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Inoculation of soil with an Isoproturon degrading microbial community reduced the pool of "real non-extractable" Isoproturon residues

Xiaomin Zhu[a,b,c,*], Reiner Schroll[b], Ulrike Dörfler[b], Baoliang Chen[a,*]

[a] Department of Environmental Science, Zhejiang University, Hangzhou 310058, China
[b] Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany
[c] University of Chinese Academy of Sciences, Beijing 100036, China

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ABSTRACT

During pesticides degradation, biogenic non-extractable residues ("apparent NER") may not share the same environmental fate and risks with the "real NER" that are bound to soil matrix. It is not clear how microbial community (MC) inoculation for pesticides degradation would influence the NER composition. To investigate degradation efficiency of pesticides Isoproturon (IPU) and NER composition following MC inoculation, clay particles harboring MC that contains the IPU degrading strain, *Sphingomonas* sp., were inoculated into soil receiving ^{14}C-labeled IPU addition. Mineralization of IPU was greatly enhanced with MC inoculation that averagely 55.9% of the applied ^{14}C-IPU was consumed up into $^{14}CO_2$ during 46 days soil incubation. Isoproturon degradation was more thorough with MC than that in the control: much less amount of metabolic products (4.6% of applied IPU) and NER (35.4%) formed in MC treatment, while the percentages were respectively 30.3% for metabolites and 49.8% for NER in the control. Composition of NER shifted with MC inoculation, that relatively larger amount of IPU was incorporated into the biogenic "apparent NER" in comparison with "real NER". Besides its well-recognized role on enhancing mineralization, MC inoculation with clay particles benefits soil pesticides remediation in term of reducing "real NER" formation, which has been previously underestimated.

1. Introduction

The phenylurea herbicides Isoproturon (IPU) are an important group of pesticides in agricultural practice to control broad leaf weeds in cereal crops and has been applied extensively worldwide (Hussain et al., 2015). The phenylurea pesticides generally have relatively high water solubilities and low tendencies to sorb within soil matrix (e.g. lgK_{ow} and K_{oc} for IPU in Table 1), thus leading to high capability of leaching from soil matrix thereby resulting in frequent detection in groundwater (Sorensen et al., 2003). Isoproturon has been worldwide detected in soil, groundwater, surface water, or even in drinking water exceeding the threshold values (e.g., European Union threshold is 0.1 μg L^{-1}) (WHO, 1996; Spliid and Koppen, 1998; Hussain et al., 2017). Isoproturon possesses half-life between 3 and 200 days in soil and even longer (exceeding one year) in water (Walker et al., 2001; Wang et al., 2016; Bollmann et al., 2017; Zeng et al., 2017). Intermediate metabolites are often smaller and more polar than their parent compounds and are more likely to get in groundwater (Fenner et al., 2013). Once entered into environment, the metabolic products of IPU can cause adverse toxicity even worse than their parent IPU (Sorensen et al., 2003), thus leading to potential environmental risks for aquatic organisms in groundwater and plants, and also as endocrine disrupting chemicals to mammals (Orton et al., 2009). Considering the associated risks, EU decided to ban the use of IPU, back in 2016 (Commission Implementing Regulation (EU) 2016/872, 2016). However, the detection of pesticides and their metabolic products in environment long after their phase-out shows their persistence, and thus raises potential risks associated with food and ecosystem safety (Fenner et al., 2013; Gavrilescu et al., 2015).

Nevertheless, besides the spraying to deeper soil layers, leaching to groundwater, or plant uptake, fate of pesticides includes sorption to soil matrix (soil minerals or soil organic matter, SOM) and degradation by light, chemical processes, or by microorganisms (Fenner et al., 2013). Isoproturon degradation was enhanced with the inoculation of IPU-degrading microorganisms compared with autogenic IPU degradation in soil (Li et al., 2017). Several microbial species, either in single strain or in community, both in inoculation laboratory systems and fields, reveal effective degradation of IPU (Li et al., 2016; Hussain et al., 2017). *Sphingomonas* sp., one of the bacterial species that showed efficient IPU degradation, was isolated from a soil that can degrade IPU

* Corresponding authors at: Department of Environmental Science, Zhejiang University, Hangzhou 310058, China.
E-mail addresses: zhuxm@zju.edu.cn (X. Zhu), blchen@zju.edu.cn (B. Chen).

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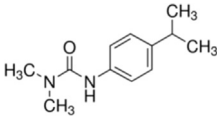
Table 1
Selected properties of Isoproturon (IPU).

Selected IPU properties	
Formula	$C_{12}H_{18}N_2O$
CAS no.	34123-59-6
IUPAC name	3-(4-isopropylphenyl)-1,1-dimethylurea
Structure	
Molecular weight	206.29 g mol^{-1}
Solubility in H_2O	70.2 mg L^{-1} (in 20 °C)
lgK_{ow}	2.46–2.84 (Nemeth-Konda et al., 2002)
K_{oc} (L kg^{-1})	174.4 (Nemeth-Konda et al., 2002)
	246.1 (Tahir et al., 2016)

with high efficiency as reported in our previous study (Kiesel, 2014). The common carrier materials (such as clay minerals or biochar) can enhance the degradation of organic pollutants (such as IPU and polycyclic aromatic hydrocarbons) because they may offer suitable habitats and function as hot spots of the inoculated microbial community or single strain (Grundmann et al., 2007; Chen et al., 2012). Besides the efficient degradation of pesticides and different organic pollutants by microbial inoculation, the bound residues or non-extractable residues (NER) are largely formed as well (Wang et al., 2010; Liu et al., 2013; Li et al., 2014, 2017). Nonetheless, how the microbial inoculation would influence the NER composition still remain unknown. Due to the lack of methodology, only few studies reported the composition of such NER focused on the proteinaceous carbon (Possberg et al., 2016), rather than the abundant polysaccharides composing microbial cell wall (Kögel-Knabner, 2002).

