extractable residues were analyzed with uniformly ring-labeled [¹⁴C]-isoproturon. Both microbial communities resulted in significantly enhanced IPU mineralization (52%–60%) and low concentrations of IPU residues in soil Dürneck (pH 7.3). The acquired IPU-mineralizing activity was persistent in the soil for at least 133 d. Initially a 9-fold higher IPU mineralization rate was attained in soil Neumarkt (pH 5.8) by inoculating MC-AS. However, no difference between treated and untreated soils was detected after IPU re-application. Both communities had negligible effect on the fate of IPU in soil Marsdorf (pH 3.8), where biodegradation of IPU was inhibited, with ≈2% IPU mineralized over 189 d. Lowering the carrier-soil ratio to 0.1% sharply reduced the IPU-mineralizing capacity of MC-AS in soil Neumarkt, however, this effect of inoculation size was less pronounced for both microbial communities in soil Dürneck. The low K_d of IPU (1.3–2.0 mL g⁻¹) indicate that IPU bioavailability is not a limiting factor of IPU degradation in the soils. Relationships between pH and maximal IPU mineralization rendered by MC-AS or MC-NS in soil closely approximate those observed in mineral salts medium, suggesting that pH is an important factor influencing biodegradation of IPU by the exogenous microbial communities. We propose that MC-AS, which has a broad pH tolerance for IPU degradation, is a promising candidate for accelerating IPU dissipation in acidic soils. The effect of inoculant density on IPU degradation is microbial community-soil specific. Using degrader microorganisms according to their physiological requirements and properties of the targeted soils may maximize the effectiveness of IPU dissipation.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Isoproturon (IPU, 3-(4-isopropylphenyl)-1,1-dimethylurea) is a worldwide extensively used phenylurea herbicide for pre- and post-emergence control of annual grass and broadleaved weeds in cereal crops (Hussain et al., 2015). The active substance is frequently detected in surface- and groundwater exceeding the

threshold concentration of pesticides in drinking water (0.1 µg L⁻¹) in EU (Fenner et al., 2013). A new report from EFSA (2015) revealed that IPU is also endocrine-disrupting, in addition to being potentially carcinogenic, which led to the ban of IPU in EU since July 2016 (Commission Implementing Regulation (EU) No 2016/872, 2016).

Degradation of IPU in agricultural soils exhibits a high spatial variability as controlled by soil pH. Greater IPU degradation usually occurred in soil with higher pH (Walker et al., 2001; Bending et al., 2003; Hussain et al., 2013). To date eleven IPU-degraders have been isolated from various agricultural soils of different sites (Table 1).

* Corresponding author.

E-mail address: renyili2009@gmail.com (R. Li).

Table 1
IPU-degrading bacteria isolated during 1999–2014.

Strain	Origin	Soil history	Soil pH	pH of IPU-MS media	pH tested in MS media	pH tested in soil	Optimal pH in MS media	Optimal pH in soil	Reference
<i>Arthrobacter globiformis</i> strain D47	UK	cereals, isoproturon, ≥ 3 yr	7.8	6.9	–	–	–	–	Cullington and Walker (1999)
<i>Sphingomonas</i> sp. strain SRS2	UK	winter barley, isoproturon, 20 yr	7.19	7.2	6.5, 7.0, 7.5, 8.0 (<7.0, >7.5, inhibited)	pH 6.26, inhibited; pH 6.5, inhibited; pH 7.5, enhanced	7.5	7.5	Sørensen et al. (2001)
<i>Arthrobacter</i> sp. N2	France	garden path soil, diuron, 3 yr	–	6.5, 6.6	–	–	–	–	Tixier et al. (2002) and Widehem et al. (2002)
<i>Sphingomonas</i> sp. strain F35	UK	winter barley, isoproturon, 20 yr	7.1–7.5	–	6.5, 7.0, 7.5, 8.0 (<7.0, >7.5, inhibited)	–	7.5	–	Bending et al. (2003)
<i>Methylophila</i> sp. TES	France	winter wheat, isoproturon, 10 yr	7.4	–	–	–	–	–	El Sebai et al. (2004)
<i>Sphingobium</i> sp. strain YBL1	China	herbicide plant field, phenylurea herbicides, 10 yr	–	–	–	4.6, inhibited; 6.7, 8.3, enhanced	–	6.7	Sun et al. (2009)
<i>Sphingobium</i> sp. strain YBL2	China	herbicide plant field, phenylurea herbicides, 10 yr	–	–	3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 (≤ 6.0 , ≥ 8.0 , inhibited)	See above	7.0	6.7	Sun et al. (2009)
<i>Sphingobium</i> sp. strain YBL3	China	herbicide plant field, phenylurea herbicides, 10 yr	–	–	–	See above	–	6.7	Sun et al. (2009)
<i>Pseudomonas aeruginosa</i> strain JS-11	–	wheat, isoproturon	–	7.2 \pm 0.2	–	–	–	–	Dwivedi et al. (2011)
<i>Sphingomonas</i> sp. strain SH	France	rape seed/winter wheat/barley, isoproturon, 10 yr	7.66–7.83	6.6	5.5, 6.5, 7.5, 8.5 (≤ 6.5 , ≥ 8.5 , inhibited)	–	7.5	–	Hussain et al. (2011)
<i>Sphingomonas</i> sp. strain AK1	Germany	–	7.2 (pH _{CaCl2})	7.2	–	–	–	–	Kiesel, 2014

– not available/not investigated. Soil pH in the table were measured with 1:1 soil-water ratio, unless specified.

All these IPU-degrading bacteria were exclusively isolated from weakly alkaline soils (pH 7.1–8.1) and showed a narrow optimal pH range of 7.0–7.5 for IPU degradation (Table 1). Attempts to enhance IPU dissipation in acidic soils using these strains were hampered by the incapability of the microbes to degrade IPU at low pH (Bending et al., 2003; Grundmann et al., 2007; Sun et al., 2009). Therefore, microorganisms able to degrade IPU under acidic conditions are needed.

Laboratorial soil bioaugmentation experiments were frequently conducted with monocultures of xenobiotic-degrading bacteria for simplicity reasons (e.g., cell enumeration or activity evaluation). However, increasing attention have been paid nowadays to microbial consortia which exhibit promising application potential in various fields including bioremediation (Brenner et al., 2008; De Roy et al., 2014). Communication between species and division of functions in constituent microbial groups enable complex microbial communities to undertake complicated tasks that cannot be realized by pure cultures (Brenner et al., 2008). Sørensen et al. (2002) found that the ultimate IPU-degrading strain *Sphingomonas* sp. strain SRS2 could only mineralize IPU intensively in mineral salts medium in the presence of certain non-IPU-degrader bacteria that supply *Sphingomonas* sp. strain SRS2 with methionine for IPU degradation. Devers-Lamrani et al. (2014) reported synergistic degradation of another phenylurea herbicide diuron by cooperation between two partial diuron-degraders, *Arthrobacter* sp. BS2 and *Achromobacter* sp. SP1, in which strain BS2 transformed the parent compound to 3,4-dichloroaniline and strain SP1 mineralized the intermediate to CO₂ ultimately. Similar findings were reported in the biodegradation of linuron (Dejonghe et al., 2003). Due to the various advantages of microbial communities, we used microbial consortia rather than isolated strains in this study to degrade IPU in soils.

Efficacy of microbes to enhance degradation of organic contaminants in soil has been extensively studied since decades e.g., as reviewed by Dejonghe et al. (2001) and Owsianiak et al. (2010), however, little is known about the sustainability of the introduced pollutant-degrading function in soil. Persistence of the degradative potential determines the extent and thoroughness of pollutant removal from soil, especially when the chemicals are not readily available due to soil sorption. Sustainable degradative function in soil may eliminate the necessity of re-inoculation of the degraders and allow the soil to be subjected to inevitable repeated pollutant exposure (e.g., regular pesticide application in agriculture).

In the present study we investigated the potential and functional sustainability of two IPU-mineralizing microbial communities enriched from an acidic soil (pH 5.8) and a neutral soil (pH 7.2), respectively, in three other agricultural soils with contrasting pH. The effect of inoculation size, IPU sorption and pH on the fate of IPU are studied. The hypotheses are (i) IPU-mineralizing microbial communities originated from the acidic soil and the neutral soil have different optimal pH for IPU degradation: pH 5.8 for the community from the acidic soil and pH 7.2 for community from the neutral soil; (ii) IPU degradation is enhanced by the microbial communities in soil with pH close to the pH of soils harboring the microbial communities; (iii) IPU bioavailability is not a limiting factor for IPU degradation in the soils.

2. Materials and methods

2.1. Chemicals

Uniformly ring-labeled [¹⁴C]-IPU (¹⁴C-IPU, purity > 95%, specific radioactivity 9.96 kBq μ g⁻¹) was purchased from GE Healthcare (Amersham Place Little Chalfont, UK). Non-labeled IPU (purity

99.0%) and its metabolites, 3-(4-isopropylphenyl)-1-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU), 3-[4-(2-hydroxyisopropylphenyl)]-1-methylurea (2-OH-MDIPU) and 4-isopropylaniline (4-IA) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Scintillation cocktails (Ultima-Flo™ AF, Ultima Gold™ XR and Permafluor® E+) and Carbo-Sorb® E were purchased from PerkinElmer (Waltham, USA). Acetonitrile (HPLC grade) was purchased from Carl Roth (Karlsruhe, Germany). All other chemicals used in this study were obtained from Merck (Darmstadt, Germany) unless specified.

¹⁴C-IPU standards for the specific experiments were prepared by mixing ¹⁴C-IPU and non-labeled IPU proportionally in methanol (Table S1, Supporting Information).

2.2. Soils and carrier material

Three agricultural soils were used for the inoculation experiments. Physico-chemical characteristics of the soils are shown in Table 2. The two major reasons for selecting these soils are: (i) The soils had contrastingly different soil pH. pH is an important factor controlling IPU degradation in soils (Table 1). To investigate the effect of pH on IPU degradation by the inoculants, soils with contrasting pH were used. pH of the selected soils ranges from 3.8 to 7.3, which covers the pH (5.0–6.8) of most agricultural soils (Blume et al., 2016); (ii) The soils had naturally low IPU-mineralizing capability (Folberth et al., 2009). It makes little sense to inoculate IPU degraders into a soil which can degrade IPU intensively by themselves. And there will be probably no chance to see the effect of inoculation. Therefore, soil with low IPU-degradative potential were used. After sampling, the soil material was air-dried, sieved to ≤2 mm and conserved at –20 °C. Two weeks before the incubation experiment, approximately 750 g (dw) of each soil were defrosted at 4 °C for one week, wetted close to the water potential of –15 kPa (Schroll et al., 2006) and equilibrated for another 7 days at room temperature.

Expanded clay particles (Seramis GmbH, Mogendorf, Germany) were used as carrier material to inoculate the microbial communities into soil. The particles are mainly made of kaolinite, illite and quartz. Properties of the carrier material are as follows (data from manufacturer): diameter, ≈4 mm; pore volume, >80%; pH (H₂O), ≈7; total N, 3–8 mg L⁻¹; P₂O₅, 5–10 mg L⁻¹ and K₂O, 100–120 mg L⁻¹. The dry weight of 20 carrier particles is ≈0.52 ± 0.06 g. The carrier material has a negligible IPU sorption capacity and has no effect on IPU degradation (Fig. S1, Supporting Information). The carrier material was used to inoculated 1,2,4-trichlorobenzene degrading microorganisms to soil in a previous study and showed the effect of improving the functioning of the degraders in soil (Wang et al., 2010).

2.3. Microbial inocula

Previously, two IPU-mineralizing microbial communities were enriched respectively from an acidic and a weakly alkaline agricultural soil (Table S2, Supporting Information) with high natural IPU-mineralizing capacity (Grundmann et al., 2007; Folberth et al., 2009). After being enriched from soil, the microbial consortia were

grown onto carrier particles in mineral salts media with IPU as the sole carbon source (IPU-MS medium, Table S3, Supporting Information) as described by Grundmann et al. (2007). The carrier particles with the microbial communities were transferred regularly in IPU-MS-media to check the function. The microbial consortia were cultivated using the same MS-media with 25 µg mL⁻¹ IPU and incubated (20 °C, 100 rpm, darkness) and aerated in the same manner (thrice per week). A stable IPU mineralization capacity (≈50% IPU mineralization during around 30 d) of the two microbial communities was measured and portions of the “active” particles colonized by the respective microbial communities were conserved in a –80 °C freezer. For enrichment from soil and cultivation in MS-media the microbial communities were fed with ¹⁴C-IPU Arelon prepared by mixing ¹⁴C-IPU with the Arelon formulation (AgrEvo, Frankfurt, Höchst, Germany) to a final IPU concentration of 500 mg L⁻¹. No significant differences were observed in IPU mineralization by the two microbial communities when IPU was added to the MS-media in the form of Arelon formulation or pure active ingredient, suggesting that the pesticide formulation did not have a significant effect on the IPU-mineralizing capacity of the two microbial communities. Therefore, in this study only the active ingredient (IPU) was used.

To multiply the microbial consortium established on carrier particles, 20 “active” particles of the respective microbial consortium from the freezer were thawed and inoculated into 20 mL IPU-MS media (25 µg mL⁻¹, ¹⁴C-IPU standard A) in a 100 mL Erlenmeyer flask. Approximately 80 sterilized carrier particles were added to the liquid culture to allow colonization by the microbial consortium. Four replicates were prepared for each microbial consortium. The liquid cultures were incubated at 75 rpm on an orbital shaker at 20 °C in darkness and aerated using a trapping system three times per week to measure IPU mineralization and refresh the microbes with oxygen. The flasks and trapping systems are as described in Fig. S2 (Supporting Information). Each incubator was aerated for 2 h at an air flow of 1.8 L h⁻¹. ¹⁴CO₂ evolved from mineralization of ¹⁴C-IPU was trapped with 10 mL 0.1 M NaOH solution in the flushing bottle. An aliquot of 2 mL of the trapping solution was mixed with 3 mL Ultima-Flo™ AF and measured in a liquid scintillation counter (Tri-Carb® 1900 TR, PerkinElmer, Waltham, USA). At the time when the two different microbial consortia established on carrier particles were inoculated into soil, the respective liquid culture showed a cumulative IPU mineralization of >50% and an IPU-mineralization rate of approximately 1% added IPU mass d⁻¹. The microbial communities were introduced to soils when they showed similar IPU-metabolizing activity to ensure the comparability of the two microbial-community inoculants. To give information about the relative inoculum density, DNA was extracted from 20 “active” particles of each microbial community using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, USA) according to the manufacturer's instructions and quantified with NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Sunnyvale, USA). For both MC-AS and MC-NS the concentration of DNA was 2.7 µg g dry weight carriers⁻¹ (0.07 µg DNA per carrier particle), corresponding to 0.028 µg DNA g dry weight soil⁻¹.

Table 2
Physico-chemical characteristics of soils used for the microcosm experiments.

Soil	pH CaCl ₂	Clay <2 µm %	Silt 2–63 µm %	Sand 63–2000 µm %	TOC %	Total N %	CaCO ₃ %	Water content at –15 kPa ^b %
Marsdorf ^a	3.8	9	16	75	1.4	0.1	<0.1	13.3
Neumarkt ^a	5.8	4	8	88	1.0	0.1	2.7	10.1
Dürneck	7.3	7.6	63.9	28.5	4.0	0.27	60.6	35.7

^a Folberth et al. (2009).

^b Soil moisture optimal for biodegradation (Schroll et al., 2006).

2.4. Soil microcosm experiment 1: efficacy and sustainability

2.4.1. Initial IPU application and inoculation

An IPU concentration of $5 \mu\text{g g}^{-1}$ was adopted in soil incubation experiments in this research. This IPU concentration is obtained by considering an IPU-application rate of 0.75 kg ha^{-1} (for initial and re-application of IPU, respectively; 1.5 kg ha^{-1} in total according to the herbicide manufacturers such as Syngenta, Nufarm, Bayer etc.), a soil density of 1.3 g cm^{-3} and a soil height of 1.2 cm (height of soil in the microcosm). ^{14}C -IPU standard B (150 μL) was applied to 3.5 g milled dry soil material, and mixed until the methanol evaporated. This spiked soil aliquot was added to 46.5 g (dry weight) equilibrated soil in a 250-mL amber glass bottle (diameter, 63 mm; body height 90 mm) and homogenized. The nominal IPU concentration in soil is $5 \mu\text{g g}^{-1}$ (3.6 kBq g^{-1}). After application, the soil was inoculated with 20 “active” particles of either of the two microbial consortia and mixed. The soil material was condensed to 1.3 g cm^{-3} (density of top soil in most agricultural soils, Blume et al., 2016) by pressing the soil gently with an end-bended steel spatula to a defined line on the incubator marking the volume corresponding to a soil density of 1.3 g cm^{-3} . IPU carry-over from the inocula to soil was negligible (data not shown). After inoculation, the soil was moistened with distilled water to a soil water potential of -15 kPa . Soil subjected to the same treatment, but without inoculation of microbes served as control. An overview of the experimental set-up is given in Table S4a (Supporting Information). Each soil treatment was conducted with four replicates. The soil samples were incubated at $20 \pm 1 \text{ }^\circ\text{C}$ for 133 days using the soil microcosm system depicted in Fig. S3 (Supporting Information). The incubators (each containing 50 g dw soil material) were weighed weekly and the lost water ($\approx 0.07 \text{ g}$ per week) was added to the soil to maintain the soil water potential at -15 kPa .

2.4.2. Re-application of IPU

After incubation for 133 d, 150 μL ^{14}C -IPU standard C was applied to each of the soil samples ($5 \mu\text{g g}^{-1}$, 3.4 kBq g^{-1}). An aliquot of 3.5 g (dw) soil from each incubator was used for the standard application. The procedures were the same as described above. After re-applying IPU, the soils were incubated for another 56 d under the same condition.

2.4.3. Measurement of ^{14}C -IPU mineralization

$^{14}\text{CO}_2$ evolved from mineralization of ^{14}C -IPU in soil was absorbed by 10 mL 0.1 M NaOH in the plastic beaker inside the incubator (Fig. S3, Supporting Information). NaOH was exchanged three times per week from day 0 to day 57 and from day 133 to day 189. Between day 57 and day 133 NaOH was exchanged twice per week. The radioactivity of evolved $^{14}\text{CO}_2$ was quantified by liquid scintillation counting. Degree of IPU mineralization during 0 d–57 d and 133 d–189 d were calculated as the amount of $^{14}\text{CO}_2$ in % of initially applied ^{14}C and re-applied ^{14}C , respectively. The IPU mineralization rate was calculated as $^{14}\text{CO}_2$ in $\mu\text{g IPU-mass equivalents d}^{-1} \text{ g dry soil}^{-1}$. Mineralization rates after initial and second ^{14}C -IPU application were calculated according to the specific radioactivity of ^{14}C -IPU standard B and ^{14}C -IPU standard C, respectively.

