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Incorporation of pesticides by soil micro-organisms as a way of bound residues formation

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Abstract

We studied the ¹⁴C-tagged biochemical compartments such as proteins, lipids and polysaccharides from bacterial and fungal cultures supplied with ¹⁴C- pesticides and ¹⁴C-glucose. We showed that ¹⁴C incorporation depends both on the substrate structure and on the nature of the microorganism. Both bacterial and fungal cells incorporated glucose ¹⁴C mainly into cell wall proteins. By contrast, glyphosate ¹⁴C was mainly incorporated into cytoplasm carbohydrates by fungi. On the other hand, 2,4-dichlorophenoxyacetic acid ¹⁴C was mainly found in the carbohydrate fractions of the bacteria walls.

Keywords

Pesticides; Soil; Bound residues; Microflora

Introduction

The fate of pesticides in soils is mostly regulated by retention. As most bio-physico-chemical processes evolve with time, parts of pesticide residues become non-ex- tractable using classical

extraction techniques. These so-called bound residues are often regarded as a means of pesticide dissipation. Nonetheless the authors have demonstrated the release of bound residues by microbial activity and physical changes of soil constituents, and in turn, their uptake by plants and earthworms, thus raising a toxicological concern (Scheunert and Schroder 1998).

Due to the non-extractability of bound residues, radiolabelled pesticides are needed to follow the fate of the active molecule and its degradation products into various soil organic compartments. For instance, using degradative techniques such as pyrolysis and chemical hydrolysis, and pesticides labelled with ^{13}C or ^{15}N , Guthrie et al. (1999), Loiseau et al. (2000), Loiseau and Barriuso (2002), and Dignac et al. (2003) showed a partial release of bound residues. Their results showed that a major part of bound residues were compounds of chemical structure similar to that of the parent compound.

The main mechanisms proposed to explain the formation of bound residues imply usually binding with soil organic matter, either by covalent binding with pesticides and degradation products (Bollag et al. 1992; Dec et al. 1997), or by sequestration into the three dimensional structure of humic substances (Guthrie et al. 1999). A third mechanism of pesticide stabilisation involves the incorporation of residues into microbial biomass. Here, pesticides are more or less degraded and participate in the building of cellular components. In that way, micro-organisms can use the pesticide carbon for their metabolism, as recently shown for phenanthrene by Johnsen et al. (2002). Other processes of residue stabilisation may exist, such as trapping into membrane structures. The two last pathways of incorporation are as yet unknown in the case of pesticides. Their impact on the overall process of stabilisation has to be assessed.

Here we report the use of ^{14}C -labelled pesticides to study the incorporation of pesticide carbon by micro-organisms, and to identify the biochemical compartments in which bacterial and fungal cultures stabilise the ^{14}C -carbon from selected pesticides and from glucose.

Experimental

Micro-organisms and incubation conditions

Experiments were conducted using (1) the Gram- bacterium *Alcaligenes xylosoxidans* isolated from an agricultural plot located in Burgundy and generously provided by INRA, Dijon, France; (2) the fungus *Trametes versicolor*, a basidiomycete from the ATCC collection, strain N°32745, and (3)

the ascomycete *Fusarium solani* iso-ated from a silt loam under meadow in Versailles, France.

The bacterial strain was grown in a deionised water solution of: 2.7 g K_2HPO_4 , 2.82 g Na_2HPO_4 , 0.123 g $MgSO_4 \cdot 7H_2O$, 0.028 g $FeSO_4 \cdot 7H_2O$, 0.016 g $ZnSO_4 \cdot 7H_2O$, 0.174 g $K_2SO_4 \cdot 7H_2O$, 0.147 g $CaCl_2 \cdot 2H_2O$, and 5 g glucose per litre. Fungal strains were grown in a solution containing 2 g KH_2PO_4 , 0.14 g $CaCl_2 \cdot 2H_2O$, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.07 g $FeSO_4 \cdot 7H_2O$, 0.046 g $ZnSO_4 \cdot 7H_2O$, 0.035 g $MnSO_4 \cdot 7H_2O$, 0.007 g $CuSO_4 \cdot 5H_2O$, 2.3 g tartrate di-Na, 1.842 g tartrate di- NH_4^+ , 5 g glucose, and 1 g yeast extract. The pH values for the culture media were 6.9 and 6.5 for bacteria and fungi, respectively.

All chemicals and solvents were of analytical grade. The radiochemical purity of the labelled substrates was up to 98%. ^{14}C -glucose, uniformly ring labelled ^{14}C -2,4 dichlorophenoxyacetic acid (2,4-D) and phosphonomethyl labelled- ^{14}C -glyphosate were supplemented with unlabelled homologues and incorporated in liquid media as sterile mixtures to obtain a final concentration of 10 mg L^{-1} with a final activity of 333 kBq L^{-1} .

