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Formation of 2,4-D bound residues in soils: New insights into microbial metabolism

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Highlights

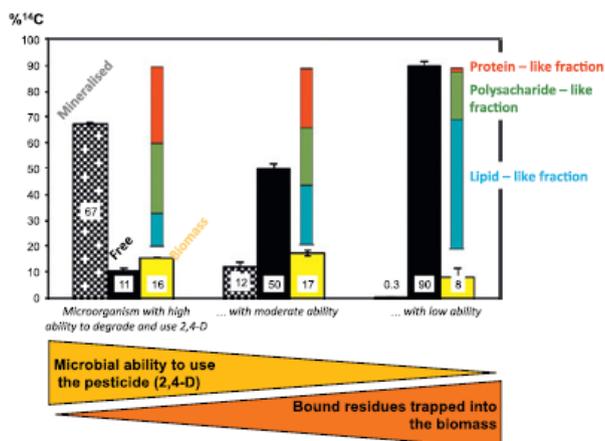
Microorganisms are involved in the formation of bound residues from the pesticide 2,4-D

Microorganisms use part of 2,4-D to produce biomass

A part of bound residues is into polysaccharides, lipids and protein biomolecules

The protein-enriched fraction is a good indicator of the microbial use of 2,4-D for bound residue formation

Graphical abstract



Abstract

The microbial contribution to the formation of bound residues in soils is studied by characterizing the metabolic activity of three microorganisms (*Trametes versicolor*, *Fusarium solani* and *Ralstonia eutropha*) on ^{14}C -2,4-dichlorophenoxyacetic acid (2,4-D) during incubation in synthetic liquid media and soil. A fractionation protocol was applied to quantify the ^{14}C -2,4-D that was incorporated into the biomass among biomolecular-like fractions. Successive fractionation of microbial biomass was implemented to break up and quantify the methanol/dichloromethane fraction (corresponding to the ^{14}C -lipid-like fraction), the trichloroacetic acid fraction (or hydrolysed ^{14}C -polysaccharide-like fraction) and the acid hydrolysable fraction (or the hydrolysed ^{14}C -protein-like fraction). Relevant differences in the 2,4-D degradation and biomass radioactivity distribution among the three microorganisms were found. The ^{14}C -protein-like fraction was the most consistent biomass fraction for reflecting the pesticide use capacity of the microorganisms under liquid and soil conditions. 2,4-D and its metabolite 4-chlorophenol were detected in methanol/dichloromethane and trichloroacetic acid fractions of the biomass of microorganisms exhibiting a low capacity to mineralize 2,4-D, thus proving that the microbial participation in the formation of bound residues while conserving the initial pesticide structure under natural soil conditions may be intimately associated with the lipid- and polysaccharide-like constituents. The fractionation protocol differentiates between ^{14}C that is incorporated into biomass as a biomolecular constituent and the pesticide or its metabolites that accumulate in the biomass and thus correspond to the stricto sensu definition of bound residues.

Introduction

Degradation studies of pesticides usually reveal difficulties in extracting parent pesticides and their transformation products from soil, mainly because of chemical interactions with soil constituents. These interactions can be responsible for the formation of non-extractable residues, which are often called “bound residues” (Führ et al., 1998). According to IUPAC “Bound residues are chemical species originating from chemicals, i.e. pesticides, that cannot be extracted by methods that do not significantly modify the chemical nature of these residues” (Roberts, 1984). They represent a fraction of the chemicals in soils, plants or animals that persist in the matrix in the form of parent substance or transformation product(s) after conventional extraction (Führ et al., 1998).

Radiolabelled pesticides are classically used to quantify the amount of bound residues in soils, and the measured bound residues correspond to the non-extractable radioactivity, which is often determined by sample combustion and liquid scintillation counting. Nevertheless, this methodological approach does not distinguish non-extractable pesticides, or their metabolites trapped in soil constituents, from the non-extractable ^{14}C that is incorporated into the newly synthesized biomolecules via microbial activity. Thus, the IUPAC definition remains theoretical but not operational (Barriuso et al., 2008; Kästner et al., 2014).

The amount of bound residues in soils is closely related to the nature of the pesticide (Barriuso et al., 2008), soil physicochemical properties (Mac Rae, 1986) and soil microbial community (Lerch et al., 2009). The formation of bound residues is commonly considered a detoxification phenomenon because of the decrease in residue availability. However, questions still remain concerning the possible temporal release of trapped pollutants and the long-term consequences of their accumulation in soil. Without better knowledge of the mechanisms governing pesticide retention and transformation in soils and carbon transformation from pesticide to natural compounds in soil, differentiation between bound residues and biogenic residues will remain theoretical concepts (Kästner et al., 1999). To precisely determine the chemical nature of bound residues in relation to formation pathways, different procedures have been proposed. Thus, $^{13}\text{C}(^{15}\text{N})$ -NMR allows the direct non-disruptive observation of bound residues (Benoit and Preston, 2000; Berns et al., 2005). Other disruptive techniques, which range from alkaline or acidic extractions (Loiseau and Barriuso, 2002) to pyrolysis (Northcott and Jones, 2000), also allow information to be obtained on the chemical structure of bound residues. Part of the non-extractable radioactivity of pesticides, which is generally considered to comprise bound residues, results from the pesticides' incorporation into the microbial biomass via the synthesis of new bimolecular compounds (Nowak et al., 2011, 2013). From the point of view of ecological risk assessment, these biogenic bound residues do not pose any environmental risk. Thus, Kästner et al. (2014) proposed a classification of bound residues in relation to their formation pathway: (type 1) sorption or trapping within soil organic matter, (type 2) linked by a covalent bond, and (type 3) biogenic bound residues from conversion into new biomass biomolecules.