The formula used to calculate the IPU-mineralization rate is as below.

$$\text{MR}_{\text{IPU}} = \frac{\text{CM}_t - \text{CM}_{t-1}}{\text{R}_{\text{spec.IPU}} * t * m}$$

MR_{IPU} : mineralization rate of IPU ($\mu\text{g d}^{-1} \text{ g dry soil}^{-1}$)

CM_t : cumulative IPU mineralization (kBq) as $^{14}\text{CO}_2$ at sampling time t (d)

CM_{t-1} : cumulative IPU mineralization (kBq) as $^{14}\text{CO}_2$ at sampling time $t-1$ (d), the time point previous to time t

$\text{R}_{\text{spec.IPU}}$: specific radioactivity of the ^{14}C -IPU standard

t : time (d) between two adjacent sampling times ($t-1$ and t)

m : dry mass of soil (g)

2.4.4. Analysis of ^{14}C -extractable residues (^{14}C -ER)

After incubation the soil was extracted with methanol at $90 \text{ }^\circ\text{C}$, 10 MPa using an accelerated solvent extractor ASE[®] 200 (Thermo Fisher Scientific, Sunnyvale, USA) equipped with 33-mL extraction cells. The ASE[®] extraction program is as follows: Preheat, 1 min; Heat and pressurize, 5 min; Static, 1 min; Flush, 80% of the cell volume; Cycles, 3 (from static to flush); Purge, 300 s; End relief, 1 min. Validity of this method for IPU extraction from soils had been confirmed previously by a recovery rate of 100% and absence of formation of artefacts (Kühn, 2004; Schroll and Kühn, 2004). For each soil sample, $\approx 25 \text{ g}$ (dw) soil was extracted. Radioactivity of ^{14}C -ER was determined by measuring two aliquots (1 mL each) of crude extracts mixed with 4 mL Ultima Gold[™] XR in the liquid scintillation counter. Soil was stored at $-20 \text{ }^\circ\text{C}$ if analysis could not be done immediately.

To prepare the samples for HPLC, the extracts were evaporated at $55 \text{ }^\circ\text{C}$, 15 kPa to 3–8 mL and diluted with distilled water to 250 mL. The solution was cleaned by SPE (Bond Elut ENV, 200 mg, 3 mL, Agilent Technologies, Santa Clara, USA) at a flow rate of $\approx 5 \text{ mL min}^{-1}$. The SPE columns were dried with nitrogen and stored at $-20 \text{ }^\circ\text{C}$. Before HPLC analysis the cartridges were eluted with 10 mL methanol. The radioactivity of the eluates was determined by measuring two aliquots (100 μL each) with 5 mL Ultima Gold[™] XR in the liquid scintillation counter. The eluates were evaporated ($55 \text{ }^\circ\text{C}$, 15 kPa) to dryness and re-dissolved in an appropriate volume of methanol to achieve a concentration of 42–167 $\text{Bq } \mu\text{L}^{-1}$ in the samples, depending on the radioactivity of the eluates. Twenty μL of each sample was injected into a ^{14}C -HPLC system (Schroll and Kühn, 2004). The HPLC was equipped with an L-4250 UV/VIS detector (240 nm, Merck-Hitachi, Darmstadt, Germany) and a LB 506 C-1 HPLC radioactivity monitor (Berthold Technologies, Bad Wildbad, Germany). ^{14}C -signals were acquired by a RadioStar 4.6.0.0 software on a computer connected to the HPLC. The HPLC gradient is presented in Table S5 (Supporting Information). Peaks in the HPLC chromatograms below the quantification limit of the ^{14}C -HPLC (20 Bq per 20 μL) were not integrated.

2.4.5. Analysis of ^{14}C -non-extractable residues (^{14}C -NER)

The soil material was milled intensively after ASE[®] extraction. Aliquots of $\approx 400 \text{ mg}$ soil were combusted using a Model 307 Sample Oxidizer (PerkinElmer, Waltham, USA) as described by Schroll and Kühn (2004). Radioactivity of ^{14}C -NER was determined by measuring the trapping solvent from the sample oxidizer by liquid scintillation counting. For each soil replicate, three aliquots were combusted.

2.5. Soil microcosm experiment 2: effect of inoculation size

Soil Neumarkt and soil Dürneck were inoculated with only two “active” carrier particles (carrier-soil ratio 0.1%) to test the effect of inoculation size on IPU degradation in the soils. A summary of the experimental design is given in Table S4b (Supporting Information). ^{14}C -IPU application (^{14}C -IPU standard D), microbial inoculation and soil incubation were performed as described in Section 2.4. The soils were incubated for 55 d. Measurement of ^{14}C -IPU mineralization and ^{14}C -residues analysis after incubation were performed as described above.

2.6. Sorption and desorption of IPU

Sorption and desorption of IPU in the three soils were conducted according to the OECD guideline (OECD, 2000) at a soil-solution ratio of 1:5 in parallel batch experiments. ^{14}C -IPU standard E was dissolved in 0.01 M CaCl_2 to prepare the ^{14}C -IPU working solution with an IPU concentration of $1 \mu\text{g mL}^{-1}$, 17.5 Bq mL^{-1} . For each soil, 4 g soil (dry weight) was weighed into a 30 mL Nalgene™ Teflon tube (Thermo Fisher Scientific, Sunnyvale, USA) and added with 20 mL ^{14}C -IPU working solution. The samples were incubated at 60 rpm min^{-1} on an over-head shaker at $20 \pm 1 \text{ }^\circ\text{C}$. In total, 21 replicates were prepared with each soil. At time points 1 h, 2 h, 4 h, 21 h, 24 h, 48 h and 72 h after the start of shaking, three replicates were centrifuged at $4500\times g$, $20 \text{ }^\circ\text{C}$ for 20 min using a Sorvall RC6 Plus centrifuge with an SH-3000 rotor (Thermo Fisher Scientific, Sunnyvale, USA). Two 1-mL aliquots of the supernatant from each sample were mixed with 4 mL Ultima Gold™ XR and measured in the liquid scintillation counter to determine the radioactivity in the liquid. Three samples with 20 mL ^{14}C -IPU working solution but without soil were subjected to the same treatment to measure the adsorption of IPU to the testing tubes. IPU adsorption was calculated as the difference (%) between the radioactivity in the liquid before and after shaking with soil.

The partition ratio K_d of IPU was calculated as below:

$$K_d = \frac{C_S}{C_A}$$

K_d : partition ratio of IPU in soil (mL g^{-1})

C_S : concentration of IPU in soil ($\mu\text{g g}^{-1}$)

C_A : concentration of IPU ($\mu\text{g mL}^{-1}$) in aqueous phase (supernatant)

The partition ratio of IPU related to soil organic carbon (TOC) content, K_{oc} , was calculated according to the following formula, where C_{oc} is TOC content in % of soil mass.

$$K_{oc} = \frac{K_d}{C_{oc}}$$

Desorption of IPU was conducted with the soil samples centrifuged after shaking for 72 h in the sorption experiment. Twenty mL IPU-free 0.01 M CaCl_2 was added to the tube after removing the supernatant. The soil was re-suspended in the solution and incubated on the overhead shaker at 60 rpm min^{-1} , $20 \pm 1 \text{ }^\circ\text{C}$ for another 144 h. After 24 h, 48 h, 72 h and 144 h the samples were centrifuged, and the supernatant was replaced by 20 mL CaCl_2 solution. ^{14}C in the supernatant was measured in the same way as for the sorption experiment.

2.7. Effect of pH on IPU mineralization

The effect of pH on IPU mineralizing capacity of the two microbial consortia (MC-AS and MC-NS) was studied in IPU-MS media with the same pH values as the tested soils. Briefly, four “active” carrier particles (microbial inocula from Section 2.3) were inoculated into 20 mL IPU-MS media supplemented with ^{14}C -IPU standard F ($5 \mu\text{g mL}^{-1}$) with pH 3.8, 5.8, 7.2 or 8.0 in a 100 mL Erlenmeyer flask. For each microbial consortium–pH combination, three replicates were prepared. The liquid cultures were incubated for 31 days at 75 rpm on an orbital shaker at $20 \pm 1 \text{ }^\circ\text{C}$ in darkness and aerated three times per week to measure ^{14}C -IPU mineralization. The incubators and aeration system are as described in Section 2.3.

2.8. Statistics

Statistical analysis (Duncan test, $p < 0.05$) is performed using IBM® SPSS® Statistics Version 20 (IBM Corporation, New York, USA) to determine the significance of differences between cumulative IPU mineralization, IPU mineralization rates, ^{14}C -ER content, ^{14}C -NER content, relative proportions or concentrations of IPU residues in different soil treatments. All figures used in this study were produced using OriginPro 2015 (OriginLab Corporation, MA, USA).

3. Results and discussion

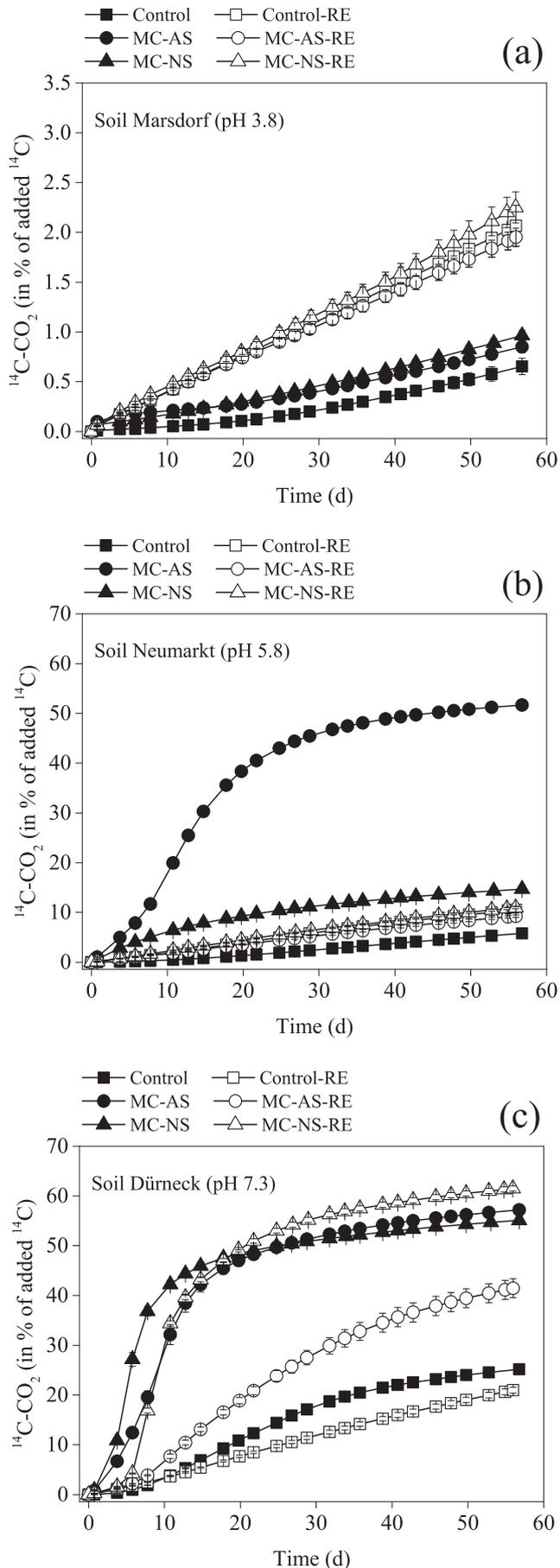
3.1. IPU mineralization

IPU mineralization rate in soil Neumarkt (pH 5.8) was greatly enhanced by inoculation of the IPU-mineralizing microbial community enriched from soil Cunnersdorf, the acidic soil (MC-AS, Fig. 1b). About 52% IPU was mineralized in this soil over 57 d, while 6% was mineralized in the control. The IPU-mineralizing microbial community enriched from soil Feldkirchen, the neutral soil (MC-NS) increased the IPU mineralization rate in soil Neumarkt to a lower degree, and the rate decreased since the start of incubation (Fig. S4b). This finding agrees with the report of Grundmann et al. (2007) that the IPU-mineralizing capability of MC-NS was inhibited in acidic soil.

In soil Dürneck (pH 7.3) inoculated with MC-AS or MC-NS, IPU mineralization was considerably accelerated (Fig. 1c). More than 55% IPU was mineralized in these soils over 57 d. The highest IPU mineralization rate ($0.44 \mu\text{g d}^{-1} \text{ g dry soil}^{-1}$) was measured in soil Dürneck amended with MC-NS (Fig. S4c). Previously, MC-NS has shown a high and stable IPU mineralizing capacity in soils with pH 6.5–7.5 where about 50% IPU was mineralized during 46 d (Grundmann et al., 2007). MC-AS mineralized 57% applied IPU in this soil during 57 d, similar to the amount of IPU mineralized in soil Neumarkt (pH 5.8) inoculated with the consortium, showing that IPU mineralizing capability of the community is not affected by pH within the range of 5.8–7.3. Considering that pH of most arable soils fall between 5.0 and 6.8 (Blume et al., 2016) and the optimal pH range for all isolated IPU-degraders up to date are 7.0–7.5 (Table 1), MC-AS may contribute significantly to IPU mineralization in a broader range of pH which has not been realized up to now by other IPU-degrading microorganisms.

IPU was mineralized extremely slow ($\approx 0.001 \mu\text{g d}^{-1} \text{ g dry soil}^{-1}$) in Marsdorf soil (pH 3.8), regardless of inoculation of IPU degraders and re-application of IPU (Fig. S4a). In total, about 2% IPU was mineralized during 189 days. It is assumed that the extremely low soil pH (3.8) is beyond the physiological tolerance of the IPU-degrading microbial communities, therefore, the inoculated IPU-degraders lost their activity in this soil.

Upon IPU re-application, no differences were observed between un-inoculated and inoculated Neumarkt soil (Fig. 1b). And the rates remained at a very low level ($\approx 0.01 \mu\text{g d}^{-1} \text{ g dry soil}^{-1}$) until the end of incubation (Fig. S4b). The disappearance of enhanced IPU-mineralizing activity in the soil inoculated with MC-AS might be due to the die-off of the inoculant microorganisms over the long incubation period without IPU as extra carbon sources (“starvation phase”, from day 57 to day 133). IPU probably serves as an advantageous C and energy source for MC-AS in soil Neumarkt as the indigenous microbial biomass showed a low IPU mineralizing activity (11.0% in 189 d). It is supposed that once IPU was consumed (57 d after initial IPU application), MC-AS lost the advantage over the native microbes and competition between MC-AS and indigenous microbes were intensified. Nevertheless, mineralization of IPU in soil Neumarkt by MC-AS in the initial phase is the first report of IPU mineralization in a moderately acidic soil enhanced by



inoculation of microbial degraders. Further work should be conducted to investigate the IPU-degradative potential of MC-AS in acidic soils with diverse physico-chemical characteristics to determine the factors controlling IPU degradation by MC-AS in acidic soils (to verify whether pH is the determinant factor).

For soil Dürneck, an intensified $^{14}\text{CO}_2$ flux was detected in the inoculated soils after re-applying IPU, however, the maximal rates were lower than before (Fig. S4c). The inconsistency in the sustainability of the IPU-mineralization activity in soil Neumarkt and soil Dürneck might be related to the different survival rates of the inoculated IPU-degrader microbes in the soils after incubation for 133 d. It is assumed that during the long-term soil incubation, large portion of readily available nutrients (e.g., dissolved organic matter, DOM) in the soil might have been consumed and therefore restrict the activity of the microbes. Soil microorganisms may attack and liberate formerly undissolved soil organic matter and live on them. Soil containing more SOM (e.g., soil Dürneck, TOC, 4.0%) offers a larger potential pool of C and N sources for the soil microbiome to sustain in the long run, therefore, microbial activity in such soils were probably less impaired after 133 days in comparison to soil with less SOM (e.g., Neumarkt, 1.0%). The high and sustainable IPU-mineralizing activity of the microbial communities in soil may allow exhaustive dissipation of IPU in the soil and eliminate the risk of repeated IPU application to the soil.

3.2. Fate of ^{14}C -IPU in soils

Distribution of ^{14}C -IPU residues in the soils varied significantly ($p < 0.05$) in soil Neumarkt and soil Dürneck depending on soil types and the inoculum. After double IPU application and incubation for 189 d, no significant differences were observed in the ^{14}C -distribution in Marsdorf (pH 3.8) soils (Fig. 2). ^{14}C -ER amounted to approximately 80% of the total applied ^{14}C (Fig. 2), which was dominantly comprised of IPU (88%, Table 3). Only a small portion of the parent compound was transformed to MDIPU (Table 3). The negligible IPU mineralization and remarkably high concentration of IPU in ^{14}C -ER (Table 4) indicate that biodegradation of IPU is strongly inhibited in this soil. Presently, there is few report about IPU biodegradation in soil with such low pH. However, such “extremely strongly acidic” soil are not common in agricultural soils; pH (CaCl_2) of arable soils frequently fall between pH 5.0–6.8 (Blume et al., 2016).

The smallest amount of ^{14}C -ER (25%) and ^{14}C -NER (39%) in soil Neumarkt (pH 5.8) were measured in the soil inoculated with MC-AS (Fig. 2). This amount of ^{14}C -ER probably derived from the re-applied, not mineralized ^{14}C -IPU. MC-NS exerted inconspicuous effect on the fate of IPU in soil Neumarkt, as compared to the control. Two unidentified metabolites (Unknown 2 and Unknown 3) were solely detected in soil Neumarkt and constituted a major portion of ^{14}C -ER (52%–63%, Table 3). The high metabolites-IPU ratio in this soil and high residual concentrations (Table 3) suggest an intensive transformation of IPU with limited mineralization in these soils. The accumulation of metabolites, rather than being further mineralized to $^{14}\text{CO}_2$ is likely due to the absence of IPU-degraders or short persistence time of the IPU-mineralizing

Fig. 1. IPU mineralization in soils Marsdorf (a), Neumarkt (b) and Dürneck (c) inoculated or uninoculated with the IPU-mineralizing microbial communities on twenty carrier particles. Solid symbols: IPU mineralization from day 0–57 (57 d after initial IPU application to the soils); open symbols: IPU mineralization from day 133–189 (56 d after re-application of IPU to the soils). Error bars represent standard deviations of four soil microcosms. Control: incubated uninoculated soil; MC-AS: the IPU-mineralizing microbial community enriched from the acidic soil Cunnersdorf; MC-NS: the IPU-mineralizing microbial community enriched from the neutral soil Feldkirchen.