The bound residues or NER are "chemical species originating from pesticides, used according to good agricultural practice, that are non-extracted by methods which do not significantly change the chemical nature of these residues" as defined by International Union of Pure and Applied Chemistry (IUPAC) (Roberts, 1984). The bound residues are largely located in the humin fraction of soil humic substances, and smaller portions in humic and fulvic acids as well (Liu et al., 2014). The binding of pesticides to organic matter can occur by sorption (Van der Waal's forces, hydrogen bonding, and hydrophobic bonding), electrostatic interactions (charge transfer, ion exchange or ligand exchange), covalent bonding, or combinations of these reactions (Bollag et al., 1992; Gevao et al., 2000). The pesticides and their metabolites can be released or leached back from soil, when soil physiochemical properties change, for example, soil moisture and organic carbon content, further posing environmental risks, especially with increasing temperature or degraded soil, which turns up more frequently in climate change global scenario (Noyes et al., 2009). Nonetheless, the IUPAC definition for NER may underestimate the microbial participation in IPU degradation, because the NER may include some portion of biogenic components that are built-up by soil microbes with IPU or its metabolites, especially in case of microbial remediation. Considering the biogenic "apparent NER" off from the total NER, it will provide essential knowledge for better understanding of benefits obtained from microbial remediation for pesticides.

We hypothesize greater percentage of biogenic "apparent NER" relative to "real NER", thus lowering the environmental risk, when IPU degrading microbial community is inoculated in soil. To test the proposed hypothesis, the soil for itself lack of effective IPU degradation ability was inoculated with microbial community capable of degrading IPU efficiently. Applying ^{14}C-labeled IPU to soil, analysis was performed to directly trace the mineralization (= formation of $^{14}CO_2$) and formation of its ^{14}C-metabolites during degradation. This study aims to partition the "apparent NER" from the "real" ones with hydrolysis, highlighting the advantage of microbial remediation strategy for pesticides degradation in addition to well-known benefits of enhanced mineralization by microbial community. We believe that, it would be a step forward in terms of estimating the risk of pesticide residues in soil, with clear quantitative differentiation between "apparent NER" and "real NER" for real methodology.

2. Materials and methods

2.1. Chemicals

^{14}C-labeled 3-(4-isopropylphenyl)-1,1-dimethylurea (^{14}C-IPU, > 98%, with specific radioactivity of 9.96 kBq μg^{-1}; International Isotope, Munich, Germany) was uniformly labeled with ^{14}C on the benzene ring. The non-labeled IPU (99.9%), as well as metabolites 3-(4-isopropylphenyl)-1-methylurea (Monodes-IPU, 99.5%) and 3-(4-isopropylphenyl)-urea (Dides-IPU, 99.5%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). 3-[4-(2-hydroxyisopropylphenyl)]-1-methylurea (2-OH-Mono, 91.4%) was provided by Agrevo (Frankfurt-Hoechst, Germany). These metabolites were used as reference substances for identifying IPU metabolites in soil with HPLC. High purity Milli-Q water (18.2 MΩ cm) from Merck Millipore, Darmstadt, Germany, was used. The scintillation cocktails Ultima Flo AF (for NaOH solution trapped $^{14}CO_2$), Ultima Gold XR (for methanol solution or water), and Ultima Gold AB (for trifluoroacetic acid solution) were purchased from PerkinElmer, Massachusetts, USA. Sodium hydroxide solution (0.1 mol L^{-1}) used to trap CO_2 in mineralization experiment, high purity methanol used for ASE extraction and other steps, and trifluoroacetic acid (TFA) solution for hydrolysis, were obtained from Merck, Darmstadt, Germany.

2.2. Soil sampling and pre-treatments

Top soil (0–20 cm) was sampled at Konjisce (Longitude 15.821°, Latitude 46.710°, and Altitude 230 m) in Slovenia, a site with a temperate climate. The soil samples (Konjisce soil) were sieved through 2 mm mesh to remove stones, plant leaves and roots, small animals and so on, and finally stored at − 20 °C in dark before experimental use. Physical and chemical characteristics of Konjisce soil were found to be as clay 7%, silt 60%, sand 33%, pH ($CaCl_2$) 7.1, TOC 1.9%, total N 0.2% (Folberth et al., 2009), water content as 35.50% at − 15 kPa. The Konjisce soil was selected for this experiment because it has poor native degradation ability of IPU (only 14.4% of total applied ^{14}C-IPU was finally mineralized to $^{14}CO_2$ within 46 days), which enabled us to enhance the degradation of IPU via the application of microbial community (Folberth et al., 2009). The Konjisce soil was taken out from − 20 °C to 4 °C for one week and then to room temperature (20 ± 1 °C) for another week prior to experiments, in order to help native microorganisms to adapt experimental climatic conditions. Once the temperature adaptation achieved, soils samples were homogenized thoroughly from which five replicates were taken to measure the optimal water content (corresponding to water content at a water tension of − 15 kPa) using Kaolin box (08.02 Eijkelkamp, Giesbeek, Netherlands). This is as a result of our previous study which found a maximum mineralization of pesticides at water potential of − 15 kPa (corresponding to pF2.18) regardless of various soil types and different pesticides (Schroll et al., 2006). Kaolin box test revealed the optimal water content of 35.50% for Konjisce soil at − 15 kPa water potential. After Kaolin box test, extra water was added into soil samples to obtain 75% of special water pool (SWP, soil water contents within this range result in an increasing mineralization until an optimum, calculated as the difference of soil water content when soil water potential is between − 15 kPa and − 20 MPa) (Schroll et al., 2006) for another week of equilibration. For Konjisce soil, soil water content at 75% SWP was 26.94%.