Under natural soil conditions, quantifying the persistence of pesticides and their potentially bioactive metabolites is important to estimate real risk (Charnay et al., 2004). One of the most important processes that define pesticides persistence under soil conditions is microbial degradation activity. Specifically, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and its metabolites are

critically affected by microorganisms, including soil bacteria (e.g., *Ralstonia eutropha*; Short et al., 1992) and white root fungi (e.g., *Trametes versicolor*; Yadav and Reddy, 1993; Ahn et al., 2002). The degradation implies the partial or complete transformation of the parent compound. The initial mechanism of bacterial degradation is the side-chain cleavage to 2,4-dichlorophenol, whereas fungi can provoke a direct ring hydroxylation via the substitution of chlorine by a hydroxyl (Hägglom, 1992; Kumar et al., 2016). During 2,4-D metabolism by microorganisms, it is expected that a portion of the pesticide will be used in the synthesis of biomolecules, such as lipids, polysaccharides and proteins, to form biogenic bound residues. Some of them can be used as biomarkers of specific microbial pathways of pesticide degradation (Charnay et al., 2004; Nowak et al., 2011; Kästner et al., 2014).

In the present study, we examine the 2,4-D biodegradation process carried out by three microorganisms maintained in synthetic liquid cultures or inoculated in soil and the incorporation of labelled herbicide into the biomass of the most important extractable biomolecules (lipids, polysaccharides and proteins). We aim to determine the microbial biomass contribution to the formation of bound residues and to identify the distribution profiles of bound residues among selected strains of fungi and bacteria. 2,4-D is a pesticide used worldwide and the biodegradation mechanisms are well known that allows us to relate specific metabolic pathways with the consequences on bound residues formation pathways depending on the specific microorganism activity.

Materials and methods

Microorganisms

Two filamentous fungi, *Trametes versicolor* (ATCC 32745 from the UMR ECOSYS collection) and *Fusarium solani* (isolated from a silt loam under a meadow in Versailles, France), and one bacterial strain, *Ralstonia eutropha* (JMP 134 from the collection of UMR Agroecology, Dijon), were used. They were maintained in pure liquid cultures according to Charnay et al. (2004). The liquid medium for both fungal strains was (in g L⁻¹) K₂HPO₄ 2.0, CaCl₂ × 2H₂O 0.14, MgSO₄ × 7H₂O 0.7, FeSO₄ × 7H₂O 0.07, ZnSO₄ × H₂O 0.046, MnSO₄ × H₂O 0.035, CuSO₄ × H₂O 0.007, glucose 5.0, yeast extract 1.0, and tartrate 2.3. The liquid medium for *Ralstonia eutropha* was (in g L⁻¹) K₂HPO₄ 1.5,

KH₂PO₄ 0.5, (NH₄)₂SO₄ 0.001, MgSO₄ × 7H₂O 0.21, yeast extract 0.005, the addition of 0.5 mL in 100 mL of liquid medium of a solution containing (in g L⁻¹) ZnSO₄ × 7H₂O 0.4, MgCl₂ × 4H₂O 0.02, boric acid 0.01, CoCl₂ × 6H₂O 0.05, CuSO₄ 0.2, NiCl₂ × 6H₂O 0.01, FeSO₄ × 7H₂O 0.5, and EDTA 0.25. All chemicals were of analytical grade, and water was MilliQ water from Waters appliance.

Soil

The topsoil (0–10 cm) from an agricultural field (Feucherolles, neighbourhood of Paris, France) was sampled. The soil is representative of the agricultural soils of the centre of France. It was a cambisol (FAO classification) with a pH of 6.9 and the following textural and chemical composition (in g kg⁻¹): clay 150, silt 783, sand 67, organic carbon 11, and total nitrogen 1.08.

Incubations conditions with 2,4-D

2,4-D solution in sterile water was prepared by mixing uniformly ring labelled ¹⁴C-2,4-D (radiochemical purity up to 98%) and unlabelled 2,4-D to reach a final concentration of 5 mg L⁻¹ of 2,4-D and a final activity of 333 Bq mL⁻¹. Inoculation of liquid media with microorganisms was done for *T. versicolor* and *F. solani* with 2 implants (10 mm diameter) from a solid pre-culture and for *R. eutropha* with 100 µL of an exponential suspension (OD₆₆₀ = 1.266). The inoculated liquid media were incubated in the dark at 28 °C, kept for 10 days in static conditions for fungi, and shaken for bacteria. Sterile controls without microorganism inoculation were prepared and incubated in similar conditions. Each assay comprised 12 replicates.

2,4-D mineralization and incorporation into soils was studied using non-sieved and sieved (2 mm) fresh soil. The soil samples were only inoculated with *R. eutropha*. Two grams (dry weight) of soil sample were placed in 25 mL culture tubes and inoculated with 20 µL of an exponential pre-cultured suspension (OD₆₆₀ nm = 1.266) of *R. eutropha*. Sterile sieved soil samples (dried at 105 °C for 3 days) were used as controls. The inoculated tubes were incubated in the dark for 10 days at 28 °C. Then, the precultured soil was rinsed twice with saline solution (NaCl 0.9%) and suspended in 25 mL of MilliQ water. Then, 1.26 mL of a MilliQ aqueous solution of ¹⁴C-2,4-D was applied to the soil samples to obtain a final 2,4-D concentration of 5 mg kg⁻¹ and 333 Bq g⁻¹. After the inoculation and application of the 2,4-D solution, soil moisture was adjusted to 80% of its water holding

capacity using sterile MilliQ water.

Fungal and bacterial liquid cultures and soil assays were incubated in hermetic one-litre sealed jars containing a glass bottle with 10 mL of NaOH (2 M) for ^{14}C -CO₂ trapping.