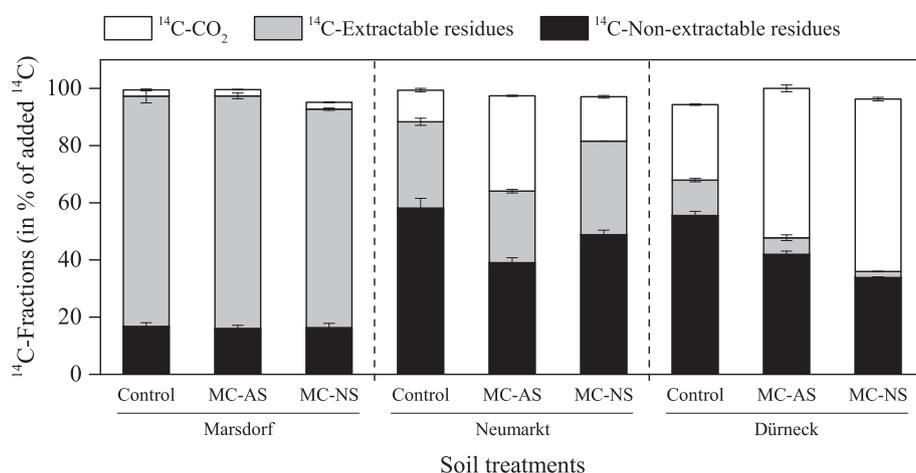


Fig. 2. Distribution of applied ^{14}C (from double ^{14}C -IPU application) in soils Marsdorf, Neumarkt and Dürneck inoculated or uninoculated with the IPU-mineralizing microbial communities on twenty carrier particles after incubation for 189 days. Error bars represent standard deviations of four soil microcosms. Abbreviations are as described in Fig. 1.

Table 3

Relative proportions of ^{14}C -IPU metabolites** in ^{14}C -extractable residues of soils Marsdorf, Neumarkt and Dürneck after incubation for 189 d. Figures after \pm represent standard deviations of four soil microcosms. Different letters indicate significant differences between soil treatments (Duncan test, $p < 0.05$).

Soil treatments	Relative proportions of IPU metabolites (as % of ^{14}C -extractable residues)						
	2-OH-MDIPU	MDIPU	IPU	4-IA	Unknown 1	Unknown 2	Unknown 3
Retention time (min)	12.13 \pm 0.09	18.91 \pm 0.05	20.17 \pm 0.05	21.60 \pm 0.06	13.14 \pm 0.09	16.70 \pm 0.12	25.06 \pm 0.07
Soil Marsdorf (pH 3.8)							
Control	nd	8.0 \pm 0.4 de	88.3 \pm 0.6 a	nd	1.6 \pm 0.3 f	*	nd
MC-AS	nd	7.8 \pm 0.2 de	88.4 \pm 0.2 a	nd	2.1 \pm 0.3 f	nd	nd
MC-NS	nd	8.1 \pm 0.4 de	88.1 \pm 0.6 a	nd	1.3 \pm 0.4 f	*	nd
Soil Neumarkt (pH 5.8)							
Control	*	9.0 \pm 0.4 d	17.3 \pm 1.0 e	2.9 \pm 0.3 b	6.0 \pm 0.7 d	17.5 \pm 0.8 b	37.3 \pm 1.9 c
MC-AS	*	8.8 \pm 0.5 d	23.4 \pm 2.6 d	*	5.5 \pm 0.7 d	12.8 \pm 0.5 c	39.0 \pm 1.5 b
MC-NS	*	7.0 \pm 0.3 e	13.9 \pm 0.5 f	3.6 \pm 0.1 a	3.6 \pm 0.1 e	18.2 \pm 0.5 a	44.8 \pm 0.8 a
Soil Dürneck (pH 7.3)							
Control	14.8 \pm 1.3 b	33.1 \pm 1.5 a	24.1 \pm 1.5 d	nd	17.9 \pm 0.6 a	*	nd
MC-AS	21.3 \pm 0.6 a	22.2 \pm 2.7 b	28.7 \pm 0.1 c	nd	15.9 \pm 1.0 b	*	nd
MC-NS	3.0 \pm 1.2 c	15.6 \pm 0.6 c	57.1 \pm 4.4 b	nd	8.2 \pm 1.5 c	*	nd

nd: not detected. *: detected, but below quantification limit (20 Bq per 20 μL). **: Substances with concentrations below the quantification limit in all soils are not presented in this table.

Table 4

Concentrations of IPU metabolites** in soils Marsdorf, Neumarkt and Dürneck after incubation for 189 d. Figures after \pm represent standard deviations of four soil microcosms. Different letters indicate significant differences between soil treatments (Duncan test, $p < 0.05$).

Soil treatments	Concentration of IPU metabolites in soil (μg IPU-mass equivalents/g dry soil)						
	2-OH-MDIPU	MDIPU	IPU	4-IA	Unknown 1	Unknown 2	Unknown 3
Retention time (min)	12.13 \pm 0.09	18.91 \pm 0.05	20.17 \pm 0.05	21.60 \pm 0.06	13.14 \pm 0.09	16.70 \pm 0.12	25.06 \pm 0.07
Soil Marsdorf (pH 3.8)							
Control	nd	0.56 \pm 0.02 a	6.22 \pm 0.22 a	nd	0.11 \pm 0.02 b	*	nd
MC-AS	nd	0.55 \pm 0.02 ab	6.18 \pm 0.12 a	nd	0.15 \pm 0.02 a	nd	nd
MC-NS	nd	0.53 \pm 0.02 b	5.72 \pm 0.14 b	nd	0.09 \pm 0.03 bc	*	nd
Soil Neumarkt (pH 5.8)							
Control	*	0.23 \pm 0.003 d	0.44 \pm 0.03 c	0.07 \pm 0.01 b	0.15 \pm 0.01 a	0.44 \pm 0.03 b	0.94 \pm 0.07 b
MC-AS	*	0.18 \pm 0.01 e	0.49 \pm 0.04 c	*	0.12 \pm 0.02 b	0.27 \pm 0.01 c	0.82 \pm 0.06 c
MC-NS	*	0.19 \pm 0.01 e	0.38 \pm 0.01 c	0.10 \pm 0.004 a	0.10 \pm 0.003 b	0.50 \pm 0.01 a	1.22 \pm 0.02 a
Soil Dürneck (pH 7.3)							
Control	0.13 \pm 0.01 a	0.28 \pm 0.02 c	0.21 \pm 0.03 d	nd	0.15 \pm 0.01 a	*	nd
MC-AS	0.09 \pm 0.02 b	0.09 \pm 0.03 f	0.12 \pm 0.02 d	nd	0.07 \pm 0.02 c	*	nd
MC-NS	0.003 \pm 0.001 c	0.015 \pm 0.001 g	0.055 \pm 0.004 d	nd	0.01 \pm 0.001 d	*	nd

nd: not detected. *: detected, but below quantification limit (20 Bq per 20 μL). **: Substances with concentrations below the quantification limit in all soils are not presented in this table.

microbial communities inoculated into the soil.

The quantity of ^{14}C -ER in Dürneck soil (pH 7.3) were significantly less than in the other two soils. Almost no ^{14}C was

extractable from soil Dürneck inoculated with MC-NS. The trace amount of IPU and IPU metabolites remaining in the soil indicate that IPU was mineralized exhaustively by MC-NS. Generally,

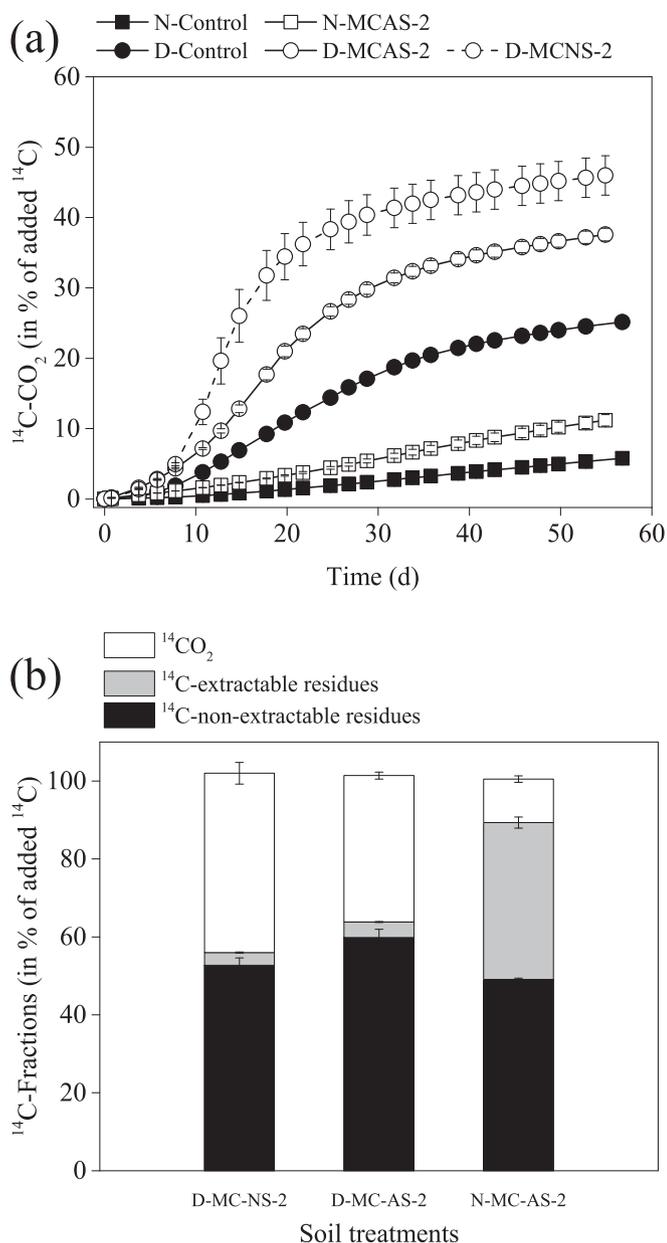


Fig. 3. IPU mineralization (a) and ^{14}C -mass balances on day 55 (b) in soil Neumarkt (N) and soil Dürneck (D) inoculated with the IPU-mineralizing microbial communities on two carrier particles. Error bars represent standard deviations of four soil microcosms. Abbreviations are as described in Fig. 1.

increased IPU mineralization is associated with decreased concentrations of IPU residues and increased relative portions of the parent compound. The ^{14}C -mass balances in all soils were between 94.3% and 100.3%.

The composition of $^{14}\text{C-ER}$ (Tables 3 and 4) gives important information on thoroughness of IPU degradation and formation of IPU metabolites. Degradates of organic pollutants are of environmental concern because they are often found as toxic as or more toxic than their parent compounds and some metabolites were more frequently detected in the environment than their parent compounds (Boxall et al., 2004; Escher and Fenner, 2011). For instance, the IPU-metabolite, 4-IA, was 600 times more toxic than IPU as determined by Microtox[®] (Tixier et al., 2002). Toxicity of IPU metabolites such as MDIPU and DDIPU are getting increasing attention in risk assessment of IPU (EFSA, 2015). In this study,

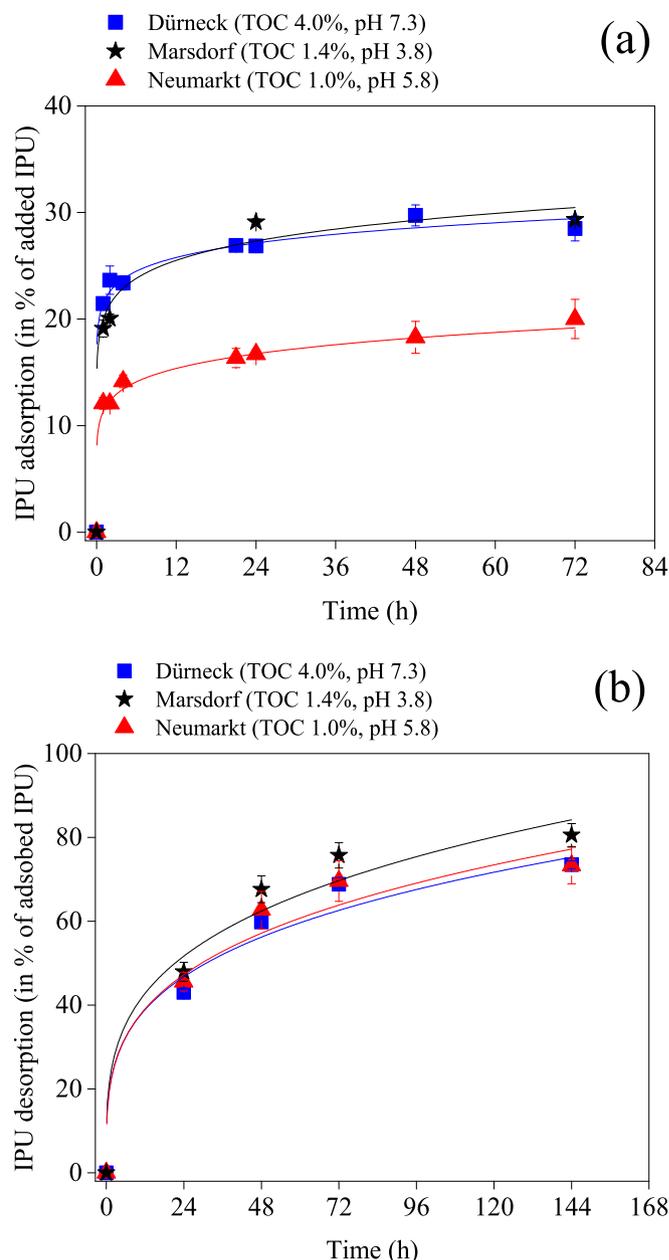
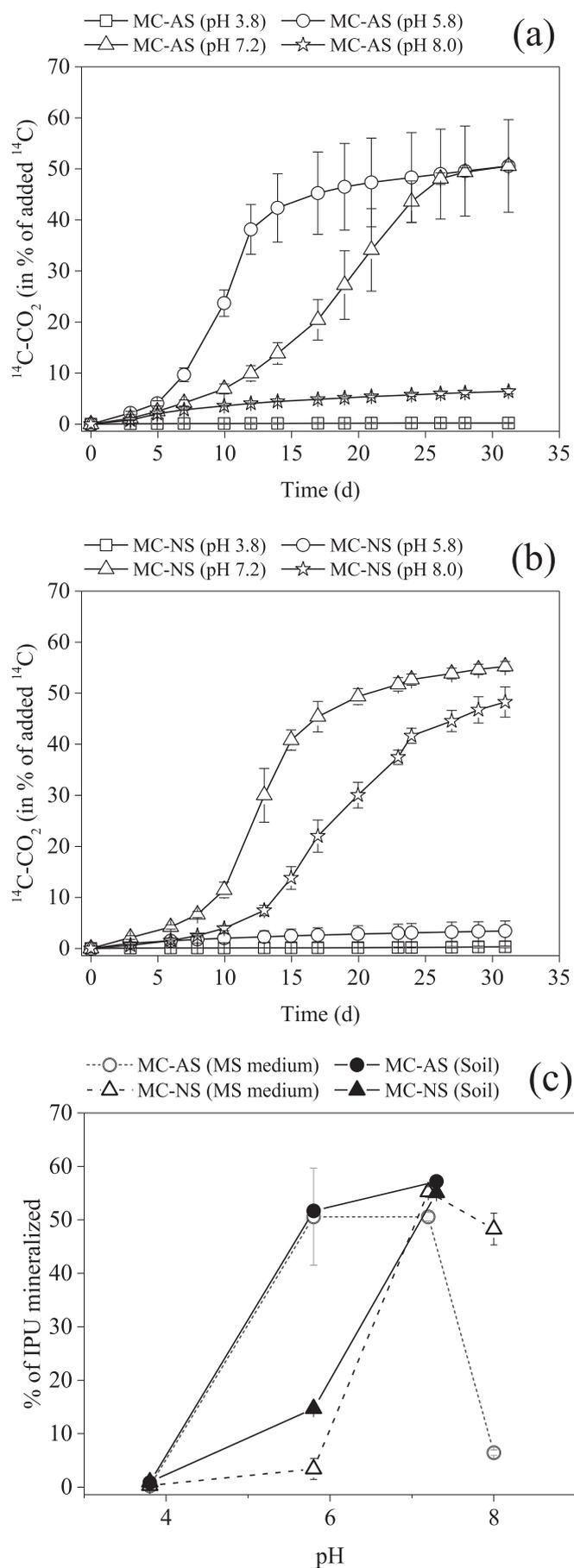


Fig. 4. Sorption (a) and desorption (b) of IPU in soils Marsdorf, Neumarkt and Dürneck. Error bars represent standard deviations of three experimental replicates.

concentrations of IPU or IPU-metabolites were significantly decreased in soil Neumarkt amended with MC-AS and in soil Dürneck amended with either of the microbial communities (Table 4), suggesting enhanced dissipation of IPU-residues by inoculation of the microbial communities.

The proportion of $^{14}\text{C-NER}$ in Neumarkt and Dürneck soils was significantly higher than in Marsdorf soil, ranging from 34% to 58%. The composition of $^{14}\text{C-NER}$ was not analyzed in this study. Generally NER exert a low environmental risks than ER because of the reduced mobility (Barraclough et al., 2005). However, the bound chemicals may be released and mobilized due to the change of soil properties e.g., change of redox state due to anoxic-oxic alterations (Liu et al., 2013), or by the activities of the soil microorganisms such as turnover of soil organic matter (Barraclough et al., 2005). Recent progress in the nature of NER unraveled that NER



formed during metabolic biodegradation (usually coupled with intensive mineralization) of organic pollutants in soil are mainly comprised of biomolecules (e.g., amino acids, fatty acids and amino sugars etc.) originated from anabolism of xenobiotics by the degrader microorganisms. Using ^{13}C -isotope label, Nowak et al. (2010, 2013) demonstrated that almost all NER formed during the metabolic degradation of 2,4-D and ibuprofen in soil were composed of biogenic residues from incorporation of the xenobiotics. Formation of biogenic NER was also observed in soil where degradation of the pollutant was partially restricted. Poßberg et al. (2016) reported that non-extractable bio-residues amounted to at least 26% of NER formed by degradation of ^{14}C -bromoxynil in a soil over 56 d. This type of environmental innocuous non-extractable residues originated from the necromass of xenobiotic degraders is defined as bioNER (Kästner et al., 2014) to distinguish from “real” NER defined by IUPAC, which are formed by xenobiotic parent compounds and/or metabolites (Roberts, 1984). It is assumed that the microbial communities causing greater IPU mineralization resulted in formation of more bioNER and less “real” NER, therefore, reduced the environmental risks of IPU residues in the soils.