2.3. Microbial community

A microbial community with a high IPU-mineralization capacity from which the key degrader of IPU isolated and identified as *Spingomonas* sp. (Grundmann et al., 2007; Kiesel, 2014) was used in this study to enhance ^{14}C-IPU degradation in soil. The whole microbial community harboring the IPU-degrading *Spingomonas* sp. strain was attached on expanded clay particles (Grundmann et al., 2007) serving as microbial hot spots for experimental use. Isoproturon degradation ability of the microbial community was tested with ^{14}C-labled IPU before the incubation experiment with following procedures. In each incubation flask (100 mL-Erlenmeyer flask), 25 mL mineral salt medium (Sorensen et al., 2001) and IPU (25 mg L^{-1}) as sole energy and C source was added for liquid culture preparation. Forty clay particles attached with the microbial community were mixed with 40 fresh clay particles in each of three liquid mediums, to enlarge the quantity of microbial community on clay particles for experimental use. The liquid culture was incubated at 100 rpm in dark at 20 °C for around 20 days on an orbital shaker (3005, GFL, Burgwedel, Germany), and aerated three times per week. Two CO_2 traps (each with 10 mL 0.1 mol L^{-1} NaOH) for each liquid culture were installed during the aeration for ^{14}C-IPU mineralization measurement with liquid scintillation counter (Tri-Carb 1900 TR, Canberra-Parkard GmbH, Schwadorf, Austria) (Schroll et al., 2006). Based on measured radioactivity, cumulative mineralization curves and mineralization rate curves were drawn to show the degradation of ^{14}C-labeled IPU. Once proximately 60% of applied ^{14}C-IPU was mineralized into $^{14}CO_2$ and the maximum mineralization rates were achieved (up to 2.6–4.7% per day), clay particles with attached microbial community were harvested from the liquid culture for soil incubation experiment. All the glassware and other equipment used for microbial community transfer process were autoclaved at 121 °C for one hour and sterilized on flame during the transfer.

2.4. Soil incubation with microbial community attached on clay particles

Non-labeled IPU standard mixed with ^{14}C-labeled IPU standard (both dissolved in methanol) were used for soil application with a radioactivity of 6944 Bq g^{-1} dry soil and a mass concentration of 5.05 μg IPU g^{-1} dry soil. For each replicate of ^{14}C-IPU treatment, 80 μL of IPU standard (4340 Bq μL^{-1}) was applied with a Hamilton syringe to 3.5 g oven dried soil in a small beaker. Each soil replicate was homogenized very well for 1 min with a spatula, and stood for several minutes to evaporate the methanol. The soil subsample was mixed with 46.5 g (dry weight) equilibrated soil in brown bottles for one minute. Twenty clay particles (dry weight 0.1 ± 0.01 g per 20 particles, water content 41.1 ± 6.1%) with attached microbial community were transferred from liquid medium to soil and mixed well for one minute, referring to as MC treatment. Soil samples with same amount of ^{14}C-IPU application without MC inoculation were used as control, to reveal the role of native soil microorganisms in IPU degradation. Five replicates were set up for both the control and MC treatment. Soil samples in incubation bottle were compacted to a soil density of 1.3 g cm^{-3} (a common soil density in most of the agricultural soils) and adjusted to a water potential of –15 kPa (corresponding to a soil water content of 35.50% for Konjisce soil).

2.5. Mineralization of ^{14}C-labeled Isoproturon

For mineralization, 9 mL of 0.1 mol L^{-1} NaOH solution in brown bottle was attached to trap $^{14}CO_2$ that would be emitted from soil during ^{14}C-IPU mineralization. A needle was inserted through the rubber cap which enables slow air flow into the brown bottle, as required for living demand of microorganisms in the soil. The incubation bottles were stored in dark at 20 ± 1 °C, covered with Aluminium foil to prevent photo-degradation of IPU. The NaOH solution inside from the brown bottles was collected three times per week with pipette into plastic bottles for radioactivity measurement with liquid scintillation counting. For that, the aliquots of NaOH solution were mixed with Ultima Flo AF scintillation cocktail. Based on radioactivity of CO_2 emission, the mineralization rate and the cumulative mineralization were calculated. Finally, on the 46th day after application, when the cumulative mineralization reached the plateau phase, which means that the accelerated mineralization had nearly ended, the incubation experiment was stopped.

2.6. Chloroform fumigation for ^{14}C-labled living microbial biomass determination

Soil aliquots (two replicates of 2 g) were taken freshly (i.e., immediately after finishing incubation process), for chloroform fumigation to measure the ^{14}C-microbial biomass. Clay particles were separated from soil and treated for fumigation with same method. Water content of soil was measured at the same time by drying soil aliquots in oven at 105 °C for 24 h. After the fumigation, soil samples and clay particles were extracted with 20 mL 0.5 mol L^{-1} K_2SO_4 to determine the living microbial biomass. At the same time, soil aliquots without fumigation were directly extracted with K_2SO_4, for the calculation of living microbial biomass. Two milliliter of extracted solution was mixed with 3 mL Ultima Gold XR for radioactivity measurement in liquid scintillation counter.