Biomass fractionation and biomolecular constituent isolation

Biomass fractionation was carried out using a methodology adapted from Charnay et al. (2004) as illustrated in Fig. 1. The biomass in liquid cultures was harvested and separated from the water-soluble fraction, corresponding to the culture medium, using filtration (Whatman GF/ A1 μm) for fungi and centrifugation (4000g, 4 °C, 30 min) for bacteria. The recovered biomasses were rinsed 3 times with 10 mL of MilliQ water and were pooled with the water-soluble fraction. A water-soluble fraction was recovered from soil assays by 3 successive extractions with MilliQ water and centrifugation (4000g, 4 °C, 30 min).

The bacterial biomass of *R. eutropha* recovered from the liquid culture and the soils containing the total biomass were disrupted by sonication (200 W, 20 min). Fungal biomass in liquid cultures was disrupted by liquid nitrogen crushing and sonication (200 W, 30 min). All disrupted solid fractions were freeze-dried before biomolecular fractionation (Fig. 1).

Lipid-like fraction

Ten millilitres of a dichloromethane/methanol (3/1) solution was added to 20 mg of each freeze-dried microbial biomass. They were shaken (24 h, 16 rpm) and sonicated (60 W, 30 min), and the liquid and solid fractions were separated by centrifugation (4000g, 4 °C, 30 min). The extraction was carried out twice.

Polysaccharide-like fraction

The solid residues obtained after dichloromethane/methanol extraction were dried under soft conditions (20 °C, 24 h). Twenty millilitres of trichloroacetic acid (10% w/v) was added to the dried biomass obtained from liquid cultures, shaken (1 h), and sonicated (60 W, 30 min), and the liquid and solid fractions were separated by centrifugation (4000g, 20 °C, 30 min). The extraction was repeated twice. The dried soils were treated with 2 mL of trichloroacetic acid (10% w/v), sonicated (60 W, 30 min), adjusted to 12 mL with trichloroacetic acid (10% w/v), and shaken (1 h, 16 rpm), and the liquid and solid fractions were separated by centrifugation (4000g, 4 °C, 30 min). After

centrifugation, the solid pellets were rinsed with 10 mL of trichloroacetic acid (10% w/v) and suspended using a vortex shaking.

Protein-like fraction

After trichloroacetic acid extractions, the solid pellets were hydrolysed with 20 mL of HCl (3 M) by heating at reflux for 16 h and were then concentrated under vacuum at 80 °C. The hydrolysates were separated from the biomass solid residue by filtration (Whatman HVLP, 0.45 µm). In the case of soil samples, hydrolysates were separated from the soil residues, which contained the residual incorporated radioactivity, by centrifugation (4000g, 4 °C, 30 min). In the case of solid samples, an additional humic fraction was recovered via the neutralization of acid hydrolysates at pH 7 and centrifugation of humic material precipitate. This step is necessary to clean the extracts before HPLC analysis.

Analytical procedures

During all incubations, NaOH-containing CO₂ traps were renewed daily, and ¹⁴C-CO₂ from the 2,4-D mineralization was measured by the scintillation counting of three 50 µL-aliquots of each fraction (Packard Ultima Gold XR as scintillation cocktail, Packard Tri-Carb 2100 TR liquid scintillation counter).

After biomass fractionation, the ¹⁴C content in the solid residues (residual non-extracted biomass, soils and humic fractions) was measured in triplicate after combustion using an Oxidizer 307 Packard. The combusted ¹⁴C-CO₂ was trapped in 5 mL of an organic base (Carbosorb E Packard) mixed with 20 mL of scintillation cocktail (Packard Permafluor E+). The radioactivity was measured by liquid scintillation counting.

Semi-quantitative analysis by HPLC was done on water extracts and pooled extracts of each biomolecular fraction. One millilitre of each liquid extract was filtered (Whatman HVLP, 0.45 µm) and analysed by HPLC on a Waters Nova-Pak C18 column (60 Å, 4 µm, 4.6 × 250 mm, Waters Milford, MA, USA) using a Waters appliance equipped with automatic injection and a 996-photodiode array detector coupled on-line with a continuous-flow radioactivity detector (Packard-Radioamatic Flo-One A-500). UltimaFloAP was used as the scintillation cocktail with a flow of 3 mL min⁻¹. The mobile phase (1 mL min⁻¹) was methanol:water:acetic acid (A [48:50:2] and B [78:20:2]). The anisocratic method used included a proportion of mobile phase A (100%, 40%,

40%, 100% changed at 0, 15, 18 and 20 min, respectively). Analytical standards of 2,4-D and its metabolites (2-chlorophenol [2-CP], 4-chlorophenol [4-CP], 2,4-dichlorophenol [2,4-DCP], and phenol, as a reference for hydroxylated-dehalogenated ring) were used to identify the degradation products in the extracts.

Statistical analysis

One-way analysis of variance was used to evaluate significant differences ($p < 0.05$) between radioactivity concentration means in all sub-samples. Multiple range tests were used to analyse the radioactivity differences between biomolecular fractions. Variance components analysis was used to estimate microbial and/or soil contribution to explain the biomolecular fraction-radioactivity differences.

Results and discussion

Overall view of the degradation of ^{14}C -2,4-D by fungi and bacteria in liquid medium

The distribution of ^{14}C at the end of incubations in liquid cultures is reported in Table 1. It presents carbon mineralized, residues in biomass and soluble radioactivity remaining in the culture media. The total recovered radioactivity ranged from 81.9% to 98.3% of applied radioactivity. The highest ^{14}C -2,4-D mineralization was observed in *R. eutropha* cultures, in accordance with the general high ability of bacteria to use the pesticide as a carbon source. This bacterial degradation was accompanied by a total disappearance of 2,4-D from the culture media, which only contained unidentified radioactivity. Approximately 15% of the ^{14}C of 2,4-D was found to be associated with bacterial biomass.