Bacteria were grown in 90 mL of liquid solution in 250-mL Erlenmeyer flasks under shaking and aerobic conditions in the dark for 7 days at $28 \pm 1 \text{ }^\circ\text{C}$. The 250-mL flasks were placed together with two vials containing 10 mL 10-N NaOH to trap the CO_2 , into 1-L sealed jars. For fungal strains, the 250-mL flasks containing 17 mL of solution were incubated statically at $28 \text{ }^\circ\text{C}$ for 10 days, using the same experimental setup. All the cultures were stopped at the end of the exponential growing phase. The evolved $^{14}CO_2$ was quantified, after mixing 500 mL of NaOH with 500 mL of water and 4 mL of scintillation cocktail, by counting with a Packard tri-Carb 2100 TR scintillation counter. Bacterial cells were harvested by 30 min centrifugation at 2,410g. Fungal biomass was filtered on Whatman GF/A 1 mm. The biomass was washed three times with distilled water. Radioactivity remaining in the culture media and rinsing water was measured by liquid scintillation counting of 500- μL aliquots. Biomass radioactivity was determined by combustion of 100-150 mg freeze-dried fractions using a Packard 307 oxidizer.

Biomass fractionation

Bacterial cells were disrupted for 20 min using a Prolabo 200-W sonicator. Fungal biomass was crushed in liquid nitrogen, then sonicated for 2x30 min at 60 W. The bacterial and fungal lysates were centrifuged for 45 min at 2,410g to separate cytoplasm and cell walls. Walls were freeze-dried.

The procedure illustrated Fig. 1 was then applied on cytoplasm or walls to sequentially separate the biochemical compartments. The determination of the amount of proteins, lipids and polysaccharides in the biomass was based on the physico-chemical properties of these biochemical compartments. Lipids were firstly extracted from the cytoplasmic fraction (50 mL) using a mixture of 50 mL dichloromethane and methyl alcohol 3/1 v/v. After a 24-h separation, the solvent was evaporated under gentle vacuum. In parallel, freeze-dried walls (20 mg) were extracted by 10 mL solvent mixture, and then sonicated for 30 min, before evaporation of the solvent. Polysaccharides were then extracted from the cytoplasmic fraction (30 mL) or cell walls (15 mg) with trichloroacetic acid (final concentration 10%) by 1 h shaking and 30 min sonication. Finally, proteins were obtained from cytoplasm (25 mL) or walls (100 mg) by hydrolysis with 50 mL 3-N hydrochloric acid. The radioactivity was measured in each fraction all along the procedure by liquid scintillation counting or combustion as described above.

Results and discussion

Metabolisation of ^{14}C -pesticides

Bacterial and fungal cultures were incubated with ^{14}C -2,4-dichlorophenoxyacetic acid (2,4-D) and phosphonomethyl labelled- ^{14}C -glyphosate to study the incorporation of ^{14}C into the microbial biomass. As shown on Fig. 2, about 50% of ^{14}C -2,4-D was mineralised by the *Alcaligenes* bacteria and *Trametes* fungus. 12% ^{14}C -2,4-D was incorporated into the fungal biomass versus only 4% in the bacterial biomass. Concerning the incubation of ^{14}C -glyphosate with the *Trametes* fungi, only 8% was mineralised, 16.5% being incorporated into the fungal biomass.

These results concerning the incorporation of labelled compounds have to be linked to the microbial size, which was always eight-fold higher in the case of fungal cultures than bacterial ones. That could mean that *Alcaligenes* bacteria incorporated 2,4-D 1,000-fold higher than *Trametes*. This difference was related to the metabolic pathways of these strains. *Alcaligenes* bacteria are able to use the herbicide 2,4-D as a carbon source, as reported by Gunalan and Fournier (1993). On the contrary, *Trametes* and many other fungi mineralise organic pollutants without using them as a carbon source.

For all pesticide studied, the amounts incorporated into the microbial biomass were low, reaching less than few milligrams per gram of microbial biomass dry weight. That result could be explained

by the presence of glucose in the culture. Indeed, glucose is a substrate easier to metabolise than pesticides.

Stabilisation of pesticides in the biochemical compartments

After incubation of bacteria and fungi with ^{14}C -pesticides, the microbial biomass was fractionated into lipids, polysaccharides and proteins to study the distribution of ^{14}C into those biochemical compartments. The results shown in Fig. 3 compare the stabilisation of ^{14}C -pesticides to that of glucose, an easily metabolisable substrate. Looking at the results concerning the distribution of glucose in the bacteria *Alcaligenes*, we noted that lipids, polysaccharides and proteins represented respectively about 11, 39 and 50% of biomass dry weight. These results were in accordance with data of the literature (Madigan et al. 1994).

The situation differed in the case of the fungus *Fusarium* because of a low value observed for polysaccharides incorporated in the cytoplasmic fraction (12%). One explanation should be related to the low efficiency of the protocol to extract polysaccharides, which are highly trapped in the polymers of fungal walls. Nevertheless, carbon from glucose seemed preferentially stored as wall's proteins in both bacteria and fungi.