3.3. Effect of inoculant density

Inoculating soil with the microbial consortia on carrier particles at a carrier-soil ratio of 0.1% (2 particles: 50 g dry weight soil) accelerated IPU mineralization in soil Neumarkt and soil Dürneck to different extents. IPU mineralization in soil Dürneck inoculated with the microbial communities were 13%–21% higher than in the control (Fig. 3a). Inoculating two carrier particles with MC-AS had a small effect on IPU mineralization in soil Neumarkt, but still twice more IPU was mineralized (Fig. 3a). A marginal level of ^{14}C -ER (3.3%–4.0%) was measured in the amended Dürneck soil with high IPU mineralization, while 40% applied ^{14}C were still extractable in soil Neumarkt inoculated with MC-AS (Fig. 3b). ^{14}C -NER amounted to 50%–60% of the applied ^{14}C in all inoculated soils (Fig. 3b). The ^{14}C -mass balances in the soils at the end of incubation (day 55) was 100.2%–102.0%.

Inoculating 2 instead of 20 particles with MC-AS resulted in almost 5-fold lower IPU mineralization in soil Neumarkt. However, this effect was less remarkable in soil Dürneck inoculated with either MC-AS or MC-NS. The impact of inoculant density on degradation of xenobiotics in soils has been extensively studied (Duquenne et al., 1996; Cullington and Walker, 1999; Karpouzias and Walker, 2000; Rousseaux et al., 2003). In bioaugmentation a critical loading of inoculant cells is required to ensure the inoculum efficacy. For instance, a notable mineralization or removal of the parent compounds were only observed when at least 10^3 – 10^8 CFU g soil $^{-1}$ degrader cells were inoculated to the soils depending on the inoculant microorganisms, pollutants and soil properties (Duquenne et al., 1996; Cullington and Walker, 1999; Karpouzias and Walker, 2000; Rousseaux et al., 2003). Higher inoculant densities generally resulted in increased degradation rates of organic pollutants in soil. In contrast, the maximal cumulative degradation of the chemicals may decrease (Cullington and Walker, 1999; Rousseaux et al., 2003) or remain unchanged (Duquenne et al., 1996; Karpouzias and Walker, 2000) with the

Fig. 5. ^{14}C -IPU mineralization by the IPU-mineralizing microbial communities enriched from the acidic soil Cunnersdorf (MC-AS) (a) and the neutral soil Feldkirchen (MC-NS) (b) in MS-media at different pH, and relationship between pH and maximal IPU mineralization by the microbial communities in MS-media and the soils (c). IPU mineralization in soils means the % of initially applied IPU mineralized during the first 57 d. Error bars represent standard deviations of three biological replicates (MS-media) or four soil microcosms.

reduced inoculation size. The divergence of inoculant density on maximal dissipation of pollutants is probably due to the variability in the (functional) sustainability of the inoculated microbes in the contaminated soils. When the inoculant microorganisms are able to establish themselves in the inoculated soil, the pollutant may allow to be mineralized or degraded thoroughly; in the other case, when the inoculant microbes has a limited residence time in the inoculated soil, the pollutants can only be mineralized or degraded to certain degrees within the persistence time of the inoculant microbes. Limited cell distribution and diffusion of pollutant towards the degrader cells may also play a role. By studying the effect of inoculant density on IPU mineralization, one could identify the lower limit of the amount of inoculants as well as the maximal mandatory inoculation size to realize a desirable remediation outcome, so that cost-effectiveness of a bioremediation approach could be ensured. In this study, inoculating MC-AS on carrier particles at a carrier-soil ratio of 0.1% is insufficient to ensure a high IPU dissipation in the acidic soil (Neumarkt), as compared to an inoculation ratio of 1%.

3.4. IPU sorption and desorption

Equilibrium of IPU sorption in the soils was reached within 72 h, during which 20%–29% IPU was adsorbed by the soil material (Fig. 4a). The majority (73%–81%) of the adsorbed IPU was allowed to be desorbed within 6 days (Fig. 4b). IPU sorption to the testing vessel is negligible (data not shown). K_d and K_{oc} values of IPU in the soils are: Marsdorf, $K_d = 2.1 \text{ mL g}^{-1}$, $K_{oc} = 147 \text{ mL g}^{-1}$; Neumarkt, $K_d = 1.3 \text{ mL g}^{-1}$, $K_{oc} = 125 \text{ mL g}^{-1}$ and Dürneck, $K_d = 2.0 \text{ mL g}^{-1}$, $K_{oc} = 50 \text{ mL g}^{-1}$.

IPU sorption in soil is positively correlated to SOM content and clay content (Boivin et al., 2005; Jarvis, 2016), while negatively influenced by pH and calcite content (Boivin et al., 2005; El Arfaoui et al., 2012). In this study, K_d of IPU is the same in soil Dürneck (TOC, 4%) and soil Marsdorf (TOC, 1.4%), suggesting the inadequacy of K_{oc} to represent IPU partition in these soils and other factors may have played a role. The low K_{oc} of IPU in soil Dürneck is probably attributed to the extremely high calcite content (61%), in comparison to soil Marsdorf (<0.1%), which reduces IPU sorption to SOM (El Arfaoui et al., 2012). The low sorption and high desorption of IPU suggest that IPU availability is unlikely a reason for the variability of IPU degradation in these three soils. These assumptions are also corroborated by the similar maximal IPU mineralization achieved in the soils and in MS-media (where IPU is 100% available, see Section 3.5).

3.5. Effect of pH

For both MC-AS and MC-NS, the highest cumulative IPU mineralization and maximal IPU mineralization rates (data not shown) were measured in MS media with the same pH as the soils where the microbial communities originally inhabited (Fig. 5a,b). Both communities showed the ability to mineralize IPU at a higher pH to a similar degree ($\approx 50\%$) as at the optimal pH. A minor portion of IPU was mineralized by MC-AS at pH 8.0 or by MC-NS at pH 5.8, and for both communities, mineralization of IPU was stalled at pH 3.8 (Fig. 5a,b). MC-AS showed a greater pH tolerance for IPU mineralization, ranging from pH 5.8 to pH 7.2. The tolerable pH range of MC-NS (7.2–8.0) is similar to the reported IPU-degraders (Table 1). As expected, IPU mineralization was maximized by MC-AS in MS-medium at pH 5.8, the same pH as the hosting soil of this microbial community. The capability of the strain to mineralize IPU at pH 7.2 indicates a high pH tolerance of this microbial community. Cumulative IPU mineralization by the microbial communities (day 0–57) in the soils related to pH approximate closely

with those obtained in the MS media (Fig. 5c). These finding suggest that pH is an important factor influencing IPU mineralization in the soils. The activity of the microbial inoculants could also be influenced by other soil factors (e.g., soil microbiome, SOM), which is shown by the divergence between IPU mineralization by MC-AS in soil Neumarkt and liquid medium with pH 5.8. Further studies investigating IPU degradation by MC-AS and MC-NS in more soils with different pH will offer comprehensive information on impact of pH on these two communities in soil.

4. Conclusion

IPU exhibited a high mobility in all tested soils as indicated by low sorption and high desorption of the compound onto soil, which suggests that IPU availability is not a limiting factor for IPU degradation in the soils. Two IPU-mineralizing microbial communities, MC-AS and MC-NS, enriched from an acidic soil and a slightly alkaline soil, respectively, showed a high potential to degrade IPU in both MS-media and soil as controlled by pH. A different tolerable pH was detected for MC-AS (pH 5.8–7.2) and MC-NS (pH 7.2–8.0). Inoculation of MC-AS or MC-NS resulted in an elevated and sustainable IPU-degradative activity in the slightly alkaline soil over a period of 189 d. MC-AS significantly accelerated IPU degradation in the acidic soil (pH 5.8), while MC-NS had a limited effect. IPU-mineralizing function of the microbial consortia was not sustainable in this soil. For both microbial communities, no significant effect of inoculation on IPU degradation was measured in soil with a pH beyond the tolerable pH range. A 10-fold reduction in the inoculation size substantially decreased IPU mineralization by MC-AS in the moderately acidic soil, but this effect was less pronounced for both communities in the slightly alkaline soil. This offers a hint that the economical competence of carrier-based bioaugmentation approaches could be enhanced by reducing inoculation size without substantial loss of effectiveness. MC-AS, which expressed a high IPU-degradative potential in a relatively broad range of pH (pH 5.8–7.2), may be a promising candidate for enhancing IPU degradation in acidic soils, that has not been achieved by the other reported IPU-degraders. This study suggests that the physiological requirements or limitations of the degrader microorganisms (e.g., sensitivity to pH) and the physico-chemical properties of contaminated soil (e.g., pH) should be considered when selecting bioagents for soil bioaugmentation: a proper match of these two may maximize the outcome of bioaugmentation.

Acknowledgements

The PhD study of R. Li was funded (Grant No. 57076385) by German Academic Exchange Service (DAAD). Consumables and facilities were offered by Helmholtz Zentrum München. The authors appreciate the comments from the reviewers and the editor which helped to improve the quality of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2016.08.022>.

References

- Barracough, D., Kearney, T., Croxford, A., 2005. Bound residues: environmental solution or future problem? *Environmental Pollution* 133, 85–90.
- Bending, G.D., Lincoln, S.D., Sørensen, S.R., Morgan, J.A.W., Aamand, J., Walker, A., 2003. In-field spatial variability in the degradation of the phenyl-urea herbicide isoproturon is the result of interactions between degradative *Sphingomonas* spp. and soil pH. *Applied and Environmental Microbiology* 69, 827–834.
- Blume, H.-P., Brümmer, G.W., Fleige, H., Horn, R., Kandeler, E., Kögel-Knabner, I.,

- Kretzschmar, R., Stahr, K., Wilke, B.-M., 2016. Scheffer/Schachtschabel Soil Science. Springer, Berlin/Heidelberg, p. 125.
- Boivin, A., Cherrier, R., Schiavon, M., 2005. A comparison of five pesticides adsorption and desorption processes in thirteen contrasting field soils. *Chemosphere* 61, 668–676.
- Boxall, A.B.A., Sinclair, C.J., Fenner, K., Kolpin, D., Maund, S.J., 2004. Peer reviewed: when synthetic chemicals degrade in the environment. *Environmental Science & Technology* 38, 368A–375A.
- Brenner, K., You, L., Arnold, F.H., 2008. Engineering microbial consortia: a new frontier in synthetic biology. *Trends in Biotechnology* 26, 483–489.
- Commission Implementing Regulation (EU) No 2016/872 of 1 June 2016 concerning the non-renewal of approval of the active substance isoproturon, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending Commission Implementing Regulation (EU) No 540/2011. *Official Journal of the European Union* L 145, 2.6.2016, 7–9.
- Cullington, J.E., Walker, A., 1999. Rapid biodegradation of diuron and other phenylurea herbicides by a soil bacterium. *Soil Biology and Biochemistry* 31, 677–686.
- De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., Boon, N., 2014. Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environmental Microbiology* 16, 1472–1481.
- Dejonghe, W., Berteloot, E., Goris, J., Boon, N., Crul, K., Maertens, S., Höfte, M., De Vos, P., Verstraete, W., Top, E.M., 2003. Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading variorox strain. *Applied and Environmental Microbiology* 69, 1532–1541.
- Dejonghe, W., Boon, N., Seghers, D., Top, E.M., Verstraete, W., 2001. Bioaugmentation of soils by increasing microbial richness: missing links. *Environmental Microbiology* 3, 649–657.
- Devers-Lamrani, M., Pesce, S., Rouard, N., Martin-Laurent, F., 2014. Evidence for cooperative mineralization of diuron by *Arthrobacter* sp. BS2 and *Achromobacter* sp. SP1 isolated from a mixed culture enriched from diuron exposed environments. *Chemosphere* 117, 208–215.
- Duquenne, P., Parekh, N.R., Catroux, G., Fournier, J.-C., 1996. Effect of inoculant density, formulation, dispersion and soil nutrient amendment on the removal of carbofuran residues from contaminated soil. *Soil Biology and Biochemistry* 28, 1805–1811.
- Dwivedi, S., Singh, B.R., Al-Khedhairi, A.A., Musarrat, J., 2011. Biodegradation of isoproturon using a novel *Pseudomonas aeruginosa* strain JS-11 as a multifunctional bioinoculant of environmental significance. *Journal of Hazardous Materials* 185, 938–944.
- EFSA (European Food Safety Authority), 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance isoproturon. *EFSA Journal* 2015 13 (8), 4206. <http://dx.doi.org/10.2903/j.efsa.2015.4206>, 99 pp.
- El Arfaoui, A., Sayen, S., Paris, M., Keziou, A., Couderchet, M., Guillon, E., 2012. Is organic matter alone sufficient to predict isoproturon sorption in calcareous soils? *Science of the Total Environment* 432, 251–256.
- El Sebai, T., Lagacherie, B., Soulas, G., Martin-Laurent, F., 2004. Isolation and characterisation of an isoproturon-mineralising *Methylophilus* sp. TES from French agricultural soil. *FEMS Microbiology Letters* 239, 103–110.
- Escher, B.L., Fenner, K., 2011. Recent advances in environmental risk assessment of transformation products. *Environmental Science & Technology* 45, 3835–3847.
- Fenner, K., Canonica, S., Wackett, L.P., Elsner, M., 2013. Evaluating pesticide degradation in the environment: blind spots and emerging opportunities. *Science* 341, 752–758.
- Folberth, C., Scherb, H., Suhadolc, M., Munch, J.C., Schroll, R., 2009. In situ mass distribution quotient (iMDQ) – a new factor to compare bioavailability of chemicals in soils? *Chemosphere* 75, 707–713.
- Grundmann, S., Fuß, R., Schmid, M., Laschinger, M., Ruth, B., Schulin, R., Munch, J.C., Schroll, R., 2007. Application of microbial hot spots enhances pesticide degradation in soils. *Chemosphere* 68, 511–517.
- Hussain, S., Arshad, M., Springael, D., Sørensen, S.R., Bending, G.D., Devers-Lamrani, M., Maqbool, Z., Martin-Laurent, F., 2015. Abiotic and biotic processes governing the fate of phenylurea herbicides in soils: a review. *Critical Reviews in Environmental Science and Technology* 45, 1947–1998.
- Hussain, S., Devers-Lamrani, M., El Azhari, N., Martin-Laurent, F., 2011. Isolation and characterization of an isoproturon mineralizing *Sphingomonas* sp. strain SH from a French agricultural soil. *Biodegradation* 22, 637–650.
- Hussain, S., Devers-Lamrani, M., Spor, A., Rouard, N., Porcherot, M., Beguet, J., Martin-Laurent, F., 2013. Mapping field spatial distribution patterns of isoproturon-mineralizing activity over a three-year winter wheat/rape seed/barley rotation. *Chemosphere* 90, 2499–2511.
- Jarvis, N., 2016. Extended sorption partitioning models for pesticide leaching risk assessments: can we improve upon the koc concept? *Science of the Total Environment* 539, 294–303.
- Karpouzias, D.G., Walker, A., 2000. Factors influencing the ability of *Pseudomonas putida* epl to degrade ethoprophos in soil. *Soil Biology and Biochemistry* 32, 1753–1762.
- Kästner, M., Nowak, K.M., Miltner, A., Trapp, S., Schäffer, A., 2014. Classification and modelling of nonextractable residue (NER) formation of xenobiotics in soil – a synthesis. *Critical Reviews in Environmental Science and Technology* 44, 2107–2171.
- Kiesel, C.A., 2014. Enhanced Degradation of Isoproturon in Soils: Sustainability of Inoculated, Microbial Herbicide Degraders, and Adaptation of Native Microbes (Ph.D. Dissertation). Technische Universität München, Munich, Germany. <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:bvb:91-diss-20141120-1188688-0-1>.
- Kühn, S.S., 2004. Bedeutung der Leistung mikrobieller Lebensgemeinschaften beim Umsatz und Abbau von Isoproturon in Böden und Möglichkeiten zur Steuerung des in-situ-Pestizidabbaus (Ph.D. Dissertation). Technische Universität München, Munich, Germany. <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:bvb:91-diss2004032911796>.
- Liu, J., Wang, Y., Jiang, B., Wang, L., Chen, J., Guo, H., Ji, R., 2013. Degradation, metabolism, and bound-residue formation and release of tetrabromobisphenol A in soil during sequential anoxic–oxic incubation. *Environmental Science & Technology* 47, 8348–8354.
- Nowak, K.M., Girardi, C., Miltner, A., Gehre, M., Schäffer, A., Kästner, M., 2013. Contribution of microorganisms to non-extractable residue formation during biodegradation of ibuprofen in soil. *Science of the Total Environment* 445–446, 377–384.
- Nowak, K.M., Miltner, A., Gehre, M., Schäffer, A., Kästner, M., 2010. Formation and fate of bound residues from microbial biomass during 2,4-D degradation in soil. *Environmental Science & Technology* 45, 999–1006.
- OECD, 2000. Adsorption–desorption Using a Batch Equilibrium Method 106. Guidelines for the Testing of Chemicals. Organisation of Economic Co-operation and Development, Paris, pp. 1–45.
- Owsianiak, M., Dechesne, A., Binning, P.J., Chambon, J.C., Sørensen, S.R., Smets, B.F., 2010. Evaluation of bioaugmentation with entrapped degrading cells as a soil remediation technology. *Environmental Science & Technology* 44, 7622–7627.
- Poßberg, C., Schmidt, B., Nowak, K., Telscher, M., Lagojda, A., Schaeffer, A., 2016. Quantitative identification of biogenic nonextractable pesticide residues in soil by 14C-analysis. *Environmental Science & Technology* 50 (12), 6415–6422.
- Roberts, T.R., 1984. Non-extractable residues in soils and plants. *Pure and Applied Chemistry* 56 (7), 945–956.
- Rousseau, S., Hartmann, A., Lagacherie, B., Piutti, S., Andreux, F., Soulas, G., 2003. Inoculation of an atrazine-degrading strain, *Chelatobacter heintzii* Cit1, in four different soils: effects of different inoculum densities. *Chemosphere* 51, 569–576.
- Schroll, R., Becher, H.H., Dörfler, U., Gayler, S., Grundmann, S., Hartmann, H.P., Ruoss, J., 2006. Quantifying the effect of soil moisture on the aerobic microbial mineralization of selected pesticides in different soils. *Environmental Science & Technology* 40, 3305–3312.
- Schroll, R., Kühn, S., 2004. Test system to establish mass balances for ¹⁴C-labeled substances in soil–plant–atmosphere systems under field conditions. *Environmental Science & Technology* 38, 1537–1544.
- Sørensen, S.R., Ronen, Z., Aamand, J., 2001. Isolation from agricultural soil and characterization of a *Sphingomonas* sp. able to mineralize the phenylurea herbicide isoproturon. *Applied and Environmental Microbiology* 67, 5403–5409.
- Sørensen, S.R., Ronen, Z., Aamand, J., 2002. Growth in coculture stimulates metabolism of the phenylurea herbicide isoproturon by *Sphingomonas* sp. strain SRS2. *Applied and Environmental Microbiology* 68, 3478–3485.
- Sun, J.-Q., Huang, X., Chen, Q.-L., Liang, B., Qiu, J.-G., Ali, S., Li, S.-P., 2009. Isolation and characterization of three *Sphingobium* sp. strains capable of degrading isoproturon and cloning of the catechol 1,2-dioxygenase gene from these strains. *World Journal of Microbiology & Biotechnology* 25, 259–268.
- Tixier, C., Sancelme, M., Ait-Aïssa, S., Widehem, P., Bonnemoy, F., Cuer, A., Truffaut, N., Veschambre, H., 2002. Biotransformation of phenylurea herbicides by a soil bacterial strain, *Arthrobacter* sp. N2: structure, ecotoxicity and fate of diuron metabolite with soil fungi. *Chemosphere* 46, 519–526.
- Walker, A., Jurado-Exposito, M., Bending, G.D., Smith, V.J.R., 2001. Spatial variability in the degradation rate of isoproturon in soil. *Environmental Pollution* 111, 407–415.
- Wang, F., Dörfler, U., Schmid, M., Fischer, D., Kinzel, L., Scherb, H., Munch, J.C., Jiang, X., Schroll, R., 2010. Homogeneous inoculation vs. microbial hot spots of isolated strain and microbial community: what is the most promising approach in remediating 1,2,4-TCB contaminated soils? *Soil Biology and Biochemistry* 42, 331–336.
- Widehem, P., Ait-Aïssa, S., Tixier, C., Sancelme, M., Veschambre, H., Truffaut, N., 2002. Isolation, characterization and diuron transformation capacities of a bacterial strain *Arthrobacter* sp. N2. *Chemosphere* 46, 527–534.