Both fungi behave differently with respect to 2,4-D degradation (Table 1). *F. solani* had the lowest 2,4-D degradation capacity, with very low mineralization and radioactivity incorporation into the fungal biomass lower than 10% of the applied radioactivity. Most of the 2,4-D, without any degradation, remained in the culture liquid. This result was expected because *F. solani* is known to lack enzymes that are able to transform the herbicide (Charnay et al., 2004). In contrast, *T. versicolor* exhibited a large ability to degrade 2,4-D with moderate mineralization, almost the

complete disappearance of 2,4-D from solution, the appearance of highly diverse metabolites (mainly 2,4-DCP and phenol), and a significant amount of radioactivity incorporated into the fungal biomass, i.e., N 20% of the initial radioactivity (Table 1). These features confirm the well-known ability of that strain for the dechlorination and oxidation of the 2,4-D (Arcand and Archibald, 1991). The high proportion of phenols identified by HPLC analysis in the culture medium of *T. versicolor* is not common in aerobic degradation. It could be related to the ability of some fungi to replace chlorine by hydroxyl; in that case, the hydroxylated metabolites would not degrade further (Häggbloom, 1992).

The ^{14}C -2,4-D degradation in synthetic liquid media was a result of microbiological activity, in accordance with previous studies (Liu and Chapman, 1984; Fukumori and Hausinger, 1993; Farhana and New, 1997; Leveau et al., 1999; Charnay et al., 2004). Several studies report a 2,4-D transformation occurring in biomass (Liu and Chapman, 1984; Fukumori and Hausinger, 1993; Leveau et al., 1999; Hawkins and Harwood, 2002). Nevertheless, in our study, the ability of the microorganisms to use radioactivity was not always associated with ^{14}C -2,4-D incorporation into biomass. For example, *R. eutropha* uses 2,4-D as a source of energy rather than incorporation into the ^{14}C biomass.

Incorporation of ^{14}C -2,4-D into the biomolecular-like fractions of fungal and bacterial biomass incubated in liquid medium

The fungal and bacterial biomass recovered after 2,4-D incubation in liquid media were sequentially fractionated, and the distribution of ^{14}C in biomolecular-like-fractions is shown Fig. 2. The high 2,4-D mineralization by bacteria *R. eutropha* resulted in the bacterial biomass with the highest polysaccharide and protein-like fractions. In the fungal biomass, when 2,4-D is highly degraded (*T. versicolor*), the radioactivity into the biomass was homogenously distributed among the three biomolecular-like fractions, with a higher proportion in the lipid-like fraction than in the bacterial biomass. However, when the fungi availability to degraded 2,4-D was low (*F. solani*), 75% of the incorporated radioactivity in the fungal biomass was associated with the lipid-like fraction, and the amount in the protein-like fraction was very low. Studies with *Cuproavidus necator* using ^{13}C -2,4-D showed a preferential use of chain-C for energy acquisition, whereas ring-C was the preferentially used carbon source for lipid synthesis (Lerch et al., 2007). The role of proteins in the formation of biogenic bound residues was noted by Nowak et al. (2011), who identified specific

labelled amino acids with C coming from the 2,4-D. This protein fraction could play an important role in the increasing persistence of biogenic bound residues of pesticides because their turnover time is much longer than that of C incorporated in lipid-like fractions (Nowak et al., 2011).

The radioactivity associated with each biomolecular fraction can correspond to novel biomolecular compounds synthesized by microorganisms or to 2,4-D and its metabolites absorbed by the biomass and co-extracted with different biomolecular fractions. To verify the chemical nature of radioactivity in each extracted biomolecular fraction, semi-quantitative chromatographic analyses of each biomolecular extracts were performed (Fig. 3). The only exception was the biomass of *F. solani* because the extracted radioactivity in the fractions was insufficient for HPLC analysis.

Control essays were also done in sterile conditions using a solution of 2,4-D to verify the chemical transformation of 2,4-D during the incubation conditions and the extraction procedures. The extractants used for lipid fraction provided a recovery of approximately 40% of the 2,4-D in this sterile control; the remainder was extracted in the next step with extractant used for the polysaccharide-like fraction. However, the trichloroacetic acid of this extractant induced a cleavage of the acid side-chain and a dechlorination of the 2,4-D molecule given a high proportion of 4-CP.

Bacterial (*R. eutropha*) and fungal (*T. versicolor*) biomass incorporated, respectively, 17 and 26% of the ^{14}C -biomass into the lipid-like fraction (Fig. 2). The HPLC analysis of the dichloromethane:methanol extracts show that a large part of the extracted radioactivity corresponded to unidentified high polar compounds that were not retained by the chromatographic C18 column (Fig. 3). 2,4-D was detected in the extracts, representing < 1% of the ^{14}C -biomass. Other metabolites were detected and found to be associated with lipid-like fractions; fungal metabolites were characterized by the accumulation of 2,4-DCP, and bacterial metabolites were characterized by the presence of 4-CP.

The biomolecular fractionation showed a direct relationship between the radioactivity recovered in the polysaccharide-like fractions and the microbial ^{14}C -2,4-D mineralization. It represented 35% of bacterial biomass radioactivity but only 24 to 28% of the fungal biomass radioactivity (Fig. 2). Only unidentified compounds were detected in the extracts, and they primarily corresponded to very polar compounds that were not retained by the chromatographic C18 column. 2,4-D and other metabolites were not detected in extracts of these fractions (Fig. 3).

As for the polysaccharide-like fractions, a direct relationship was found between the radioactivity content into the protein-like fractions and the microbial 2,4-D use capacity. The protein-like fraction represented 39% of bacterial biomass radioactivity and 26% of fungal *T. versicolor*

radioactivity but only 2% of the biomass of fungi *F. solani* (Fig. 2). The protein-like fraction can be used as an indicator of pesticide microbial use incapacity, considering the almost absent radioactivity incorporation in the protein-like fraction for *F. solani*, their poor 2,4-D mineralization capacity and the almost total 2,4-D recovery in the culture liquid remaining after incubation.