Total amounts of ^{14}C recovered were higher concerning glucose (100%) than in the case of pesticides (72-92%).

Results concerning the cultures incubated with the herbicides 2,4-D and glyphosate, which belong to different chemical families, were quite different (Fig. 3). Both pesticides were preferentially incorporated in the cytoplasm. The *Trametes* fungi incorporated these compounds mainly in the glucidic fraction extracted from the cytoplasm, amounting to about 54% for glyphosate and to 33% and for 2,4-D. In the case of the bacteria *Alcaligenes*, the incorporation of 2,4-D was more important in both the lipidic and glucidic fractions extracted from the wall.

Conclusion

A sequential protocol of biochemical fractionation was developed. It allowed to us quantify the amount of pesticides stabilised by micro-organisms through incorporation, and to identify the biochemical compartment in which carbon is preferentially incorporated by bacterial and fungal cells. In addition, we showed that the incorporation of labelled carbon depended on both the

structure of the substrate and on the nature of the micro-organism, and we observed differences between pesticides and glucose, a substrate more suitable for metabolisation. Even if the generalisation of a pesticide incorporation mechanism by the microbial biomass is not possible at this stage, it could be stated that it represented a major route in the formation of bound residues. No information is available yet on the chemical structure of the compounds linked during stabilisation and consequently on their possible toxicity. Additional studies are in progress to address these points.

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Fig. 1. Procedure of sequential biomass fractionation to separate the lipid, polysaccharide and protein fractions

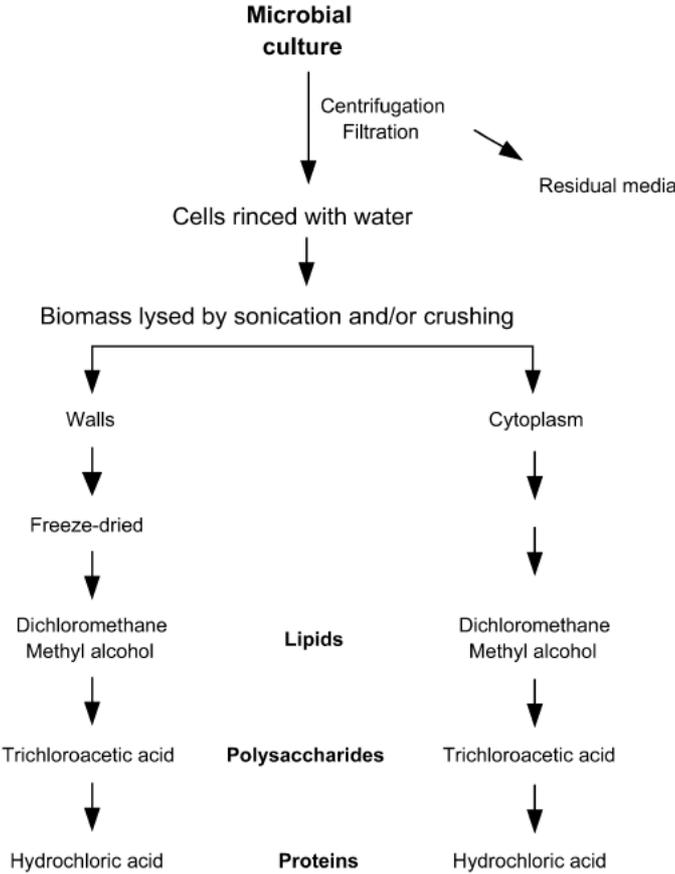


Fig. 2. ^{14}C radioactivity in the residual culture media (white), biomass (black) and evolved CO_2 (hatched) from incubations of *Alcaligenes* bacteria and *Trametes* fungi with ^{14}C -2,4 dichlorophenoxyacetic acid (2,4-D) and phosphonomethyl labelled- ^{14}C - glyphosate. The radioactivity balance at the end of the experiments in the cultures ranged from 90 to 98%

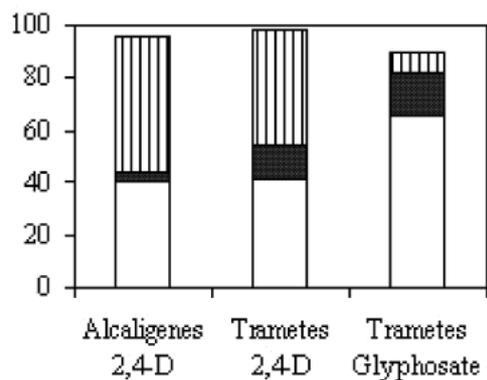


Fig. 3. Distribution of the labelled carbon in the cytoplasm (white) and cell walls (black) of the bacterial strain *Alcaligenes* (Alc), the fungal strains *Fusarium* (Fus) and *Trametes* (Tram), incubated with either ^{14}C -2,4-dichlorophenoxyacetic acid (2,4-D), phosphonomethyl labelled- ^{14}C -glyphosate (Glyp) or ^{14}C -glucose