Supplementary information of Publication II

Supporting Information: Fig. S1

To test IPU sorption on carriers, four autoclaved carrier particles was added to 10 mL sterile ^{14}C -IPU solution ($5 \mu\text{g g}^{-1}$) to simulate the inoculation size of 20 carrier particles in 50 g soil (1%) in this study. The dry weight of 20 carrier particles is 0.52 g. The relative amount of carrier particles was increased to 3% by addition of 8 sterile carrier particles. Concentration of IPU in the liquid was measured over 129 days. As shown in **Fig. S1**, carrier particles impose a negligible impact on IPU concentration in the aqueous phase. HPLC analysis of ^{14}C -residues in the filtered liquid phase revealed that IPU was not degraded by the carrier particles.

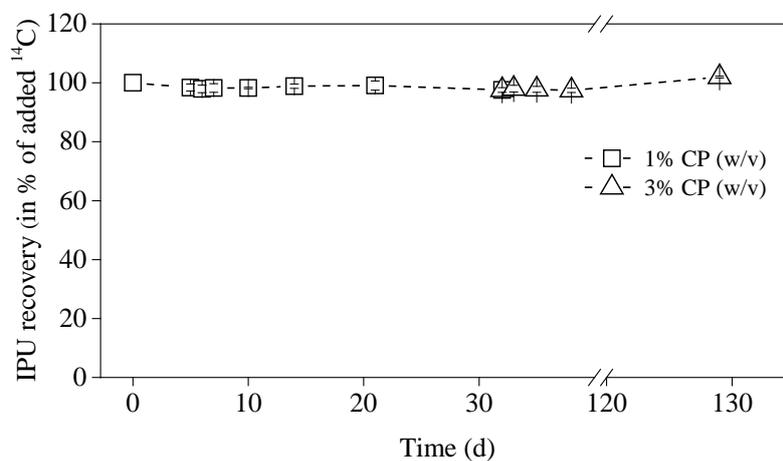


Fig. S1. Adsorption of IPU to carrier particles in sterile Milli-Q[®] water. Error bars indicate standard deviation of three replicates.

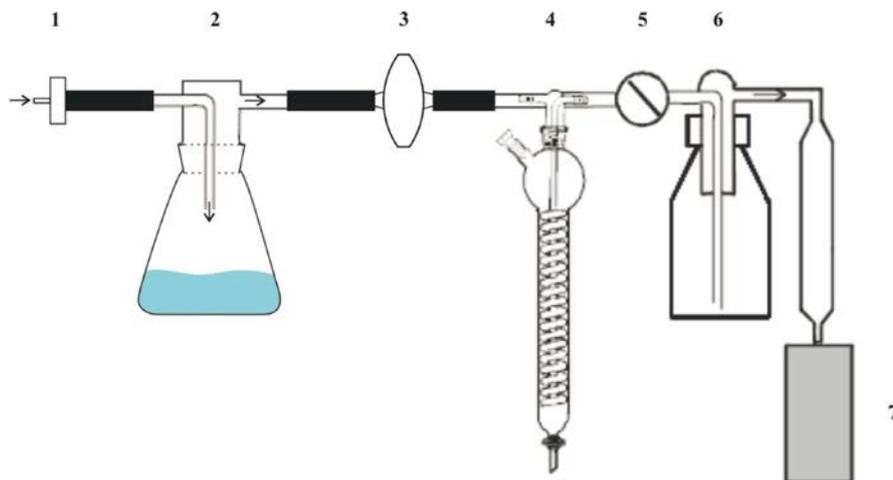
Supporting Information: Fig. S2

Fig. S2. ^{14}C - CO_2 trapping system used to aerate the liquid cultures (modified from Kiesel, 2014).

→: direction of air flow

1: sterile filter with a porosity of $0.2\ \mu\text{m}$, for filtering microbes in air

2: 100 mL Erlenmeyer flask containing 20 mL liquid culture of the microbial communities

3: sterile filter with porosity of $0.2\ \mu\text{m}$, for filtering microbes in air and connection to the flushing bottle.

The Erlenmeyer flask was closed with two clamps between **1–2** and **2–3** when the liquid culture was incubated on the shaker.

4: flushing bottle filled with 10 mL 0.1 M NaOH, for trapping ^{14}C - CO_2 from ^{14}C -IPU mineralization

5: precise regulation valve

6: Woulff bottle

7: pump

Reference

Kiesel, C. A. Enhanced Degradation of Isoproturon in Soils: Sustainability of Inoculated, Microbial Herbicide Degraders, and Adaptation of Native Microbes. Ph.D. Dissertation, Technische Universität München, Munich, Germany, 2014. <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:bvb:91-diss-20141120-1188688-0-1>

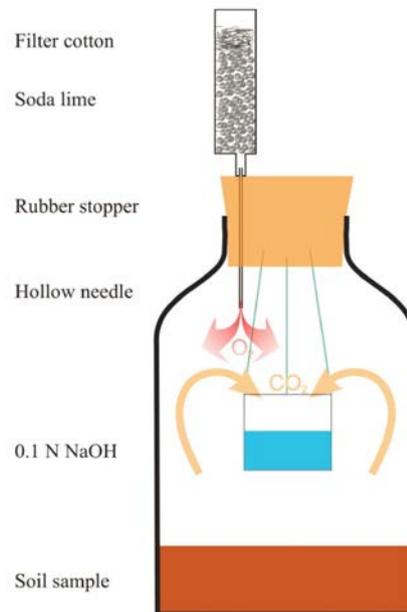
Supporting Information: Fig. S3

Fig. S3. Soil microcosm system for trapping ^{14}C - CO_2 from soil (Kiesel, 2014).

Reference

Kiesel, C. A. Enhanced Degradation of Isoproturon in Soils: Sustainability of Inoculated, Microbial Herbicide Degraders, and Adaptation of Native Microbes. Ph.D. Dissertation, Technische Universität München, Munich, Germany, 2014. <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:bvb:91-diss-20141120-1188688-0-1>

Supporting Information: Fig. S4

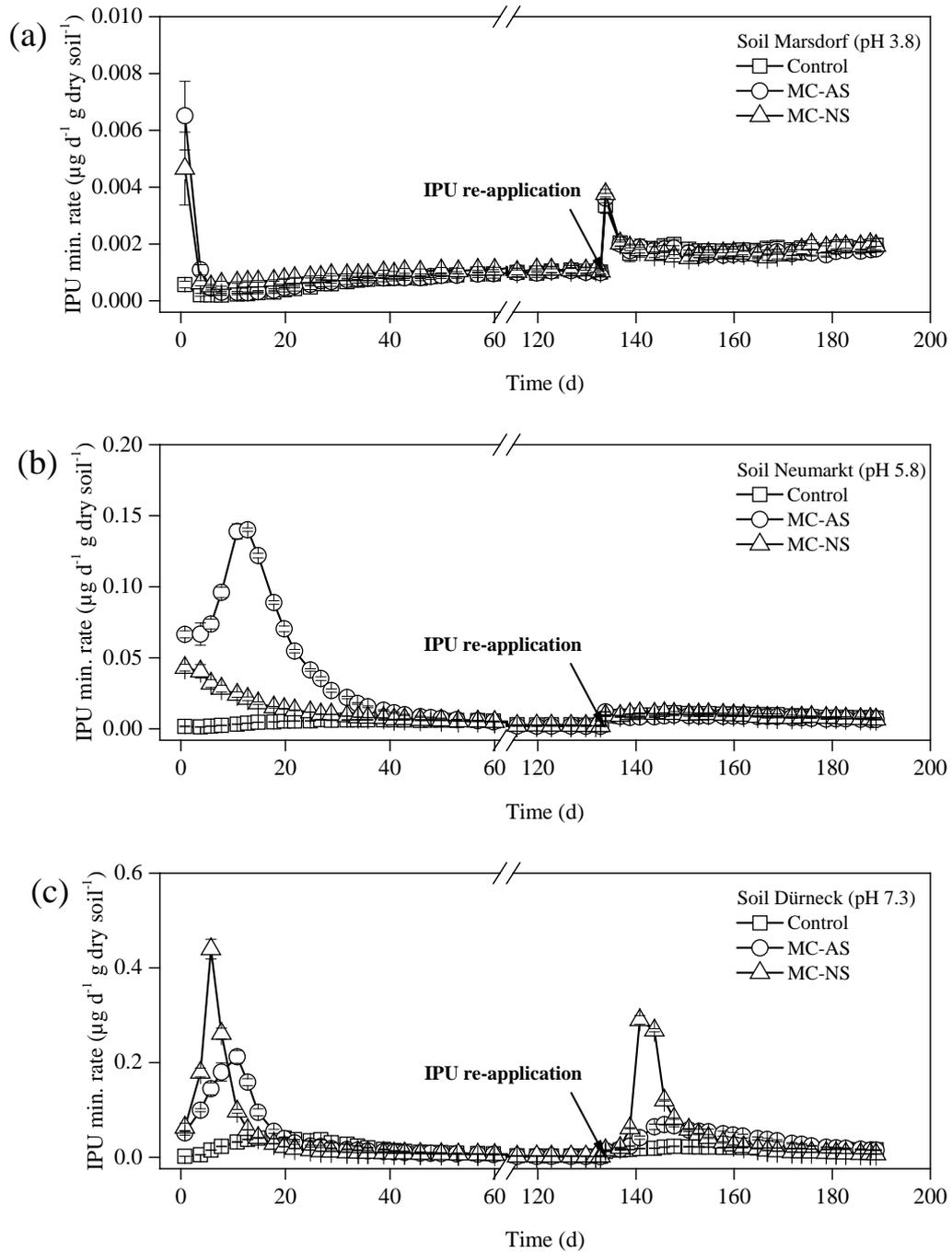


Fig. S4. IPU mineralization rates in soils Marsdorf (a), Neumarkt (b) and Dürneck (c) inoculated or uninoculated with the IPU-mineralizing microbial communities on twenty carrier particles. Error bars

represent standard deviations of four soil microcosms. Control: incubated uninoculated soil; MC-AS: the IPU-mineralizing microbial community enriched from the acidic soil Cunnersdorf; MC-NS: the IPU-mineralizing microbial community enriched from the neutral soil Feldkirchen.

Supporting Information: Table S1**Table S1**¹⁴C-IPU standards used in this study.

¹⁴ C-IPU standard	Specific radioactivity (Bq μg ⁻¹)	Experiment using the standard	IPU concentration (μg mL ⁻¹ or μg g ⁻¹)
A	6.7	Liquid culture: multiplying inocula	25
B	723	Soil microcosm experiment 1*: 1 st application	5
C	676	Soil microcosm experiment 1*: 2 nd application	5
D	326	Soil microcosm experiment 2**	5
E	17.8	IPU sorption and desorption in soil	5
F	26.0	Liquid culture: effect of pH on IPU mineralization	5

*: soil inoculated with the IPU-mineralizing microbial communities established on 20 carrier particles and incubated for 189 days.

** : soil inoculated with the IPU-mineralizing microbial communities established on 2 carrier particles and incubated for 55 days.

Supporting Information: Table S2**Table S2**

Physico-chemical characteristics of the soils originally harboring the IPU-mineralizing microbial community MC-AS (Cunnersdorf) and MC-NS (Feldkirchen) (Folberth et al., 2009).

Soil	pH CaCl ₂	Clay < 2 µm %	Silt 2–63 µm %	Sand 63– 2000µm %	TOC %	Total N %	CaCO ₃ %	Water content at –15kPa* %
Cunnersdorf	5.8	8	12	80	2.3	0.2	< 0.1	16.0
Feldkirchen	7.2	33	34	33	2.7	0.3	5.1	32.8

*: Soil moisture optimal for biodegradation (Schroll et al., 2006).

References

Folberth, C., Scherb, H., Suhadolc, M., Munch, J.C., Schroll, R., 2009. *In situ* mass distribution quotient (iMDQ) – A new factor to compare bioavailability of chemicals in soils? *Chemosphere* 75, 707–713.

Schroll, R., Becher, H.H., Dörfler, U., Gayler, S., Grundmann, S., Hartmann, H.P., Ruoss, J., 2006. Quantifying the effect of soil moisture on the aerobic microbial mineralization of selected pesticides in different soils. *Environmental Science & Technology* 40, 3305–3312.

Supporting Information Table S3

Table S3

Mineral salts media used to cultivate the two IPU-mineralizing microbial communities (Sørensen et al., 2001).

Mineral salt	Concentration (per L)
KH ₂ PO ₄	1.36 g
Na ₂ HPO ₄ * 2 H ₂ O	1.78 g
MgSO ₄ * 7 H ₂ O	0.05 g
CaCl ₂	0.01 g
H ₃ BO ₃	2.86 mg
MnSO ₄ * H ₂ O	1.54 mg
CuSO ₄ * 5 H ₂ O	0.04 mg
ZnCl ₂	0.021 mg
CoCl ₂ * 6 H ₂ O	0.041 mg
Na ₂ MoO ₄ * 2 H ₂ O	0.025 mg
pH	5.8* or 7.2**

*for the IPU-mineralizing microbial community enriched from soil Cunnersdorf (MC-AS).

**for the IPU-mineralizing microbial community enriched from soil Feldkirchen (MC-NS).

Reference

Sørensen, S.R., Ronen, Z., Aamand, J., 2001. Isolation from agricultural soil and characterization of a *Sphingomonas* sp. able to mineralize the phenylurea herbicide isoproturon. *Applied and Environmental Microbiology* 67, 5403–5409.

Supporting Information Table S4a,b**Table S4a**

Experimental design of soil microcosm experiment 1. Weight of 20 carrier particles is ≈ 0.5 g.

Treatments	Type of inoculants	Quantity of inocula	carrier to soil ratio (w/w)	No. of IPU application
Soil Marsdorf (pH 3.8)				
Control	uninoculated	–	–	2
MC-AS	carrier particles with MC-AS	0.5 g	1%	2
MC-NS	carrier particles with MC-NS	0.5 g	1%	2
Soil Neumarkt (pH 5.8)				
Control	uninoculated	–	–	2
MC-AS	carrier particles with MC-AS	0.5 g	1%	2
MC-NS	carrier particles with MC-NS	0.5 g	1%	2
Soil Dürneck (pH 7.3)				
Control	uninoculated	–	–	2
MC-AS	carrier particles with MC-AS	0.5 g	1%	2
MC-NS	carrier particles with MC-NS	0.5 g	1%	2

Table S4b

Experimental design of soil microcosm experiment 2. Weight of 20 carrier particles is ≈ 0.5 g.

Treatments	Soil	Type of inoculation	Quantity of inocula	carrier to soil (w/w)	No. of IPU application
N-MC-AS-2	Neumarkt	carrier particles with MC-AS	0.05 g	0.1%	1
D-MC-AS-2	Dürneck	carrier particles with MC-AS	0.05 g	0.1%	1
D-MC-NS-2	Dürneck	carrier particles with MC-NS	0.05 g	0.1%	1

Supporting Information: Table S5**Table S5**

HPLC gradient program for detecting IPU and IPU-metabolites.

Time (min)	Mobile phases	
	Acetonitrile (%)	10 mM NH ₄ CH ₃ CO ₂ (%)
0.0	5	95
15.0	60	40
20.0	60	40
25.0	5	95
35.0	5	95

Appendix to § 3 Results and Discussion

Appendix 1: Purification of ^{14}C -IPU

^{14}C -IPU was purified with ^{14}C -HPLC before being used in the experiments in this study. In total about 150 HPLC injections were made to obtain approximately 39 MBq ^{14}C -IPU. The radiochemical purity of the purified ^{14}C -IPU was 95.1%–98.1% (**Fig. A1**).