R. etropha showed a high proportion of radioactivity in the protein-like fraction. 2,4-D and its metabolites were not detected in extracts of this fraction, whereas unidentified and unretained polar compounds corresponded to hydrolysed amino acids (Fig. 3). The absence of chemical structures related to 2,4-D is expected because the drastic conditions of acid hydrolysis. The rapid incorporation of tracers (^{13}C , ^{15}N) from pesticides as constituents of proteins has been shown previously (Nowak et al., 2011). The protein-like fraction of the fungal biomass from *T. versicolor* is also composed of chromatographic unretained polar compounds (Fig. 3). This fungus is well known for its capability to partially degrade 2,4-D through its high dechlorination and oxidation ability (Arcand and Archibald, 1991). The 2,4-D metabolites produced by *T. versicolor* are mainly recovered in the culture liquid, with a low use of 2,4-D carbon to produce biomass.

The corresponding results found on the protein-like fractions corroborate that this fraction is the most appropriate biological marker for biomass construction using 2,4-D as a carbon source (Nowak et al., 2011).

Degradation of ^{14}C -2,4-D in soil

The distribution of ^{14}C -2,4-D in incubated soils, including mineralization, water-soluble radioactivity and residues remaining in soil is shown in Table 2. The total recoveries of applied radioactivity ranged from 81.9 to 98.8%.

Incubation of sterile soil showed a very low 2,4-D mineralization, which indicated relatively well-maintained sterile conditions during soil incubation. In this sterile incubation, 84% of radioactivity was solubilized by water, indicating that this water extraction can be used to characterize the 2,4-D residues in soils; non-water soluble residues represented 6% of the applied radioactivity (Table 2). Sterile soil inoculated with *R. etropha* showed 65% 2,4-D mineralization, similar to the mineralization rate under liquid medium; this high mineralization was also found when 2,4-D was incubated directly with non-sterile soil (Table 2). Endogenous soil microorganisms showed 2,4-D mineralization kinetics with a lag phase of 2 days, followed by a rapid increase in the mineralization

rate (Fig. 4). The lag phase is interpreted as the adaptation time of the degrading microorganisms. In contrast, 2,4-D mineralization on sterile and non-sterile soil inoculated with *R. eutropha* started immediately, without any lag phase. Mineralization rates were rapid at the beginning and slowed progressively during incubation. Thus, the bacterial strain maintained its 2,4-D mineralization activity from the onset of soil inoculation, whereas endogenous soil microorganisms required an adaptation period to exploit 2,4-D mineralization. A slight slowing of mineralization was observed when *R. eutropha* was inoculated on non-sterile soil (Fig. 4). This could be interpreted as the result of a competition between endogenous microorganisms and inoculated *R. eutropha*. This high capacity of ^{14}C -2,4-D mineralization observed for *R. eutropha* here, in both solid and liquid conditions, is consistent with previous reports and supports its ability to use the pesticide as a carbon source (Charnay et al., 2004). Their elevated and rapid ^{14}C -2,4-D microbial degradation activity is in accordance with the reported results (Liu and Chapman, 1984; Fukumori and Hausinger, 1993; Farhana and New, 1997; Leveau et al., 1999; Charnay et al., 2004). The *R. eutropha* cultures had a significant increase in radioactivity residues remaining in soil after water extraction; some of this radioactivity can be considered to be associated with soil microbial biomass (biogenic bound residues). The non-extractable radioactivity under soil conditions, between 29 and 38% of the initial ^{14}C -2,4-D (Table 2), was greater than expected from biomass production under liquid conditions. The observed differences suggest an increase in 2,4-D use for biomass production, and/or an increase in the physical and chemical retention of 2,4-D and its degradation products on the soil constituents.

Distribution of ^{14}C -2,4-D into the biomolecular-like fractions of incubated soil

Most of the residual radioactivity remaining in soil after water extraction in incubation under sterile conditions was solubilized with the extractant of the lipid-like fraction (Fig. 5); the extracted radioactivity was mainly 2,4-D (Fig. 6). Under soil incubations with active micro-organisms, this fraction showed the lowest radioactivity recoveries, remaining below 10% of the residual radioactivity remaining in soils (Fig. 5). HPLC analysis showed that the solubilized radioactivity was mainly unidentified compounds, with a low amount or a complete absence of 2,4-D and the presence of 2,4-DCP (Fig. 6). Lipid constituents play an important role in the incorporation of tracers into the microbial biomass. In the metabolic functioning of microorganisms, pesticides tracers are first incorporated into lipids (mainly via synthesis of phospholipids), then into amino

acids and protein synthesis (Nowak et al., 2011, 2013).

The proportion of the polysaccharide-like fraction ranged between 9 and 15% of the radioactivity remaining in soils. The highest amount was found when *R. eutropha* was inoculated on the sterile soil. However, the proportion of this biomolecular-like fraction was lower than that observed when *R. eutropha* was incubated in liquid conditions; similar to the liquid conditions, the radioactivity in extracts of the polysaccharide-like fraction was mainly unidentified chromatographic unretained polar compounds.

The magnitude of protein-like fraction is directly correlated to the microbial 2,4-D use capacity. Its proportion was homogenous for all soil incubations in the presence of active microorganisms, with N 45% of the radioactivity remaining in the soil extracted as a protein-like fraction (Fig. 5). This elevated amount is consistent with the values published under other conditions, as estimated by measurements of individual amino acids labelled with ^{13}C coming from ^{13}C -pesticides (Nowak et al., 2011; Girardi et al., 2013). For sterile soil incubation, the radioactivity recoveries in extracts of the protein-like fraction were very low (6% of the radioactivity remaining in soil), corresponding to the compounds retained by the chromatographic C18 column (2-CP and other unidentified compounds). The microbial use of 2,4-D had a significant effect in the protein-like fraction in which the proportion of radioactivity remaining in soil was the highest; the radioactivity extracted in these fractions was mainly chromatographic unretained polar compounds, which may have corresponded to the amino acid.