2.7. Soil extraction and analysis of Isoproturon metabolic production

Right after fumigation of soil samples, the remaining soil of each treatment and the control were transferred to metal columns for accelerated solvent extraction (ASE) (Dionex, Thermo Fisher, MA, USA) to analyze IPU metabolites qualitatively and quantitatively. The extraction was performed with methanol at 90 °C under 100 mbar according to ASE program (total time of extraction was 20 min), followed by determination of radioactivity in the extracts (including metabolites and parent IPU) with scintillation counter. Succession extraction was conducted to extract IPU and its metabolites, until the fourth extraction accounted for less than 10% radioactivity of extracted sum. The ASE extract was evaporated with rotary evaporator (Büchi Rotavapor R-114, Flawil, Switzerland) to remove methanol which could cause damage to solid phase extraction (SPE) cartridges in next cleaning step. Solid phase extraction was performed with Bond Eluent ENV cartridge (Bond Elut-ENV, 200 mg/3 mL, Agilent Technologies, Santa Clara CA, USA), to adsorb IPU and its metabolites in the cartridge and to get rid of impurity. Adsorbed IPU and its metabolites were later eluted by methanol, evaporated again into a small amount in HPLC vial, and prepared for HPLC analysis. Acetonitrile and HPLC water (using 20 mmol L^{-1} Ammonium Acetate [NH_4Ac] as a buffer) were used as liquid phase for HPLC, and the column was LiChrospher 100 RP-18 (5 μm) HPLC cartridge (Merck Millipore, Darmstadt, Germany). Twenty microliter sample solution was injected into HPLC and run at a flow rate of 1.0 mL min^{-1} with L-6200 gradient pump (Merck-Hitachi, Darmstadt, Germany). The metabolites (sum of labeled and non-labeled ones) were detected with UV–visible detector (Merck-Hitachi L-4250, Darmstadt, Germany) coupled to HPLC, at a wave length of 240 nm; while the ^{14}C-labeled metabolites were determined with HPLC-coupled radioactivity monitor (Berthold LB 506, Bad Wildbad, Germany). Isoproturon metabolites were identified with the help of non-labeled metabolites according to their retention times and the quantification of metabolites in samples was done according to the radioactive-detector-signal.

2.8. Quantification of NER, “apparent NER”, and “real NER”

Soil samples after ASE extraction were collected in plastic bags and shortly stored at − 20 °C for total NER and “apparent NER” analysis. Quantification of NER was conducted by soil combustion with the

Sample Oxidizer (Packard Model 307, PerkinElmer, Massachusetts, USA). Five replicates each around 250–300 mg of each soil sample was combusted after water content determination. Hydrolysis of soil samples with trifluoroacetic acid (TFA) was conducted to determine the "apparent NER" (Amelung et al., 1996). For each sample three replicates of each 10 g soil was hydrolyzed with 100 mL TFA (4 mol L^{-1}) at 105 °C for 4 h. By TFA hydrolysis following same condition, the biogenic bound residues (i.e. the "apparent NER") will be cut down into smaller molecules (polysaccharide into monosaccharide, proteins into amino acids, etc.) and will be released into the solution (Amelung et al., 1996). After hydrolysis, two aliquots of 100 µL sample solution were taken out with pipette, mixed with 15 mL Ultima Gold AB plus 5 mL Milli-Q water to determine radioactivity.

2.9. Data analysis

Mass balance of IPU was calculated on ^{14}C basis. Percentages of radioactivity accounting for the applied radioactivity from the mineralization, metabolites, total NER are summed up and give recovery rates of 95.9 ± 1.2% for MC and 93.2 ± 0.4% for the control on a ^{14}C basis. Total NER were further partitioned into "apparent NER" and "real NER", and assigned into mass balance calculation as well.

3. Results and discussion

3.1. Enhanced mineralization of ^{14}C-labeled Isoproturon with MC

The cumulative mineralization and mineralization rate curve during 46 days of incubation are shown in Fig. 1**A**, which indicates that the mineralization of ^{14}C-IPU with MC inoculation were significantly enhanced in comparison to control experiment without MC inoculation (Fig. 1**A**). Treatment MC showed a sigmoid curve of cumulative mineralization and speeding-up mineralization rate at the beginning of the incubation (before the 10th day), which is the representative for metabolic degradation. During the logarithmic phase when the mineralization rate reached its maximum (0.30 ± 0.04 µg IPU g^{-1} soil d^{-1} at day 7.8), the cumulative mineralization of IPU rapidly increased (from 0.45 ± 0.10 µg g^{-1} soil to 1.03 ± 0.18 µg g^{-1} soil in two days); while at the same time the mineralization rate of control was only 0.012 ± 0.002 µg g^{-1} soil d^{-1} (Fig. 1**B**). Generally, mineralization rate of control was low and stable throughout the whole incubation period, being an average 0.014 ± 0.001 µg g^{-1} soil d^{-1}. The cumulative mineralization of control indicates the background mineralization caused by native microorganisms in soil, with a co-metabolic way of mineralization. The background mineralization of soil reached 0.66 ± 0.01 µg g^{-1} soil (that is 13.1 ± 0.2% of the applied IPU) throughout 46 days, identical as reported in previous study for Konjisce soil (with 14.4% cumulative mineralization in 46 days) (Folberth et al., 2009). The final cumulative mineralization after 46 days was 2.82 ± 0.05 µg g^{-1} soil (that is 55.9 ± 0.9% of the applied IPU amount) in MC, which was much higher than the control, revealing that the native IPU mineralization capacity of Konjisce soil was considerably enhanced by inoculation with the IPU-degrading microbial community.