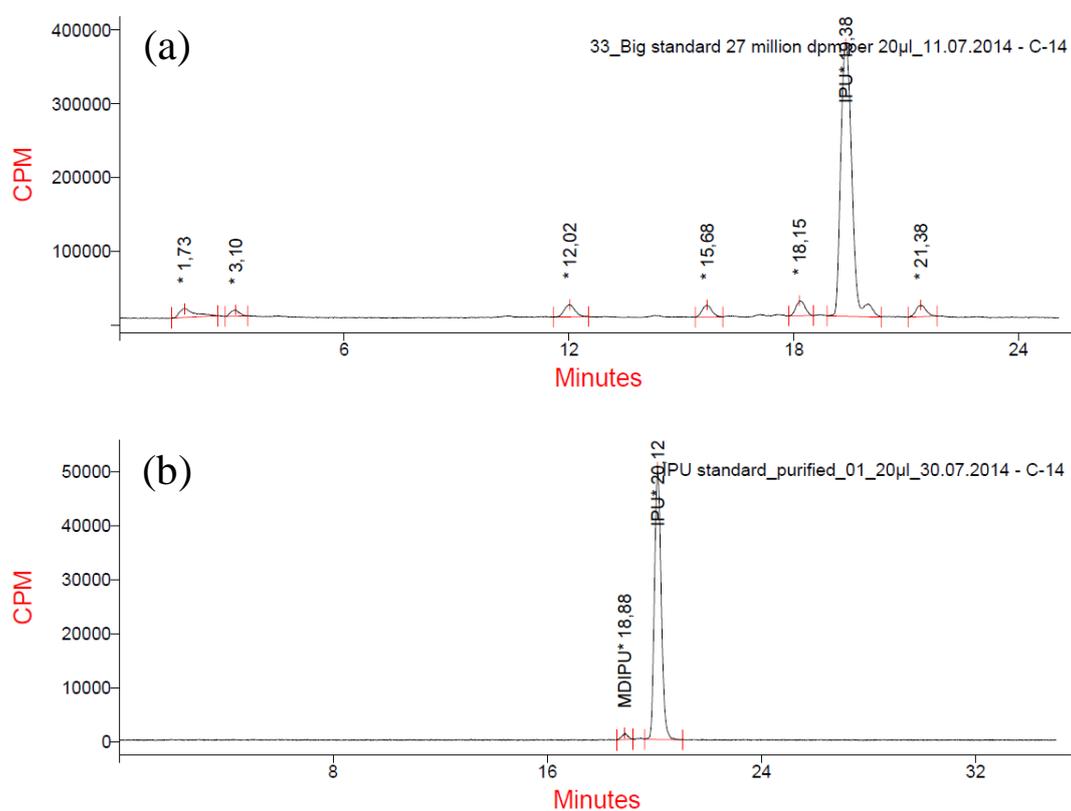


Fig. A1. Radiochemical purity of ^{14}C -IPU before (a, radiochemical purity 81.6%) and after HPLC purification (b, radiochemical purity 98.1%).

Appendix 2: IPU sorption to carrier particles

To test IPU sorption on carriers, four autoclaved carrier particles was added to 10 mL sterile ^{14}C -IPU solution ($5 \mu\text{g g}^{-1}$) to simulate the inoculation size of 20 carrier particles in 50 g soil (1%) in this study. The dry weight of 20 carrier particles is 0.52 g. The relative amount of carrier particles was increased to 3% by addition of 8 sterile carrier particles. Concentration of IPU in the liquid was measured over 129 days. As shown in **Fig. A2**, carrier particles impose a negligible impact on IPU concentration in the aqueous phase. HPLC analysis of ^{14}C -residues in the filtered liquid phase revealed that IPU was not degraded by the carrier particles.

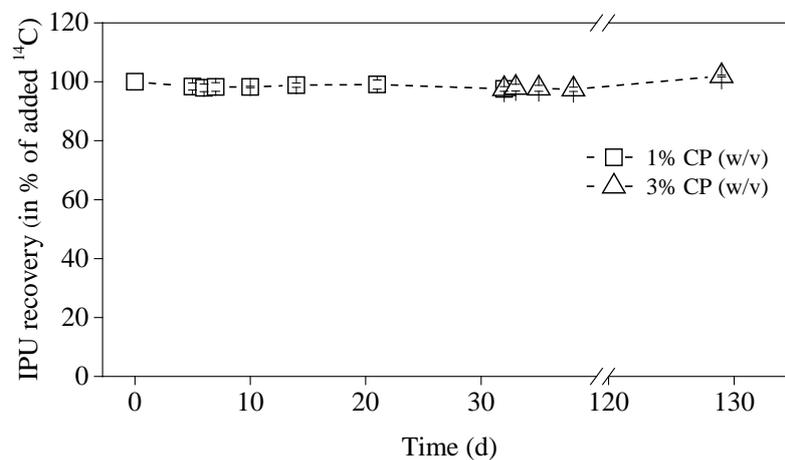


Fig. A2. Adsorption of IPU to carrier particles in sterile Milli-Q[®] water. Error bars indicate standard deviation of three replicates.

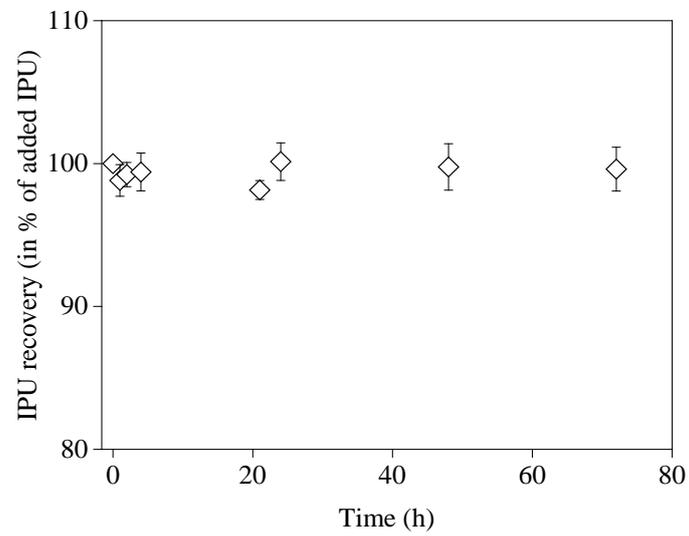
Appendix 3: IPU sorption to Teflon vials (Section 2.7)

Fig. A3. ^{14}C -IPU recovery in Teflon vials without soil. Error bars represent standard deviations of three replicates.

Appendix 4: Microbial diversity of MC-AS and MC-NS

To analyze the microbial diversity of MC-AS and MC-NS, the microbial communities established on the carrier particles were cultivated in MS-media with ^{14}C -IPU and non-labeled IPU, respectively. ^{14}C -IPU mineralization was measured in liquid cultures with ^{14}C -IPU and served as references for the IPU-mineralizing activity of MC-AS and MC-NS cultivated with non-labeled IPU, which was used for microbial community structure analysis using illumina[®] sequencing. Both microbial consortia mineralized around 50% over 28 d (**Fig. A4**). The composition of the MC-AS and MC-NS were shown in **Fig. A5**.

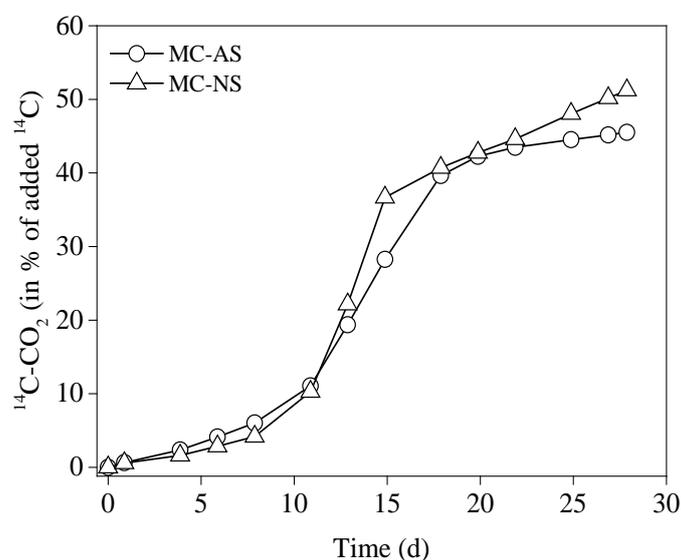


Fig. A4. ^{14}C -IPU mineralization by MC-AS and MC-NS in MS media used as references for the IPU-mineralizing function of MC-AS and MC-NS cultivated in MS-media with non-labeled IPU for DNA extraction (illumina[®] sequencing).

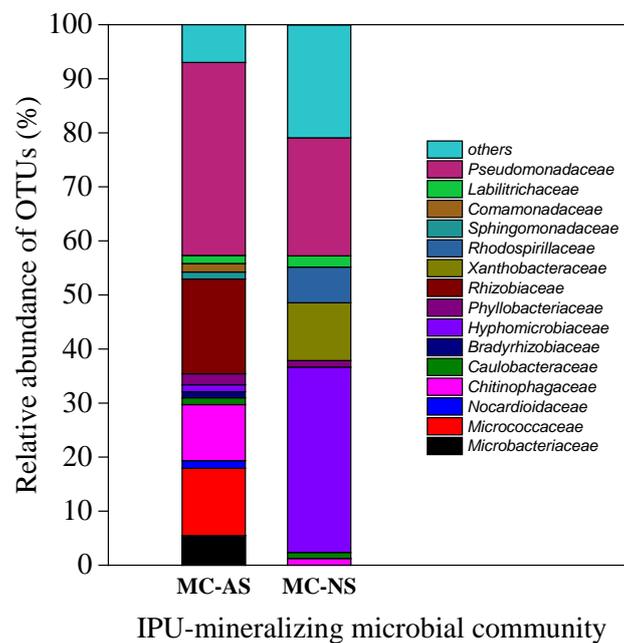


Fig. A5. Taxonomic compositions of MC-AS and MC-NS on family level determined by illumina[®] sequencing of 16S rRNA genes. The group “others” in the figure represents the OTUs with a relative abundance of < 1% and OTUs that are not annotated up to family level.

Appendix 5: ^{14}C -mass balance of Liquid culture incubation experiment II: Effect of pH (Section 2.9)

As shown in **Fig. A6**, pH determines the fate of IPU in liquid cultures of both MC-AS and MC-NS. Around 50% IPU was mineralized by MC-AS in MS-media with pH 5.8 or 7.2 and by MC-NS in MS-media with pH 7.2 or 8.0. In liquid cultures with pH beyond these two values, IPU was mineralized to a much lower degree (MC-AS-pH 8.0 and MC-NS-pH 5.8) or not mineralized (pH 3.8). Most of ^{14}C -IPU (> 88%) remained in these liquid media without being transformed or mineralized. For both MC-AS and MC-NS, the amount of ^{14}C in suspended solids and carrier particles increased with higher IPU mineralization, while ^{14}C -residues remaining in filtered liquid media decreased. Low ^{14}C -recoveries (75%–82%) were obtained in liquid cultures where IPU was mineralized intensively, while liquid cultures with low IPU mineralization showed a good ^{14}C -mass balance of 98%–104%. ^{14}C in suspended solids and carrier particles of liquid cultures with high IPU mineralization amounted to 16%–18% of initially applied ^{14}C , which was 4–7 times more than the value in liquid cultures with limited IPU mineralization.

In this experiment, suspended solids obtained by filtration (0.22 μm) were considered to be comprised of free cells living in the liquid phase. Increase of ^{14}C in suspended solids and active carrier particles suggests incorporation of C from IPU into microbial biomass. The low ^{14}C -recovery in these liquid cultures might be due loss of ^{14}C via formation of volatile ^{14}C -compounds which could not be trapped by NaOH during aeration of the liquid cultures. The poor recoveries of ^{14}C -IPU in liquid culture has also been measured in other experiments (personal communication with Dr. U. Dörfler). Unfortunately, no proper trapping solutions has been identified being able to absorb the volatile substances.

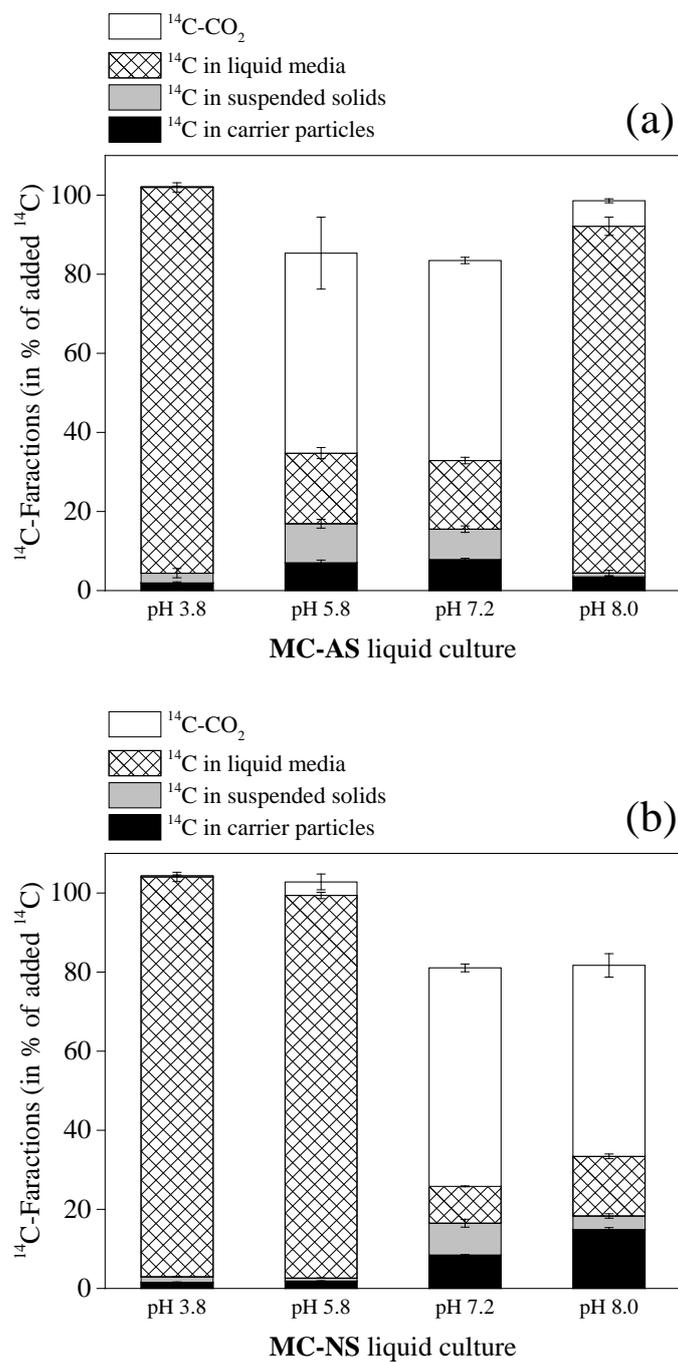


Fig. A6. Fate of ^{14}C -IPU in liquid cultures of MC-AS (a) and MC-NS (b) after incubation for 31 d. Error bars indicate standard deviations of three replicates.

Appendix 6: Functional sustainability of MC-NS in soil “Konjšče”

Soil “Konjšče” inoculated with MC-NS established on two carrier particles (soil-carrier ratio 0.1%) displayed a sustainable IPU-mineralizing activity over 189 d. The maximal IPU mineralization rate ($0.45 \mu\text{g d}^{-1} \text{g dry soil}^{-1}$) detected after re-application of IPU was even higher than the maximal rate ($0.36 \mu\text{g d}^{-1} \text{g dry soil}^{-1}$) measured after initial IPU application (**Fig. A7**). Within 57 d after the 1st and 2nd IPU application, similar amounts of IPU were mineralized (60%–65%) in the soil (**Fig. A8**). On day 189 only 4% of totally added ^{14}C was extractable; the ^{14}C -mass balance was 100% (**Fig. A9**). This persistent and stable IPU-mineralizing function suggest that the IPU-metabolic traits introduced into the soil via the microbial community (MC-NS) could maintain in the soil microbiome, either remaining in the original hosting microbes or being transferred to the endogenous soil microorganisms. In a former study it was shown that the IPU-mineralizing activity of MC-NS could remain stable in another agricultural soil over 322 d (Kiesel, 2014). The sustainable IPU-mineralizing activity shows that the decrease of IPU mineralizing rate in the soil treated with MC-NS via the various inoculating approaches (**Part I, Fig. 1b**) was not because of the decay of IPU degrader cells, but the decrease of IPU availability.

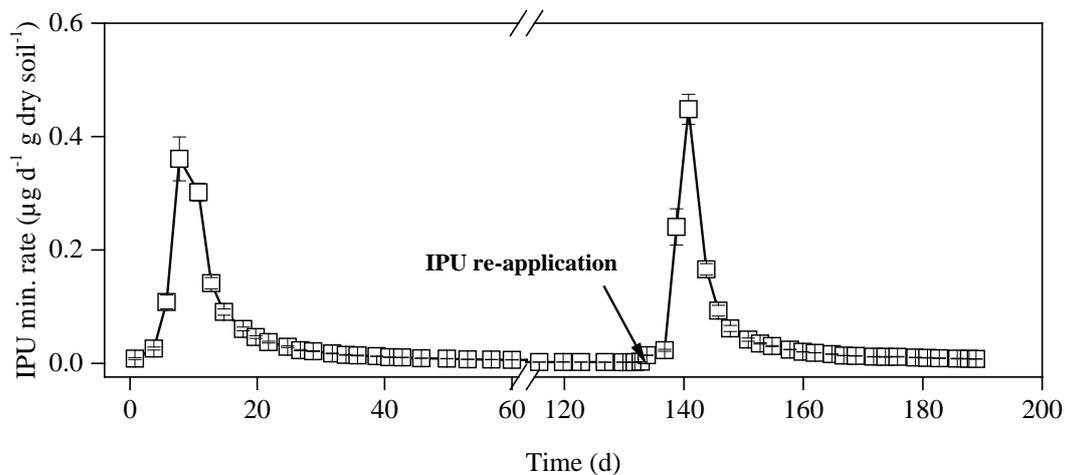


Fig. A7. ^{14}C -IPU mineralization rate during 189 d in soil Konjišče inoculated with MC-NS at a carrier-soil ratio of 0.1% (2 carrier particles in 50 g dw soil). ^{14}C -IPU was applied to the soil on day 0 and day 133. Error bars indicate standard deviations of four replicates.