The increase resulting from microbial use of 2,4-D is consistent with results obtained from liquid cultures. This behaviour confirms that the protein-like fractions are the most appropriate biological markers for biomass construction using 2,4-D as a carbon source in both solid and liquid conditions. The microbial activity is reflected in an increase in chromatographic unretained radioactivity, which corresponds in part to hydrolysed amino acids. This confirms the important role of amino acids in the incorporation of tracers coming from pesticides and their use as indicators of biogenic bound pesticide residues (Nowak et al., 2011; Wang et al., 2016).

Conclusions

Biological activity in soils determines the fate of pesticides via the degradation process. In addition to partial degradation from metabolite production, parts of pesticides can be used for

microorganisms as a carbon source to produce microbial biomass. However, the pesticide (or its metabolites) may be trapped in the biomass without a real transformation. 2,4-D degradation pathways are different between bacterial and fungal strains, with fungi having different capacities depending on their enzymatic equipment.

Special attention must be placed on quantifying the biomolecular fractions and their biomass incorporation in degradation studies. The biomass trapping capacity and increase in the amount of residues support the hypothesis that microbial participation in pesticide soil retention is through bound residue formation. The protein-enriched fraction is the most appropriate indicator of 2,4-D microbial metabolic use and biogenic bound residue formation. Furthermore, the polar chromatographic unretained compounds could be an indicator of biomass transformation as a result of 2,4-D use, due to its direct relationship with mineralization. The identification of these unretained compounds must be accomplished by using other isotopic techniques, such as the ^{13}C stable isotope, thus allowing the use of other chromatographic techniques (Lerch et al., 2007; Nowak et al., 2011, 2013; Wang et al., 2016). To extend this finding to other carbon sources, soils and microorganisms, it is necessary to repeat the protocol with other pesticides and with different soils and microbial species. These results confirm that biogenic bound residues exist. Our results also increase our understanding of carbon distribution from pesticides in microbial soil biomass and suggest a biological marker as a tool for future studies.

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Table 1. Distribution of radioactivity between mineralization, microbial biomass and culture liquid remains after 10 days incubation of ^{14}C -2,4-D with uninoculated and inoculated liquid media with fungal (*F. solani* and *T. versicolor*) and bacterial (*R. eutropha*) strains. HPLC analysis of the liquid media included 2,4-D and its main metabolites (2,4-DCP, 4-CP, 2-CP, and phenol as dehalogenated compound) and unidentified radioactivity unretained by the chromatographic column (UR).

	Mineralized ($^{14}\text{CO}_2$)	Recovered in biomass	Remaining in liquid culture medium % of initial radioactivity ^{14}C -2,4-D	HPLC analyses of liquid medium	Total radioactivity recovered
Non-inoculated	0.1 ± 0.6	-	93.8 ± 0.8	86.7 ± 2.1 (2,4-D)	93.9 ± 1.4
Fungi					
<i>Fusarium solani</i>	0.3 ± 0.3	7.8 ± 3.8	89.4 ± 0.5	85.9 ± 2 (2,4-D)	97.5 ± 4.6
<i>Trametes versicolor</i>	7.9 ± 0.4	24.3 ± 1.0	49.7 ± 0.5	28.9 ± 1.4 (Phenol) 13.1 ± 2.1 (2,4-DCP) 1.9 ± 0.8 (2-CP) 2.5 ± 1.1 (4-CP) 1.4 ± 0.4 (2,4-D)	81.9 ± 1.9
Bacteria					
<i>Ralstonia eutropha</i>	65.2 ± 0.4	15.5 ± 0.2	17.6 ± 0.5	1.4 ± 0.6 (UR) 0.0 ± 0.1 (2,4-D)	98.3 ± 1.1

Table 2. Distribution of radioactivity between mineralization, solid residuals after water extraction (including soil microbial biomass), and water-soluble extracts after 10 days of incubation of sterilized and non-sterilized soil, inoculated with *R. eutropha*. HPLC analyses of the water-soluble residues include 2,4-D and its main metabolites (2,4-DCP, 4-CP, 2-CP and phenol) and unidentified radioactivity unretained by the chromatographic column (UR).

	Mineralized ($^{14}\text{CO}_2$)	Remaining in soil	Counted in water extracts % of initial radioactivity ^{14}C -2,4-D	HPLC analyses of water extracts
Sterile soil	3.6 ± 0.7	6.3 ± 1.3	84.3 ± 1.2	70.2 ± 2.3 (2,4-D)
Soil	63.7 ± 0.75	34.0 ± 3.3	2.4 ± 0.2	1.9 ± 0.1 (UR)
Sterile soil + <i>R. eutropha</i>	64.9 ± 0.7	29.6 ± 1.8	6.3 ± 0.8	2.7 ± 0.4 (UR)
				2.4 ± 0.2 (2,4-D)
				0.3 ± 0.1 (2,4-DCP)
Soil + <i>R. eutropha</i>	59.6 ± 0.5	38.0 ± 1.3	2.5 ± 0.2	1.4 ± 0.1 (UR)

Fig. 1. Sequential biomass fractionation procedure for fungal and bacterial cultures in soils with ^{14}C -2,4-D as a carbon source. The procedure includes: mineralization quantified as total amount of trapped $^{14}\text{CO}_2$, water-soluble fractions (HSF), biomolecular-like fractionation of biomass, methanol:dichloromethane extraction for lipid-like fraction (Li-LF), trichloroacetic acid extraction for polysaccharide-like fraction (Po-LF), and acid hydrolysis for protein-like fraction (Pr-LF), and finally residual radioactivity in soils (SR) or biomass (BR).