The microbial community including the key degrader can utilize IPU as carbon and energy source, defined as metabolic way of degradation; while in the control samples with no specifically degrading MC, the small amount of IPU degradation was ascribed to co-metabolic way (Zhang et al., 2012). Inoculation with MC attached on clay particles efficiently enhanced the degradation of IPU in soil, that more than half of applied IPU was mineralized into CO_2, with the role of MC in this study. Thus, the additionally inoculated microbes play an important role in enhancing the mineralization of pesticide in soil.

3.2. Metabolites of Isoproturon

For the treatment of MC, ASE extraction released IPU metabolic products of only 0.23 ± 0.005 µg IPU g^{-1} soil (that is 4.6 ± 0.1% of applied IPU); while for the control, much higher amount of IPU and its metabolites (that is 1.62 ± 0.17 µg g^{-1} soil, accounting for 32.1 ± 3.3% of applied IPU) could be extracted. Further following SPE purification and elution, identified IPU metabolites and some parent IPU amount to 0.15 ± 0.02 µg g^{-1} soil (that is 3.1 ± 0.5% of applied IPU) within the MC treatment, while 1.41 ± 0.04 µg g^{-1} soil (27.9 ± 0.9% of applied IPU) for IPU metabolites within the control. The SPE purification and elution following the ASE extraction result in some loss of radioactivity from the impurity and other undefined metabolic products.

It was observed that *Sphingomonas* sp. can break down the side chain of phenylurea pesticides such as Isoproturon and Diuron step by step and formed corresponding chemical compounds Monodes-IPU, Dides-IPU, 2-OH-Mono and 4-isopropylanilin (4IA) (Sorensen et al., 2001; Zhang et al., 2012). Some of the metabolites were found in this study as well, identified as Monodes-IPU, 2-OH-Mono, and Dides-IPU (Fig. 2). In control samples, Monodes-IPU, 2-OH-Mono, Dides-IPU, and some parent IPU that was left over, respectively accounted for 7.8 ± 0.3%, 0.4 ± 0.03%, 0.2 ± 0.01%, and 10.7 ± 0.4% of the applied IPU (Table 2). As that of MC treatment, only Monodes-IPU and small amounts of IPU were found, roughly accounting for 0.5 ± 0.08% and 1.8 ± 0.3% of applied IPU (Table 2), respectively.

The microbial community that was inoculated into soil contains *Sphingomonas* sp., which is able to break down the benzene ring of 4IA and can release CO_2 as the final production of total mineralization

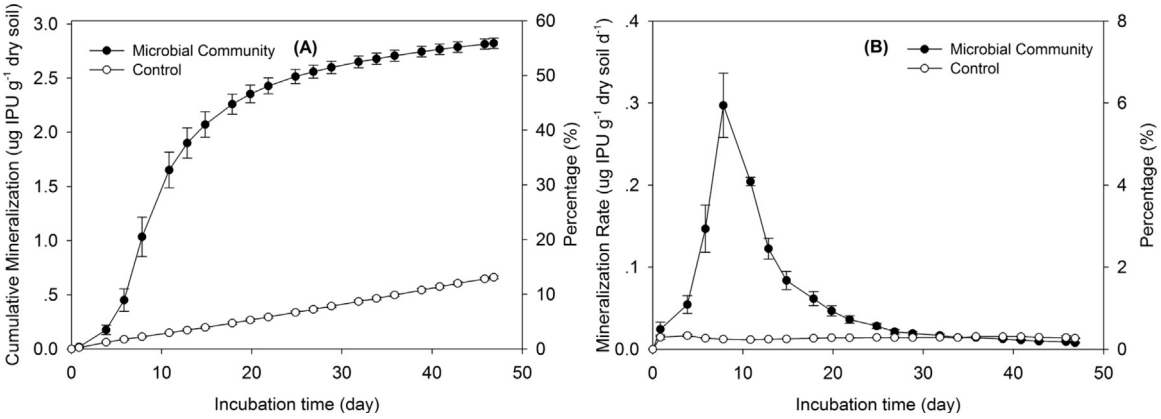


Fig. 1. Cumulative mineralization (**A**) and mineralization rate (**B**) of IPU during 46 days incubation with (solid dots) and without (hollow dots) microbial community inoculation. Data presented are IPU mass that were mineralized in the according days, in form of average value from 5 replicates with one standard deviation indicated by error bars (some of which are smaller than the symbol).

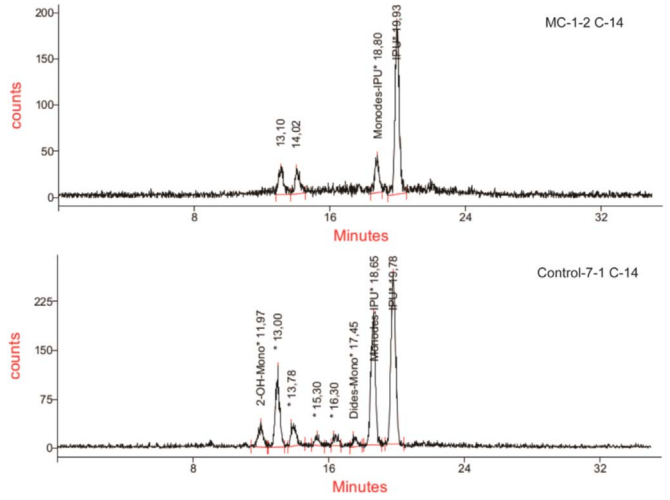


Fig. 2. HPLC-chromatograms of ASE extracted IPU metabolites detected by ^{14}C detector. MC = soil samples inoculated with microbial community; Control = soil samples without microbial community inoculation.