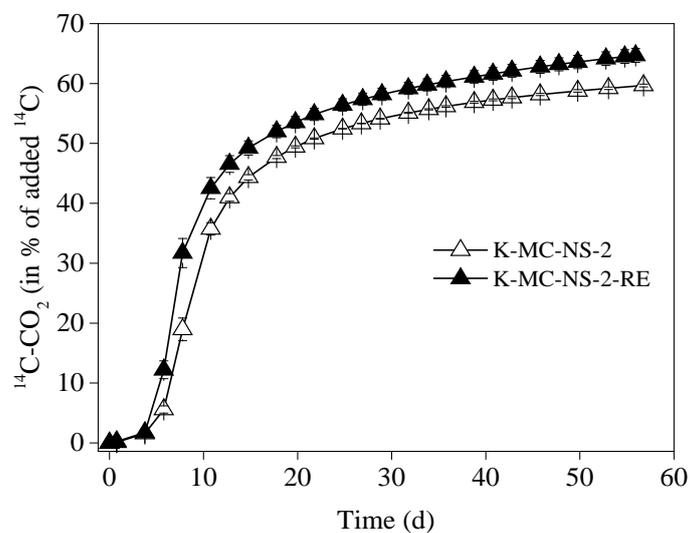


Fig. A8. ^{14}C -IPU mineralization during 57 d after initial application (on day 0, open triangles) and re-application (on day 133, closed triangles) of ^{14}C -IPU in soil Konjišče inoculated with MC-NS at a carrier-soil ratio of 0.1%. Error bars indicate standard deviations of four replicates.

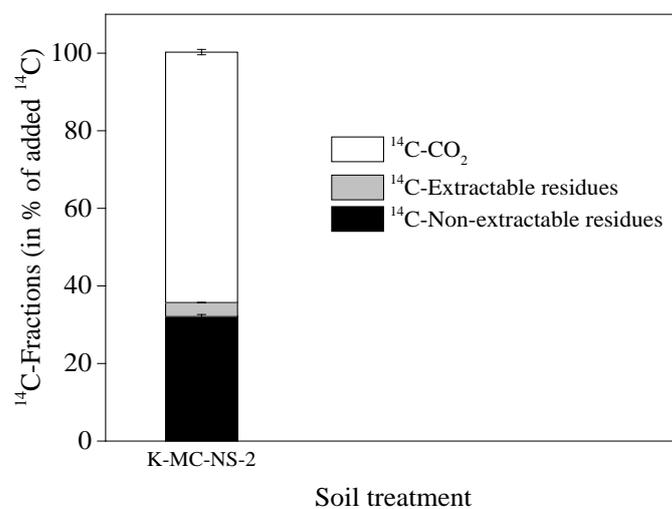


Fig. A9. Fate of ¹⁴C-IPU after 189 d in soil Konjišče inoculated with MC-NS at a carrier-soil ratio of 0.1%. ¹⁴C-IPU was applied to the soil twice, on day 0 and day 133. Error bars indicate standard deviations of four replicates.

4 General Discussion and Conclusion

In addition to the discussion about the specific experimental results in the two papers in *Chapter 3*, a general discussion and summary were made to address the scientific and societal issues within the research realm of this study.

4.1 Implications for bioaugmentation of soil pollution with organic chemicals

In Publication I (*Chapter 3*), a highly promising approach to speed up IPU degradation in agricultural soil was identified. This inoculant, consisting of a complex microbial consortium established on carrier particles, was formulated by considering the advantages of using carrier material and microbial consortia in soil bioaugmentation (*Section 1.4.1* and *Section 1.4.2*). The results of Publication I clearly showed that compared to other inoculants, the community-carrier combination degraded IPU more exhaustively and reduced the risks of non-extractable pesticide residues to a minimal level (decreased amount and biogenic properties). The inoculant also displayed a highly sustainable and stable IPU mineralizing function in different soils (Kiesel, 2014; Li et al., 2016; Li et al., 2017). In order to generalize about the universal efficacy of this approach in degradation of other chemicals, the effectiveness of this approach for soil bioaugmentation was studied with three contrasting substances that can become soil pollutants, namely 1,2,4-TCB, IPU and sulfamethazine (a veterinary antibiotic); in all cases, the microbial community established on carrier particles demonstrated significantly higher biodegradative capacity than other inoculants (Wang et al., 2010; Hirth et al., 2016; Li et al., 2017). These studies with different types of organics suggest that the superiority of microbial–community–carrier–particle complex in soil bioaugmentation is probably valid for other organic contaminants. Therefore, it is suggested that this approach can be considered as a potential priority approach when designing bioremediation remedies to decontaminate soil polluted by organics.

In Publication II (*Chapter 3*), it is investigated that the efficacy, functional stability, the economically related issue (inoculation size in terms of carrier usage per unit of polluted soil) and the abiotic factors influencing the remediation effectiveness of the highly promising approach, “microbial-community-carrier-particle” identified in Publication I. The capability of the first-reported IPU-mineralizing microbial community enriched from an acidic soil, MC-AS, was examined in this study. The results in Publication II demonstrated a high potential of the carrier-based approach using the two microbial communities to enhance IPU degradation in different soils. pH was determined as an important factor controlling IPU degradation in soils by the two microbial communities. Specific tolerable pH ranges for IPU degradation was identified for MC-AS and MC-NS, respectively. The results with reduced inoculation size showed that similar IPU degradation can be achieved with considerably less inoculants, which gives some hints about the cost-effectiveness of this approach in practical application. Publication II demonstrated that when the abiotic properties of polluted soil fit in the biological requirements of inoculant microorganisms (e.g., pH in IPU degradation by MC-AS and MC-NS), the effectiveness of bioaugmentation could be maximized. Therefore, bioaugmentation strategies should be designed according to both the eco-physiological requirements of the microbial inoculants and the physico-chemical characteristics of the contaminated environment (“tailored bioaugmentation”). This conclusion is corroborated recently by a new study on a soil-bacterium compatibility model in soil bioremediation (Horemans et al., 2016). Additionally, the importance of microbial diversity is also underlined indirectly by this study: while MC-NS showed ineffectiveness in IPU degradation under acidic conditions (e.g., soil with pH 5.8), a distinct microbial community from an acidic soil, MC-AS, had its optima to degrade IPU at the same pH.

4.2 Pros and cons of the microbial-community-carrier-particle approach

Unlike no other, the research conducted in this study has its own merits and defects. Below listed the advantages and disadvantages of the microbial-community-carrier particle approach in the context of this thesis.

Pros: The carrier-based microbial ecological approach showed highly efficient and stable pollutant-degradative activity, which are essential qualities for successful bioremediation. Moreover, as a top-down approach, to use complex soil microbial communities as a whole in bioaugmentation saves considerable time- and labor-input in isolation of specific degraders due to culturability problems. Isolation of degrader strains from soil can be tedious and unpredictable because soil microbiome is an ecological inter-connected community. The survival and growth of a degrader bacterium may depend on many co-existing microorganisms. Isolation can also be impeded by special nutrient requirements that cannot be fulfilled by the isolation media and/or specific conditions for growth. In general, the composition of substrates (SOM and mineral nutrients), soil microhabitats and soil microbiome are highly diverse and complex, which could deteriorate the isolation of specific microorganisms from soil. For instance, *Sphingomonas* sp. strain AK1, an IPU-degrader in the soil community MC-NS, was isolated approximately ten years after the microbial community was enriched from the soil. If one would only keen on using isolated key degrader strains in bioaugmentation, the availability of the effective IPU-degradative inoculant would have to be significantly postponed due to isolation and identification processes. Moreover, provided that the key degraders are available, the efficacy of isolated strains in bioaugmentation will likely be lower than microbial communities, as shown in degradation of 1,2,4-TCB, IPU and sulfamethazine in soil (Wang et al., 2010; Hirth et al., 2016; Li et al., 2017). As a result, with respect to soil bioaugmentation, microbial communities have many advantages that isolated degrader strains may not have. Even though the mechanisms of superior degradative potential of the microbial-community-carrier-particle complex are not explicitly clear up to now, it is an efficient, stable and cost-effective bioaugmentation approach.

Cons: A major challenge of the microbial-community-carrier approach is the difficulty in quantifying the inoculants, in terms of cell counts of each constituent microorganisms. In this research, the cell numbers in the four inoculant variants (Publication I) and the two IPU-mineralizing microbial consortia (Publication II) were not measured, since the focus of this study is the function (IPU degradative potential) of the inoculants rather than the growth. Nevertheless, IPU-mineralizing activity was adopted to rationalize the comparison between the inoculants. The rationale for using IPU-mineralizing activity

instead of cell numbers has been discussed circumstantially in the methodological part of Publication I (*Soil incubation and measurement of ^{14}C -IPU mineralization*), therefore, not stated tautologically here. In Publication II, DNA of the inoculants (the “active” particles) was quantified to give information about the relative biomass and inoculum density. The microbial communities were introduced to soils when they showed similar cumulative IPU mineralization and IPU-mineralization rates to ensure the comparability of the two microbial-community inoculants.

When conducting soil bioaugmentation experiments, usually cell numbers in the inocula are given to indicate the biomass and inoculum density (e.g., 10^5 inoculant cells per g soil). For well-dispersed liquid culture of bacterial monocultures, cells can be quantified properly by agar plating, microscopy or flow cytometry with assistance of relevant cell staining technologies (e.g., FISH fluorescence *in situ* hybridization). However, for a complex community growing in the form of biofilm, these methods can become cumbersome because of culturability, cell dispersion and cells extraction problems. Therefore, quantification of DNA was used as an alternative index of inoculant biomass (Elliott et al., 2010). In Publication II, the inoculants were microbial communities colonized on highly porous carrier particles in the form of biofilm (Kühn, 2004). Therefore, the quantity of DNA was used instead of cell numbers to indicate the inoculation size. Furthermore, neither the cell number nor quantity of DNA gives information about the IPU-metabolic activity of the inoculants upon inoculation. Thus, to allow a rational comparison of the two inoculants. The microbial communities were cultivated in MS-media with the same ingredients composition and IPU concentration and inoculated to the soils when they display similar cumulative IPU mineralization and IPU mineralization rates. By doing so, the IPU mineralizing potential of the two inoculant are well-comparable (see Fig. 5a,b, MC-AS-pH 5.8 and MC-NS-pH 7.2 of Publication II).

4.3 Future research topics

Besides the research conducted in this thesis, several other studies could be conducted regarding the two IPU-mineralizing microbial communities. Below are some examples:

(1) Testing the IPU degradative potential of MC-AS in more acidic agricultural soils (e.g., pH rang 5.0–6.5) to verify the capability of MC-AS to clean up IPU in acidic soils and to look into the soil parameters controlling IPU degradation by MC-AS (e.g., to verify, among the many soil physico-chemical properties, whether pH is the determinant factor for IPU degradation by MC-NS). Generally, acidic soils are not used for agriculture, more for silviculture or perhaps grassland. pH of arable soils should be kept sufficiently high (pH > 5) usually by liming to exclude toxicity of metal cations (e.g., Al³⁺, Mn²⁺) to plants (Blume et al., 2016). Therefore, occurrence of IPU in acidic soil should be rare. It would be only necessary to test the capacity of MC-AS in arable soils with pH ranging from 5 to 6.5, the pH range for IPU degradation which has not been covered by the already-obtained IPU-degraders (**Table 3**).

(2) Isolating the IPU-key degrader strain from MC-AS and identifying the IPU-metabolic pathway of this specific strain. Up to date there is no report about IPU-degrader strain isolated from acidic soils. Isolation of the key degrader strain from MC-AS may lead to discovery of a new IPU-metabolic pathway and the novel degradative genes involved.

(3) Identification of genes responsible for IPU mineralization (especially IPU ring cleavage). This kind of study will allow designing specific FISH probes or PCR primers for tracking the proliferation and activity of the specific IPU degraders introduced into the environment.

4.4 My opinion about environmental pollution resulting from agrochemicals

The pesticide dilemma seems inevitable in agriculture (except organic agriculture). Pesticides are essential for conventional agriculture to secure yield, but immediately upon application on the field the active substances form a major diffusion pollution which may exert severe threats on environmental and human health. Pesticides ensure the productivity but ruin food quality (e.g., due to intake of pesticide residues by crops or remaining pesticides residues on plant tissues) and quality of other living resources (e.g., drinking water). It would be necessary to encourage agriculture to produce less but healthier food rather than excess but low quality food, especially when food supply overreach its requirements by current agricultural technologies (Hiç et al., 2016). Even though it is perceived that the global food

deficit is because of food distribution or accessibility problem rather than food availability (productivity), to forbidden pesticides in agriculture is unrealistic. However, mitigation strategies are asked for to alleviate the risks of pesticides, such as reducing pesticide utilization by validating the application rate and application frequency, promoting pesticides with low toxicity and higher degradability or using biocontrol methods etc. Finally, as an environmental scientist it is important to keep in mind that

*All environmental technologies should be viewed as the last resort to cure environmental pollution, but not the reason to allow pollution; in the long run, **prevention is always better than remediation.***

References

- Albareda, M., Rodríguez-Navarro, D.N., Camacho, M., Temprano, F.J., 2008. Alternatives to peat as a carrier for rhizobia inoculants: Solid and liquid formulations. *Soil Biology and Biochemistry* 40, 2771–2779.
- Alletto, L., Coquet, Y., Benoit, P., Bergheaud, V., 2006. Effects of temperature and water content on degradation of isoproturon in three soil profiles. *Chemosphere* 64, 1053–1061.
- Bending, G.D., Lincoln, S.D., Sørensen, S.R., Morgan, J.A.W., Aamand, J., Walker, A., 2003. In-Field Spatial variability in the degradation of the phenyl-urea herbicide isoproturon is the result of interactions between degradative *Sphingomonas* spp. and soil pH. *Applied and Environmental Microbiology* 69, 827–834.
- Blume, H.-P., Brümmer, G.W., Fleige, H., Horn, R., Kandeler, E., Kögel-Knabner, I., Kretschmar, R., Stahr, K., Wilke, B.-M., 2016. Scheffer/Schachtschabel Soil Science. Springer, Berlin/Heidelberg, pp. 163–164.
- Boivin, A., Cherrier, R., Schiavon, M., 2005. A comparison of five pesticides adsorption and desorption processes in thirteen contrasting field soils. *Chemosphere* 61, 668–676.
- Brenner, K., You, L., Arnold, F.H., 2008. Engineering microbial consortia: a new frontier in synthetic biology. *Trends in Biotechnology* 26, 483–489.
- Commission Implementing Regulation (EU) No 2016/872 of 1 June 2016 concerning the non-renewal of approval of the active substance isoproturon, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending Commission Implementing Regulation (EU) No 540/2011. Official Journal of the European Union L 145, 2.6.2016, pp. 7–9.
- Cullington, J.E., Walker, A., 1999. Rapid biodegradation of diuron and other phenylurea herbicides by a soil bacterium. *Soil Biology and Biochemistry* 31, 677–686.

- De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., Boon, N., 2014. Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environmental Microbiology* 16, 1472–1481.
- Dejonghe, W., Berteloot, E., Goris, J., Boon, N., Crul, K., Maertens, S., Höfte, M., De Vos, P., Verstraete, W., Top, E.M., 2003. Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovorax* strain. *Applied and Environmental Microbiology* 69, 1532–1541.
- Department for Environment, Food and Rural Affairs, Pesticides Safety Directorate, 1995. Evaluation of Fully Approved or Provisionally Approve Products—Evaluation on: Isoproturon, 1995. York YO1 7PX.
- Devers-Lamrani, M., Pesce, S., Rouard, N., Martin-Laurent, F., 2014. Evidence for cooperative mineralization of diuron by *Arthrobacter* sp. BS2 and *Achromobacter* sp. SP1 isolated from a mixed culture enriched from diuron exposed environments. *Chemosphere* 117, 208–215.
- Directive 2013/39/EU of The European Parliament and of The Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Official Journal of the European Union L 226/1, 24.8.2013.
- Dwivedi, S., Singh, B.R., Al-Khedhairi, A.A., Musarrat, J., 2011. Biodegradation of isoproturon using a novel *Pseudomonas aeruginosa* strain JS-11 as a multi-functional bioinoculant of environmental significance. *Journal of Hazardous Materials* 185, 938–944.
- EFSA (European Food Safety Authority), 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance isoproturon. *EFSA Journal* 2015; 13(8): 4206, 99 pp. doi:10.2903/j.efsa.2015.4206.
- El Arfaoui, A., Sayen, S., Paris, M., Keziou, A., Couderchet, M., Guillon, E., 2012. Is organic matter alone sufficient to predict isoproturon sorption in calcareous soils? *Science of The Total Environment* 432, 251–256.