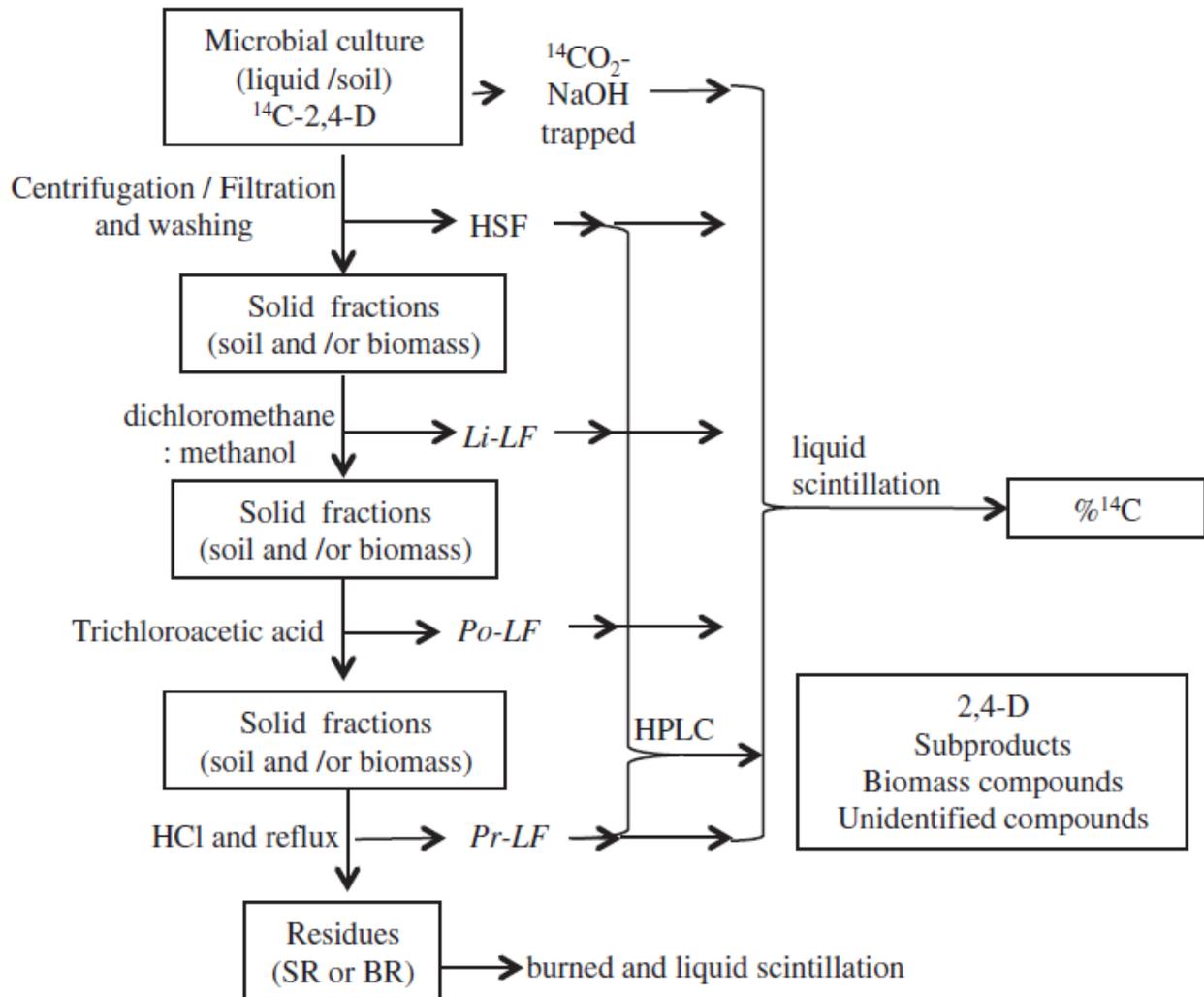


Fig. 2. Distribution of incorporated radioactivity into bacteria (*R. eutropha*) and fungi (*T. versicolor* and *F. solani*) between the biomolecular-like fractions (lipid, polysaccharide and protein-like fractions) and residual non-extractable radioactivity after 10 days of incubation in liquid conditions.

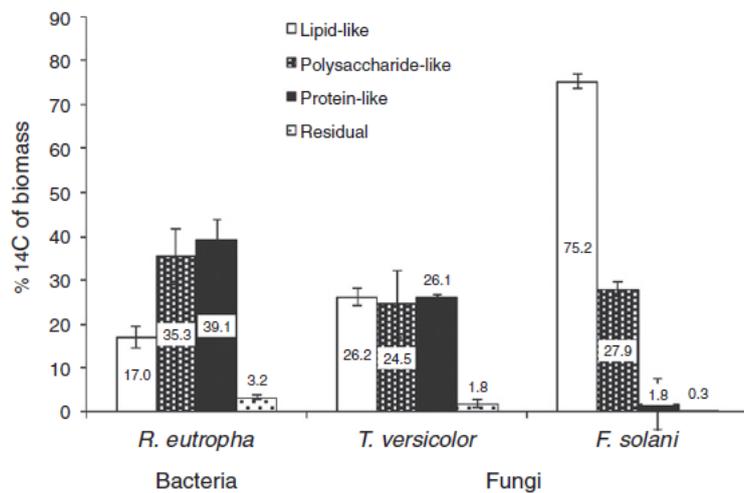


Fig. 3. Semi-quantitative HPLC analysis of extracted radioactivity associated with each biomolecular-like fractions of sterile control incubations in liquid conditions and inoculated liquid samples with *R. eutropha* and *T. versicolor* after 10 days of incubation. HPLC analysis includes 2,4-D, its main metabolites (2,4-DCP and 4-CP), unidentified compounds (UI) and unidentified radioactivity unretained by the chromatographic column (UR).