(Johannesen et al., 2003). It can be seen from the data (Table 2) that in treatment with MC, IPU was degraded more thoroughly, with only a little amount of un-degraded IPU and only one identified intermediate metabolite finally exist. On the contrary, more types of metabolites, higher content of remained IPU and intermediate metabolites were still remained in control samples, due to the lack of efficient microbial degradation. What is worth noticing is that as large as 10.7% of applied IPU exist as extractable residues in control samples (Table 2), which means that they are not closely bound to soil matrix and still has the possibility to be mineralized. However, for long term such part of extractable IPU and its metabolic residues could also be possibly bound to soil matrix where there is possibility of inefficient degradation. The aging process of IPU and its metabolites in soil may cause weak IPU mineralization by the IPU-degrading microbial strain (Johannesen et al., 2003). Without the role of IPU degrader, a considerable part of IPU or its metabolites were considered to further combine with soil matrix, forming NER as a consequence.

3.3. Apportionment of "apparent" and "real" NER of Isoproturon

Interactions between pesticides and their metabolic products (e.g. 4IA, the metabolic production of IPU degradation) with soil result in low bioavailability of IPU metabolites and the formation of bound residues or non-extractable residues (NER) (Johannesen et al., 2003). In our study, NER accounted for larger amount of IPU mass initially applied in control than that in MC inoculated soil, i.e., 2.51 ± 0.04 μg g^{-1} soil approximately (that is 49.8 ± 0.7% of applied IPU) for control and 1.78 ± 0.02 μg g^{-1} soil (35.4 ± 0.4%) for MC, respectively (Table 3). This is consistent with an outdoor lysimeter study within soils, in which the formation of NER was relatively low when the mineralization was high and vice versa, in soils with low mineralization NER formation was much higher (Grundmann et al., 2011). This indicates the significant role of soil microbial community in reducing the NER formation while enhancing the mineralization. Sorption of IPU, its metabolites, or NER on clay particles has neglect influences on the results, seeing that the quantification of NER of fresh clay particles (when they were just separated from soil after 46 days incubation) reveals only around 0.10 μg IPU–residue equivalents g^{-1} soil in clay particles in each MC sample, accounting for only 1.9% of the applied ^{14}C-IPU (Table 3).

Non-extractable residues have potential environmental risks when they are released again from soil to groundwater, when SOM break down (Barraclough et al., 2005). However, the composition of NER is not well studied due to its complex nature during formation and a lack of methodology. It was assumed that different microbial degradation capabilities of pesticides in soils might have affected the formation of NER, and laboratory experiments with these soils confirmed this assumption (Grundmann et al., 2007). Therefore, it was hypothesized that two main principal pathways for the formation of NER exist in soils receiving pesticides: one being the well-known physiochemical sorption

Table 3
Incorporation of ^{14}C into each fractions during ^{14}C-IPU degradation either with (MC) or without (control) microbial community (attached on clay particles) inoculation in soil. After 46 days incubation, clay particles were separated from soil and analyzed for ^{14}C in some fractions as well.

Treatment	Fractions	Mass basis (μg IPU g^{-1} soil)	Percentage of applied IPU (%)
MC	Mineralization	2.82 ± 0.05	55.9 ± 0.9
	Metabolites	0.23 ± 0.005	4.6 ± 0.1
	Identified metabolites	0.12 ± 0.02	2.3 ± 0.4
	NER	1.78 ± 0.02	35.4 ± 0.4
	Apparent NER	1.24 ± 0.02	24.7 ± 0.4
	Real NER	0.54 ± 0.04	10.7 ± 0.7
	^{14}C-Microbial biomass[a]	0.13 ± 0.03	2.6 ± 0.5
	SUM[b]	4.84 ± 0.06	95.9 ± 1.2
Control	Mineralization	0.66 ± 0.01	13.1 ± 0.2
	Metabolites	1.53 ± 0.05	30.3 ± 1.1
	Identified metabolites	0.96 ± 0.03	19.1 ± 0.6
	NER	2.51 ± 0.04	49.8 ± 0.7
	Apparent NER	1.46 ± 0.03	29.0 ± 0.7
	Real NER	1.05 ± 0.06	20.8 ± 1.2
	^{14}C-Microbial biomass	0.17 ± 0.01	3.3 ± 0.2
	SUM	4.70 ± 0.02	93.2 ± 0.4
Clay particles (20 per incubator)	Metabolites	0.0014 ± 0.0001	0.03 ± 0.002
	Apparent NER	0.08 ± 0.01	1.6 ± 0.2
	^{14}C-Microbial biomass	0.03 ± 0.05	0.7 ± 0.2
	Fresh combustion[c]	0.10	1.9

[a] Data present here for ^{14}C-microbial biomass was only in soil from which clay particles were already picked out.
[b] SUM = Mineralization + Metabolites + NER.
[c] Fresh combustion was conducted for the clay particles that were separated from soil, to determine the total amount of ^{14}C on clay particles just after 46 days incubation.

Table 2
Metabolic products in IPU degradation and parent IPU in soil extraction with ASE. Both percentage and mass basis are presented for each metabolites products, parent IPU, and the sum.