- El Sebai, T., Lagacherie, B., Soulas, G., Martin-Laurent, F., 2004. Isolation and characterisation of an isoproturon-mineralising *Methylophila* sp. TES from French agricultural soil. *FEMS Microbiology Letters* 239, 103–110.
- El Sebai, T., Lagacherie, B., Soulas, G., Martin-Laurent, F., 2007. Spatial variability of isoproturon mineralizing activity within an agricultural field: Geostatistical analysis of simple physicochemical and microbiological soil parameters. *Environmental Pollution* 145, 680–690.
- Elliott, D.R., Scholes, J.D., Thornton, S.F., Rizoulis, A., Banwart, S.A., Rolfe, S.A., 2010. Dynamic changes in microbial community structure and function in phenol-degrading microcosms inoculated with cells from a contaminated aquifer. *FEMS Microbiology Ecology* 71, 247–259.
- Enserink, M., Hines, P.J., Vignieri, S.N., Wigginton, N.S., Yeston, J.S., 2013. The pesticide paradox. *Science* 341, 728–729.
- European Union, 2016. Agriculture, forestry and fishery statistics (2015 edition). Publications Office of the European Union, Luxembourg, ISBN 978-92-79-57816-8. p. 118.
- Fenner, K., Canonica, S., Wackett, L.P., Elsner, M., 2013. Evaluating pesticide degradation in the environment: Blind spots and emerging opportunities. *Science* 341, 752–758.
- Folberth, C., Scherb, H., Suhadolc, M., Munch, J.C., Schroll, R., 2009. *In situ* mass distribution quotient (iMDQ) – A new factor to compare bioavailability of chemicals in soils? *Chemosphere* 75, 707–713.
- Furuno, S., Foss, S., Wild, E., Jones, K.C., Semple, K.T., Harms, H., Wick, L.Y., 2012. Mycelia promote active transport and spatial dispersion of polycyclic aromatic hydrocarbons. *Environmental Science & Technology* 46, 5463–5470.
- Gentry, T., Rensing, C., Pepper, I.A.N., 2004. New approaches for bioaugmentation as a remediation technology. *Critical Reviews in Environmental Science and Technology* 34, 447–494.
- Grube, Arthur; Donaldson, David; Kiely, Timothy; Wu La, 2011. Pesticides Industry Sales and Usage: 2006 and 2007 Market Estimates. Biological and Economic Analysis Division, Office of Pesticide

- Programs, Office of Chemical Safety and Pollution Prevention, U.S. Environmental Protection Agency, Washington, DC 20460. p. 8.
- Grundmann, S., Doerfler, U., Munch, J.C., Ruth, B., Schroll, R., 2011. Impact of soil water regime on degradation and plant uptake behaviour of the herbicide isoproturon in different soil types. *Chemosphere* 82, 1461–1467.
- Grundmann, S., Fuß, R., Schmid, M., Laschinger, M., Ruth, B., Schulin, R., Munch, J.C., Schroll, R., 2007. Application of microbial hot spots enhances pesticide degradation in soils. *Chemosphere* 68, 511–517.
- Gu, T., Zhou, C., Sørensen, S.R., Zhang, J., He, J., Yu, P., Yan, X., Li, S., 2013. The novel bacterial N-demethylase *pdmAB* is responsible for the initial step of N,N-dimethyl-substituted phenylurea herbicide degradation. *Applied and Environmental Microbiology* 79, 7846–7856.
- Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: Exploiting fungi in bioremediation of hazardous chemicals. *Nature Reviews Microbiology* 9, 177–192.
- Hiç, C., Pradhan, P., Rybski, D., Kropp, J.P., 2016. Food surplus and its climate burdens. *Environmental Science & Technology* 50, 4269–4277.
- Hirth, N., Topp, E., Dörfler, U., Stupperich, E., Munch, J.C., Schroll, R., 2016. An effective bioremediation approach for enhanced microbial degradation of the veterinary antibiotic sulfamethazine in an agricultural soil. *Chemical and Biological Technologies in Agriculture* 3, 29.
- Horemans, B., Breugelmans, P., Saeys, W., Springael, D., 2016. A soil-bacterium compatibility model as a decision-making tool for soil bioremediation. *Environmental Science & Technology* Just Accepted Manuscript DOI: 10.1021/acs.est.6b04956
- Hussain, S., Arshad, M., Springael, D., Sørensen, S.R., Bending, G.D., Devers-Lamrani, M., Maqbool, Z., Martin-Laurent, F., 2015. Abiotic and biotic processes governing the fate of phenylurea herbicides in soils: A review. *Critical Reviews in Environmental Science and Technology* 45, 1947–1998.

- Hussain, S., Devers-Lamrani, M., El Azhari, N., Martin-Laurent, F., 2011. Isolation and characterization of an isoproturon mineralizing *Sphingomonas* sp. strain SH from a French agricultural soil. *Biodegradation* 22, 637–650.
- Hussain, S., Devers-Lamrani, M., Spor, A., Rouard, N., Porcherot, M., Beguet, J., Martin-Laurent, F., 2013. Mapping field spatial distribution patterns of isoproturon-mineralizing activity over a three-year winter wheat/rape seed/barley rotation. *Chemosphere* 90, 2499–2511.
- Husson, O., 2013. Redox potential (Eh) and pH as drivers of soil/plant/microorganism systems: A transdisciplinary overview pointing to integrative opportunities for agronomy. *Plant and Soil* 362, 389–417.
- Jablonowski, N.D., Krutz, J.L., Martinazzo, R., Zajkoska, P., Hamacher, G., Borchard, N., Burauel, P., 2013. Transfer of atrazine degradation capability to mineralize aged ¹⁴C-labeled atrazine residues in soils. *Journal of Agricultural and Food Chemistry* 61, 6161–6166.
- Jarvis, N., 2016. Extended sorption partitioning models for pesticide leaching risk assessments: Can we improve upon the K_{oc} concept? *Science of The Total Environment* 539, 294–303.
- Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H.A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. *Environmental Health Perspectives* 104, 715–740.
- Kiesel, C. A., 2014. Enhanced Degradation of Isoproturon in Soils: Sustainability of Inoculated, Microbial Herbicide Degraders, and Adaptation of Native Microbes. Ph.D. Dissertation, Technische Universität München, Munich, Germany, <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:bvb:91-diss-20141120-1188688-0-1>.
- Kohlmeier, S., Smits, T.H.M., Ford, R.M., Keel, C., Harms, H., Wick, L.Y., 2005. Taking the fungal highway: Mobilization of pollutant-degrading bacteria by fungi. *Environmental Science & Technology* 39, 4640–4646.

- Kühn, S. S., 2004. Bedeutung der Leistung mikrobieller Lebensgemeinschaften beim Umsatz und Abbau von Isoproturon in Böden und Möglichkeiten zur Steuerung des in-situ-Pestizidabbaus. Ph.D. Dissertation, Technische Universität München, Munich, Germany. <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:bvb:91-diss2004032911796>.
- Larsen, L., Sørensen, S.R., Aamand, J., 2000. Mecoprop, isoproturon, and atrazine in and above a sandy aquifer: Vertical distribution of mineralization potential. *Environmental Science & Technology* 34, 2426–2430.
- Li, R., Dörfler, U., Munch, J.C., Schroll, R., 2017. Enhanced degradation of isoproturon in an agricultural soil by a *Sphingomonas* sp. strain and a microbial consortium. *Chemosphere* 168, 1169–1176.
- Li, R., Dörfler, U., Schroll, R., Munch, J.C., 2016. Biodegradation of isoproturon in agricultural soils with contrasting pH by exogenous soil microbial communities. *Soil Biology and Biochemistry* 103, 149–159.
- Ma, J., Ibekwe, A.M., Yang, C.-H., Crowley, D.E., 2013. Influence of bacterial communities based on 454-pyrosequencing on the survival of *Escherichia coli* O157:H7 in soils. *FEMS Microbiology Ecology* 84, 542–554.
- Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., Naidu, R., 2011. Bioremediation approaches for organic pollutants: A critical perspective. *Environment International* 37, 1362–1375.
- Moyano, F.E., Vasilyeva, N., Bouckaert, L., Cook, F., Craine, J., Curiel Yuste, J., Don, A., Epron, D., Formanek, P., Franzluebbers, A., Ilstedt, U., Kätterer, T., Orchard, V., Reichstein, M., Rey, A., Ruamps, L., Subke, J.A., Thomsen, I.K., Chenu, C., 2012. The moisture response of soil heterotrophic respiration: Interaction with soil properties. *Biogeosciences* 9, 1173–1182.
- OECD, 2000. Adsorption–Desorption Using a Batch Equilibrium Method 106. Guidelines for the Testing of Chemicals. Organisation of Economic Co-operation and Development, Paris, pp. 1–45.

- Orchard, V.A., Cook, F.J., 1983. Relationship between soil respiration and soil moisture. *Soil Biology and Biochemistry* 15, 447–453.
- Owsianiak, M., Dechesne, A., Binning, P.J., Chambon, J.C., Sørensen, S.R., Smets, B.F., 2010. Evaluation of bioaugmentation with entrapped degrading cells as a soil remediation technology. *Environmental Science & Technology* 44, 7622–7627.
- Paterson, S., Mackay, D., Tam, D., Shiu, W.Y., 1990. Uptake of organic chemicals by plants: A review of processes, correlations and models. *Chemosphere* 21, 297–331.
- Qiu, J., Chen, G., Xu, J., Luo, E., Liu, Y., Wang, F., Zhou, H., Liu, Y., Zhu, F., Ouyang, G., 2016. In vivo tracing of organochloride and organophosphorus pesticides in different organs of hydroponically grown malabar spinach (*Basella alba* L.). *Journal of Hazardous Materials* 316, 52–59.
- Schamfuß, S., Neu, T.R., van der Meer, J.R., Tecon, R., Harms, H., Wick, L.Y., 2013. Impact of mycelia on the accessibility of fluorene to PAH-degrading bacteria. *Environmental Science & Technology* 47, 6908–6915.
- Schjønning, P., Thomsen, I.K., Moldrup, P., Christensen, B.T., 2003. Linking soil microbial activity to water- and air-phase contents and diffusivities. *Soil Science Society of America Journal* 67, 156–165.
- Schroll, R., Becher, H.H., Dörfler, U., Gayler, S., Grundmann, S., Hartmann, H.P., Ruoss, J., 2006. Quantifying the effect of soil moisture on the aerobic microbial mineralization of selected pesticides in different soils. *Environmental Science & Technology* 40, 3305–3312.
- Schroll, R., Kühn, S., 2004. Test system to establish mass balances for ¹⁴C-labeled substances in soil–plant–atmosphere systems under field conditions. *Environmental Science & Technology* 38, 1537–1544.
- Sebäi, T., Devers-Lamrani, M., Lagacherie, B., Rouard, N., Soulas, G., Martin-Laurent, F., 2011. Isoproturon mineralization in an agricultural soil. *Biology and Fertility of Soils* 47, 427–435.

- Shi, S., Bending, G.D., 2007. Changes to the structure of *Sphingomonas* spp. communities associated with biodegradation of the herbicide isoproturon in soil. *FEMS Microbiology Letters* 269, 110–116.
- Skopp, J., Jawson, M.D., Doran, J.W., 1990. Steady-state aerobic microbial activity as a function of soil water content. *Soil Science Society of America Journal* 54, 1619–1625.
- Sonia Rodríguez-Cruz, M., Jones, J.E., Bending, G.D., 2006. Field-scale study of the variability in pesticide biodegradation with soil depth and its relationship with soil characteristics. *Soil Biology and Biochemistry* 38, 2910–2918.
- Sørensen, S.R., Ronen, Z., Aamand, J., 2001. Isolation from agricultural soil and characterization of a *Sphingomonas* sp. able to mineralize the phenylurea herbicide isoproturon. *Applied and Environmental Microbiology* 67, 5403–5409.
- Sørensen, S.R., Ronen, Z., Aamand, J., 2002. Growth in coculture stimulates metabolism of the phenylurea herbicide isoproturon by *Sphingomonas* sp. strain SRS2. *Applied and Environmental Microbiology* 68, 3478–3485.
- Spark, K.M., Swift, R.S., 2002. Effect of soil composition and dissolved organic matter on pesticide sorption. *Science of The Total Environment* 298, 147–161.
- Stark, J.M., Firestone, M.K., 1995. Mechanisms for soil moisture effects on activity of nitrifying bacteria. *Applied and Environmental Microbiology* 61, 218–221.
- Suhadolc, M., Schroll, R., Gattinger, A., Schloter, M., Munch, J.C., Lestan, D., 2004. Effects of modified Pb-, Zn-, and Cd- availability on the microbial communities and on the degradation of isoproturon in a heavy metal contaminated soil. *Soil Biology and Biochemistry* 36, 1943–1954.
- Sun, D., Hale, L., Crowley, D., 2016. Nutrient supplementation of pinewood biochar for use as a bacterial inoculum carrier. *Biology and Fertility of Soils*, 1–8.
- Sun, J.-Q., Huang, X., Chen, Q.-L., Liang, B., Qiu, J.-G., Ali, S., Li, S.-P., 2009. Isolation and characterization of three *Sphingobium* sp. strains capable of degrading isoproturon and cloning of

- the catechol 1,2-dioxygenase gene from these strains. *World Journal of Microbiology and Biotechnology* 25, 259–268.
- Tixier, C., Sancelme, M., Ait-Aïssa, S., Widehem, P., Bonnemoy, F., Cuer, A., Truffaut, N., Veschambre, H., 2002. Biotransformation of phenylurea herbicides by a soil bacterial strain, *Arthrobacter* sp. N2: Structure, ecotoxicity and fate of diuron metabolite with soil fungi. *Chemosphere* 46, 519–526.
- Tyagi, M., da Fonseca, M.M., de Carvalho, C.C.R., 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation* 22, 231–241.
- United States Environmental Protection Agency, Office of Solid Waste and Emergency Response, 2013. Superfund Remedy Report, Fourteenth Edition. Washington, D.C. 20460.
- United States Environmental Protection Agency, Technology Innovation and Field Services Division, Contaminated Site Clean-Up Information (CLU-IN) web site, <https://clu-in.org/techfocus/default.focus/sec/Bioremediation/cat/Overview/>.
- Van Dyke, M.I., Prosser, J.I., 2000. Enhanced survival of *Pseudomonas fluorescens* in soil following establishment of inoculum in a sterile soil carrier. *Soil Biology and Biochemistry* 32, 1377–1382.
- van Elsas, J.D., Chiurazzi, M., Mallon, C.A., Elhottová, D., Křišťůfek, V., Salles, J.F., 2012. Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proceedings of the National Academy of Sciences* 109, 1159–1164.
- van Veen, J.A., van Overbeek, L.S., van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews* 61, 121–135.
- Vieublé-Gonod, L., El Arfaoui, A., Benoit, P., 2016. Impact of spatial distribution of exogenous organic matter on C mineralization and isoproturon fate in soil. *Soil Biology and Biochemistry* 95, 180–188.
- Vieublé-Gonod, L., Benoit, P., Cohen, N., Houot, S., 2009. Spatial and temporal heterogeneity of soil microorganisms and isoproturon degrading activity in a tilled soil amended with urban waste composts. *Soil Biology and Biochemistry* 41, 2558–2567.

- Walker, A., Jurado-Exposito, M., Bending, G.D., Smith, V.J.R., 2001. Spatial variability in the degradation rate of isoproturon in soil. *Environmental Pollution* 111, 407–415.
- Wang, F., Dörfler, U., Schmid, M., Fischer, D., Kinzel, L., Scherb, H., Munch, J.C., Jiang, X., Schroll, R., 2010. Homogeneous inoculation vs. microbial hot spots of isolated strain and microbial community: What is the most promising approach in remediating 1,2,4-TCB contaminated soils? *Soil Biology and Biochemistry* 42, 331–336.
- Wang, F., Fekete, A., Harir, M., Chen, X., Dörfler, U., Rothballer, M., Jiang, X., Schmitt-Kopplin, P., Schroll, R., 2013. Soil remediation with a microbial community established on a carrier: Strong hints for microbial communication during 1,2,4-Trichlorobenzene degradation. *Chemosphere* 92, 1403–1409.
- Wick, L.Y., Remer, R., Würz, B., Reichenbach, J., Braun, S., Schäfer, F., Harms, H., 2007. Effect of fungal hyphae on the access of bacteria to phenanthrene in soil. *Environmental Science & Technology* 41, 500–505.
- Widehem, P., Aït-Aïssa, S., Tixier, C., Sancelme, M., Veschambre, H., Truffaut, N., 2002. Isolation, characterization and diuron transformation capacities of a bacterial strain *Arthrobacter* sp. N2. *Chemosphere* 46, 527–534.
- Yu, X.-Y., Ying, G.-G., Kookana, R.S., 2009. Reduced plant uptake of pesticides with biochar additions to soil. *Chemosphere* 76, 665–671.

Acknowledgement

First of all, I would like to thank my *Doktorvater*, Prof. Jean Charles Munch, for his continuous and strong support during my PhD study. Without your support there would be no chance for me to obtain the DAAD fellowship and further to realize the doctoral work. Thank you for your support and help in scholarship extensions, manuscript revision, thesis writing and thesis reviewing procedure. Especially at the end of my PhD your support is one of the strongest power enabling me pass through all the difficulties. Thank you!

I would like to thank Dr. Reiner Schroll for offering me this extremely interesting research topic and the access to all lab facilities and consumables without limitations to realize my work. Thank you also for your helpful discussion about experiments and results. Especially thank you for the effort to keep the lab running during the difficult times.

I would like to give my thanks especially to Dr. Ulrike Dörfler for her endless help in all aspects of my life in Germany: your help with experiments, manuscript writing and revision, your encouragement when dark time came etc. Thank you for applying for the student flats for me, the living consumable to start life in München, driving my family to the airport, the reference letters... All your help and kindness are unforgettable. Thank you!

I want to thank Natalie Hirth, my officemate, for her accompanying during the doctoral study, your contribution to the lab issues and your help in my daily life (baby-sitting, German translating, telephone calling, bike...). Your optimism and encouragement turns dark bright and bright brighter. It is my great pleasure and my great luck to have you as a colleague and share the same office. I would also like to give my gratitude to Hanyin Sun, Jose Rodrigo and Xin Jin for their accompanying. It is a lot fun and gaining experience to talk with you guys about science and life. Patrick Weiss, thank you very much for your help with the laboratory instruments and ordering, and also for your nice barbecue parties. Angelo Weiss and Dr. Michael Rothballer, thank you a lot for your help with 16S rDNA analysis and FISH.

Last but not least, I would like to give my thanks to Prof. Dr. Rainer Matyssek and Prof. Dr. Siegfried Scherer from TUM and Prof. Jan Roelof van der Meer from University of Lausanne for serving as my thesis examining committee and for carrying out the examining process so efficiently.

Finalized on 02 January 2017

Chavannes-près-Renens (Lausanne), Switzerland

Curriculum Vitae

Personal information

Name: Li, Renyi
Address: Chavannes-près-Renens (Lausanne), 1022 Switzerland
E-Mail: renyili2009@gmail.com
Phone: +41 078 636 0586
Date and place of birth: 18.11.1986, Hubei, China
Family status: married (one son)
Nationality: Chinese

Education

04.2013–09.2016 Helmholtz Zentrum München / Technische Universität München, München, Germany. PhD Biology, speciality Soil Ecology (graduate with Magna Cum Laude)
09.2008–06.2011 Huazhong Agricultural University, Wuhan, China. Master's degree Soil Science, speciality Soil Microbiology
09.2004–07.2008 Yangtze University, Jingzhou, China. Bachelor's degree Biology, speciality Biotechnology (graduate with honor)

Award

04.2013–10.2016 DAAD (German Academic Exchange Service) Research Grant for Doctoral Candidates and Young Academics and Scientists (more than 6 months), Grant No. 57076385

Training

04.2013–09.2016 Helmholtz Graduate School qualification programs (HELENA Graduate School, in cooperation with TUM Graduate School)
09.2012–01.2013 German language course, Deutschkolleg, Tongji University, Shanghai, China (DAAD Scholarship)

Professional experience

- 11.2016– Postdoc, Department of Fundamental Microbiology, University of Lausanne,
Lausanne, Switzerland
- 04.2013–09.2016 Research assistant, Institute of Soil Ecology, Helmholtz Zentrum München,
Munich, Germany
- 11.2011- 08.2012 Part-time job in local business (water purification systems), Guangxi, China
- 07.2011–10.2011 Research assistant, Department of Plant Nutrition, College of Resources and
Environmental Sciences, China Agricultural University, Beijing, China