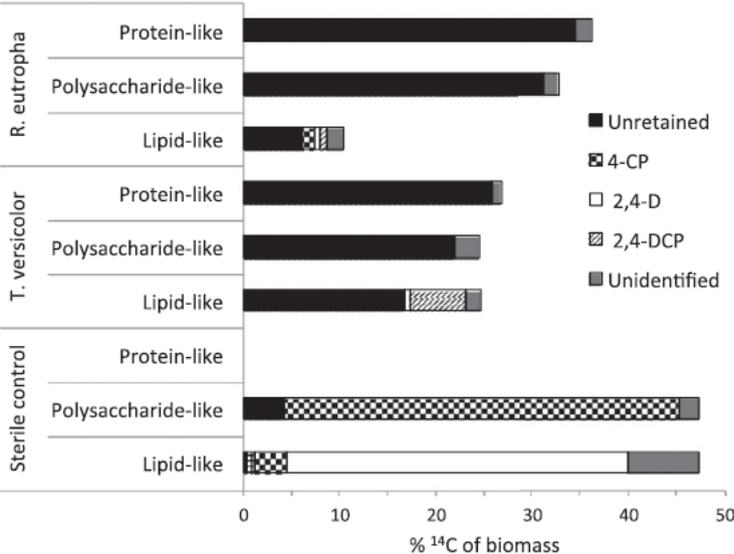


Fig. 4. Mineralization kinetics of ^{14}C -2,4-D incubated in sterile and non-sterile soil, inoculated or uninoculated with *R. eutropha* (results expressed in % ^{14}C - CO_2 of ^{14}C -2,4-D initial).

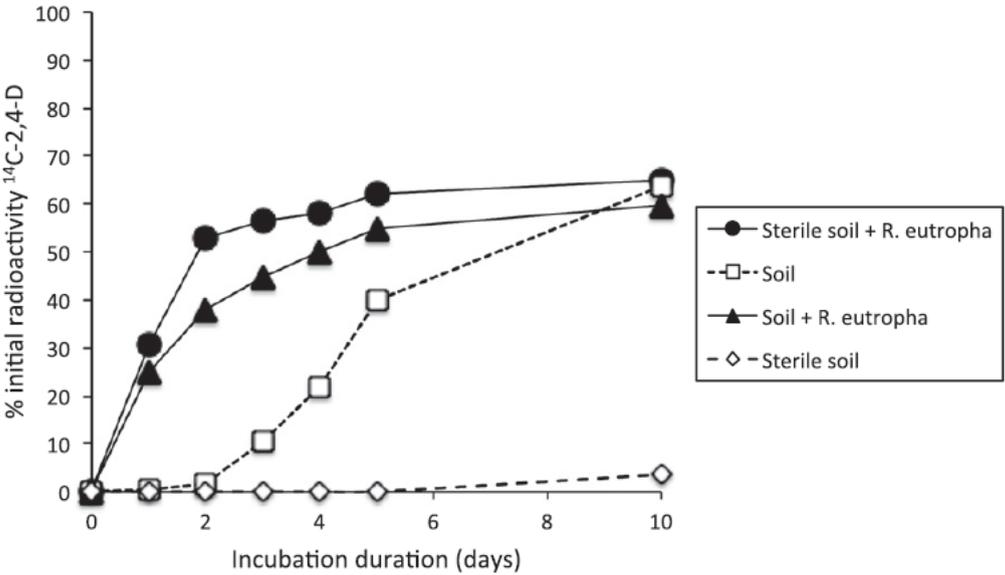


Fig. 5. Radioactivity distribution of 10 days incubated soils (sterile and non-sterile soil, and inoculated and uninoculated with *R. eutropha*) after water extraction between the biomolecular-like fractions (lipid, polysaccharide and protein-like fractions), humic fraction and residual non-extractable radioactivity.

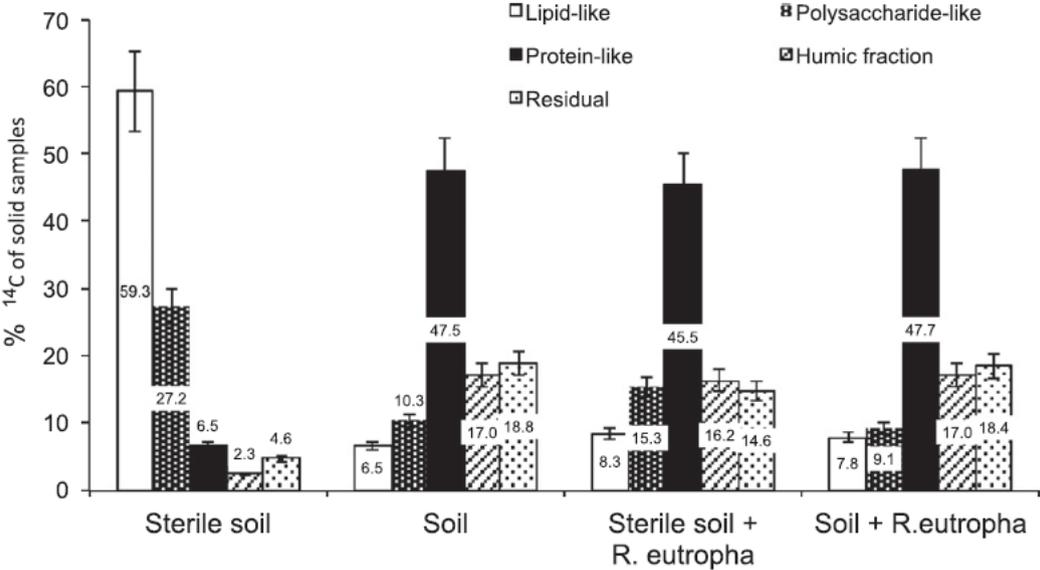


Fig. 6. Semi-quantitative HPLC analysis of extracted radioactivity associated with each biomolecular-like fraction of 10 days incubated soils (sterile and non-sterile soil, and inoculated and uninoculated with *R. eutropha*). HPLC analysis includes 2,4-D, its main metabolites (2,4-DCP, 4-CP, 2-CP and phenol, as dehalogenated compound), unidentified compounds (UI) and unidentified radioactivity unretained by the chromatographic column (UR).