Treatment	IPU	Monodes IPU	2-OH-Mono	Dides-IPU	SUM
In percentage (%)					
MC	1.8 ± 0.3	0.5 ± 0.08	ND[a]	ND	2.3 ± 0.4
Control	10.7 ± 0.4	7.8 ± 0.3	0.4 ± 0.03	0.2 ± 0.01	19.1 ± 0.7
In mass basis (μg IPU g^{-1} dry soil)					
MC	0.09 ± 0.02	0.02 ± 0.004	ND	ND	0.12 ± 0.02
Control	0.54 ± 0.02	0.39 ± 0.01	0.02 ± 0.001	0.01 ± 0.001	0.96 ± 0.03

[a] ND = not detected.

of parent pesticides and their intermediate metabolites to soil components ("real NER" is consequently formed), and another one being the microbial incorporation of pesticides and metabolites in the form of biomolecules ("apparent NER") (Grundmann et al., 2011), which may have different fate in comparison to "real NER". Thus, from these results it can be assumed that ^{14}C from the labeled pesticides can be incorporated into microbial biomolecules like homo- and heteropolysaccharides from fungal cell wall and peptidoglucan (murein) from bacterial cell wall, which contains carbohydrate and amino acid elements (Kögel-Knabner, 2002). Polysaccharides of microorganisms are relatively easy to be decomposed, forming basic units such as glucosamine, galactosamine or muranmic acid, which accumulate during decomposition in soil (Kögel-Knabner, 2002). It is estimated that 80% of sugars in soil originate from microorganisms and their residues (Gunina and Kuzyakov, 2015). Hydrolysis with TFA will cut polysaccharides into monosaccharides and release such biogenic components from soil for analysis (Amelung et al., 1996). Therefore, analysis of such ^{14}C-labeled biomolecules following TFA hydrolysis will then be a confirmation for the "apparent" or biogenic nature of the NER.

With the microbial community harboring the IPU degrading single strain *Sphingomonas* sp., it can be expected that ^{14}C labeled compounds must have been largely involved in the circulation within the microbial biomass. A relatively high amount of NER was identified in experiments with ^{14}C-labeled 1,2,4-TCB degrading microbes, thought to be ^{14}C incorporated into microbial biomass as well (Wang et al., 2010). In general, major parts of NER are found to be microbe-involved "apparent NER" rather than "real NER" in our study, especially with MC inoculation in soil, where 24.7 ± 0.4% of the applied IPU mass was incorporated into "apparent NER" (Table 3). It is interesting to see that even for control soil that did not receive the microbial community inoculation, "apparent NER" accounted for a large percentage (29.0 ± 0.7%) of applied IPU as well, which is even larger than that in the MC treatment (24.7 ± 0.4% for "apparent NER"). The formation of NER (and also the biogenic residues) normally follows a dynamic curve that they accumulate from the beginning of the incubation and reach an equilibration through the incubation (Nowak et al., 2011; Wang et al., 2017). However, it should be noted that further mineralization of NER into CO_2 was found (amounting to 8.7–9.2% of the NER) when the NER-containing soil was re-incubated with fresh soil, demonstrating that some portion of the NER still has bioavailability (Wang et al., 2017). Certain study show that bioavailability determines the fate of NER through mineralization (Wang et al., 2017). If bioavailable, the accumulation of the "apparent NER" at certain time would be an equilibration between its formation due to microbial incorporation and its dissipation due to release of the ^{14}C-labeled substances back to soil along with microbial death and cell lysis and re-utilization by other microbes. With the current data from our study no conclusion can be drawn on whether the bioavailability or the microbial activity limits the decomposition of the "apparent NER" from the control, so we do not have the answer for why the amount of "apparent NER" was higher in the control than that in the MC treatment. To clearly demonstrate this, a dynamic curve showing changes in each ^{14}C-labeled component and changes of microbial activity through time during IPU degradation, as well as a detailed analysis into the chemical composition of the "apparent NER" would be required in near future.

Although it seems that higher mineralization would not necessarily result in higher absolute content of "apparent NER" (Fig. 3), but for "real NER", MC inoculation in soil starts to show its benefits. Much lower amount of "real NER" was finally formed in MC treatment (10.7 ± 0.7% of applied IPU) than that of control (20.8 ± 1.2%) after 46 days incubation (Table 3, Fig. 3). This is in accordance with our hypothesis that the inoculation of IPU-degrading microbial community would shift the NER composition to a higher "apparent" to "real" NER ratio. The biomolecules formed with ^{14}C originated from ^{14}C-IPU will become free when microbial cells die, releasing ^{14}C from such biogenic components back to environment in form of CO_2, sugars, amino acids and so on, which can be utilized again by other microbes (Fig. 4). That being hypothesized in such way, ^{14}C from IPU will be incorporated into SOM, participating in SOM turnover thereafter. Therefore, the fate of the "apparent NER" will be absolutely different from that for the "real NER" formed with physiochemical sorption to soil matrix. As a consequence, "apparent NER" does not pose environmental risks like the "real" ones have, and our study demonstrates that soil inoculation with IPU degrading microbial community can decrease such environmental risks.

3.4. Formation of ^{14}C-labeled living microbial biomass

Living microbial biomass that was ^{14}C labeled as a result of microbial ^{14}C-IPU turnover was determined with the chloroform fumigation extraction. Expanded clay particles were applied as carrier material for microbial community because they can serve as hot spots for microbial activity, as reported earlier (Grundmann et al., 2007), and can be separated easily from soil for living microbial biomass determination by fumigation extraction. ^{14}C-Labled living microbial biomass in soil from MC treatment and the control respectively accounted for 2.6 ± 0.5% and 3.3 ± 0.2% of applied IPU (Table 3). Clay particles separated from MC treatment harbored a ^{14}C-labeled living microbial biomass that accounted for 0.7 ± 0.2% of the applied ^{14}C-IPU. When microbial biomass in clay particles and in soil from MC treatment was combined together, 3.3 ± 0.5% of applied IPU was recovered, which made the ^{14}C-labeled living microbial biomass in MC treatment identical with that in the control.

Living microbial biomass that was labeled with ^{14}C indicates that soil microbes have utilized ^{14}C from the applied ^{14}C-IPU for construction of their biomolecules. Especially for MC treatment in this study, when the degrading strain *Sphingomonas* sp. in MC uses ^{14}C-IPU for energy and growth and performs all steps of IPU degradation alone, ^{14}C is incorporated into the microbial biomass. In the absence of *Sphingomonas* sp. i.e., in control soil, the metabolically degrading microbes decompose IPU in a cooperative way. However, although the native microbes in control soil did not contain the IPU-degrader *Sphingomonas* sp., they had built almost the same amount of living microbial biomass as that in MC treatment at the end of 46 days soil incubation.

The microbial strain *Sphingomonas* sp. within this microbial community can solely conduct IPU mineralization (Kiesel, 2014). The mineralization capability of this microbial community when applied to soil is characterized by a sigmoid cumulative mineralization curve, which is in accordance with the typical growth curve when a bacterial single strain adapt themselves into a new environment and utilize the available carbon source to amplify their population, and thus it can be concluded that this IPU-degrading strain was indeed growing using IPU as a carbon source. The mineralization curve shows that ^{14}C-IPU has been turned over very fast shortly (around 20 days) after the incubation was started (Fig. 1). When the available ^{14}C-IPU was almost consumed up as a carbon source, the mineralization rate dropped to an identically low level for both MC treatment and the control (Fig. 1). At that time (46 days after incubation), when identically low mineralization rate was found for both MC treatment and the control, identical living microbial biomass was accordingly found in the both as well (Table 3).

4. Conclusions

Microbial community inoculation with carrier material in soil greatly enhanced the thorough degradation of ^{14}C-IPU (4.2 folders higher mineralization from MC treatment than that from the control). The "real NER" formation with MC inoculates was only half of control. The formation of NER, especially the "real NER" that represents the realistic environmental risks, was reduced. The amount of intermediate metabolites with MC treatment only accounted for around 15% of that in the control. That being said, inoculation with IPU-degrading

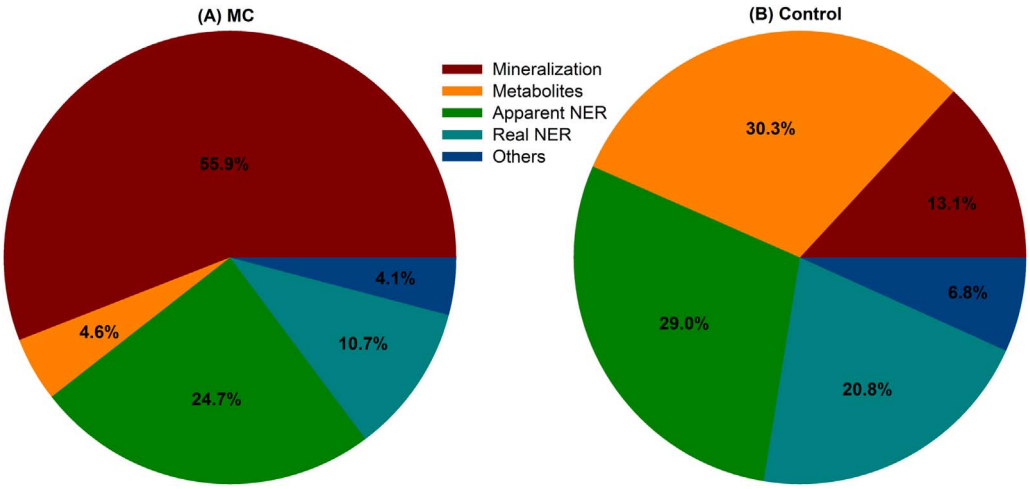


Fig. 3. ^{14}C-Mass balance calculated based on the sum of mineralization, metabolites, "apparent NER", and "real NER", respectively with (**A**: MC) and without (**B**: Control) microbial community inoculation. Some unidentified fractions and/or radioactivity loss in each experimental step are indicated as "Others". MC = soil samples inoculated with microbial community; Control = soil samples without microbial community inoculation.

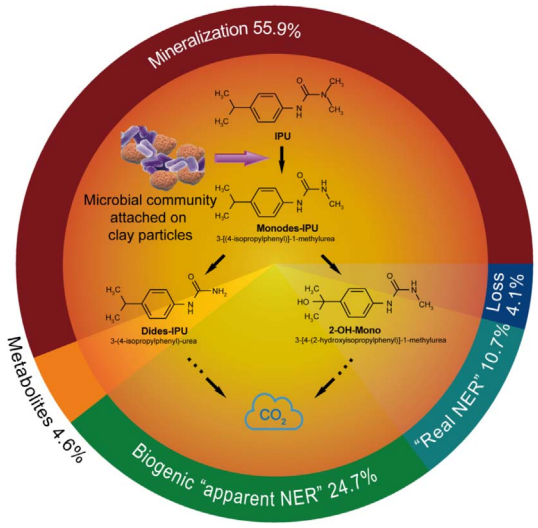


Fig. 4. The metabolic pathway and metabolites distribution of ^{14}C-IPU in soil with the inoculation of IPU-degrading microbial community.

microbial community not only enhances the mineralization, but also improves the "degradation quality" (which means less intermediate metabolites, less NER, and less "real NER") and thus reduces the possible environmental risks associated with IPU and its degradation products. This is the previous underestimated part with microbial remediation for pesticides.

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