Fate and impact of the antibiotic ciprofloxacin in soils from integrated terrestrial microcosms submitted to pig slurry amendment

Mougin Christian[1]*, Cheviron Nathalie[1], Hedde Mickael[1] and Hernandez-Raquet Guillermina[2]
(1) INRA, UR PESSAC, Route de Saint-Cyr, 78026 Versailles, FR
(2) INSA, INRA, CNRS, UMR 5504, UMR792 LISBP, 135 Avenue de Rangueil, 31077 Toulouse Cedex 4, FR
*Corresponding author: christian.mougin@versailles.inra.fr

Abstract
The antibiotic ciprofloxacin is suspected to induce significant adverse effects on soil microbial processes, with possible consequences on soil functions. Here we studied the fate and effects of ciprofloxacin on soil microbial communities, using integrated microcosms where the antibiotic was applied by spiked pig slurry amendment or direct spreading. We showed that the very weak microbial mineralization of the antibiotic occurring within 84 days of incubation, was increased during the following 84–d incubation by the activity of added earthworms. Nevertheless, ciprofloxacin stabilized in the soil, probably as non-extractable residues. It was poorly bioavailable and weakly transferred to water and wheat seedlings. At concentrations found in the agricultural environment, we were unable to demonstrate significant effects of ciprofloxacin on soil microorganisms. We conclude that additional experiments are needed to evidence effect of antibiotics residues on soil microorganisms.

Introduction
Fluoroquinolones are a major class of antibiotics worldwide used for human and veterinary medicine. Ciprofloxacin used for human medicine is excreted in urine and faeces, and enters the environment mainly through the spreading of sewage sludge. In the case of veterinary use of fluoroquinolones for cattle, pig or poultry protection, ciprofloxacin is released as the main active metabolite of the antibiotic enrofloxacin, and enters the soil through grazing livestock and manure or slurry use as fertilizers of agricultural soils [1]. The effects and fate of ciprofloxacin on the soil ecosystem are poorly known, thus underlining the ecotoxicological relevance of this antibiotic. In a first time, we assessed the fate of ^{14}C-labeled ciprofloxacin entering an agricultural soil according to several scenarios of contamination [2]. The originality of our approach was to include in microcosms soil-dwelling macroorganisms well-known for their ability in bioturbation. In a second time, we studied the effects of the antibiotic on the soil microbial community [3].
Here we synthesize our main results obtained during the ANR program “Diperpha”.

Material and Methods
Soil characteristics
The silt loam (Luvisol, FAO classification) was collected in the 10–20 cm layer of an experimental site (La Cage, INRA, Versailles, France). It comprised 21.6% sand, 61.1% silt, and 17.3% clay. Its content in organic carbon was 0.98%, and its pHwater was 7.1. Its water holding capacity (WHC) was 15.64 ± 0.21% at pF=2.5 and 6.79 ± 0.21% at pF=4.2.

Design of integrated microcosms
We used the integrated microcosms developed by our team. Four experimental treatments were prepared: non-amended control soil, soil amended with slurry spiked with low and high amounts of antibiotic and finally soil spread with acetonic solution of antibiotic. The pig slurry, referred as green slurry, was first spiked with [2-^{14}C]-ciprofloxacin for studying the fate of the antibiotic, or unlabeled antibiotic to assess its effect. Then the slurries were applied onto the soil to mimic organic amendments. The integrated microcosms incubated for 168 days under 16 h light at 20°C and 8 h darkness at 18°C. After 56 days of incubation, three seeds of wheat were sowed in each microcosm. After 84 days of incubation, four mature earthworm specimens (two *Aporrectodea caliginosa* Savigny 1826 and two *Apporrectodea longa* Savigny 1826) were introduced in each microcosm.

A similar protocol was used to assess the effects of ciprofloxacin on soil microbial communities, but unlabelled antibiotic was used and the duration of incubations was 28 days.

Analytical procedures

A stream of wet air was continuously flushed through the microcosms to allow $^{14}CO_2$ trapping in NaOH solutions. NaOH solutions were changed every 7 days and $^{14}C0_2$ was determined by liquid scintillation counting. Two soil cores were extracted to determine extractable and bound ^{14}C in the layers of the soil and soil/slurry mixtures at the beginning of the experiment, and after 84 days of incubation. At the end of the experiment (168 days of incubation), soils corresponding to the location to the previous upper and lower layers were separated, and samples were collected in each of them to measure the influence of earthworm bioturbation. Radioactive compounds were extracted from 10 g fresh soil samples during 20 min, in the presence of 40 mL acidified ACN [4]. To assess the leaching of cyprofloxacin, water was sprayed after 70 days of incubation onto the soils to mimic 20-mm rainfalls and to allow recovery of leachates. Wheat seedlings were harvested and dried after a 28-d period of growth (84 days of incubation). The radioactivity was measured in all liquid fractions (extracts, leachates) by liquid scintillation counting. Non-extractable radioactivity (soil, soil/slurry samples, and seedlings) was determined by combustion, followed by liquid scintillation counting.

Biological descriptors

Enzymatic activities were determined using spectrophotometric methods. For laccase measurements, the reaction mixture contained 1 mL soil solution (obtained from 1 g soil in 6 mL KH_2PO_4/citric acid buffer pH 3.0), and 600 µl of a 50 mM guaiacol solution. After incubation at 37 °C for 20 min, the mixture was centrifuged at 12000 rpm at 4 °C for 2 min. The formation of tetrameric guaiacol in the supernatant was measured at 470 nm.

Dehydrogenase activity (DHA; E.C. 1.1.1.1) was measured in 30-mL centrifuge tubes containing 1 mL distilled water and 250 µL aqueous solution of 1,3,5-triphenyltetrazolium chloride (TTC) at 40 g/L mixed with 1 g fresh soil. Incubation was performed at 37 °C for 16 hours. The absorbance of the supernatant (red colour in case of formazan formed) was measured at 485 nm.

For acid phosphatase (ACP; E.C. 3.1.3.2) and β-glucosidase (GLU; E.C. 3.2.1.21) activities, 16 g soil were shaken in 100 mL distilled water. Then, 125 µL of soil suspension were incubated with 25 µL 0.05 M substrate (4-nitrophenyl phosphate for the phosphatase or 4-nitrophenyl ß-D-galactopyranoside for the glucosidase) at 37 °C for one hour in multiwell plates. At the end of the incubation, 25 µL of CaCl2 (0.5 M) were added in order to stabilize humic acid, and 100 µL Tris 0,1 M pH12 were used to stop the reaction. The amount of p-nitrophenol (PNP) formed was obtained by the measurement of the supernatant absorbance at 405 nm. Bacterial and fungal biomass were determined from soil genomic DNA [3].

Results

Mineralization of ciprofloxacin in soils

The mineralization of ciprofloxacin was monitored in the four integrated microcosms (Figure 1). No mineralization occurred in the microcosms containing the reference soil. By contrast, traces of mineralisation (less than 0.01% of initial amount of radioactivity) were detected during the first week of incubation in the three microcosms containing 14C-ciprofloxacin. Then, mineralization increased very slightly to a maximal value of 0.019% of initial radioactivity at day 84 for the lower spiking amount, and 0.007–0.010% for the highest amount of contamination.

Earthworm introduction in the soil at day 84 led to a 7-fold increase of antibiotic mineralization, reaching 0.058–0.080% of initial radioactivity after the next 84-day period, depending on the microcosm considered.

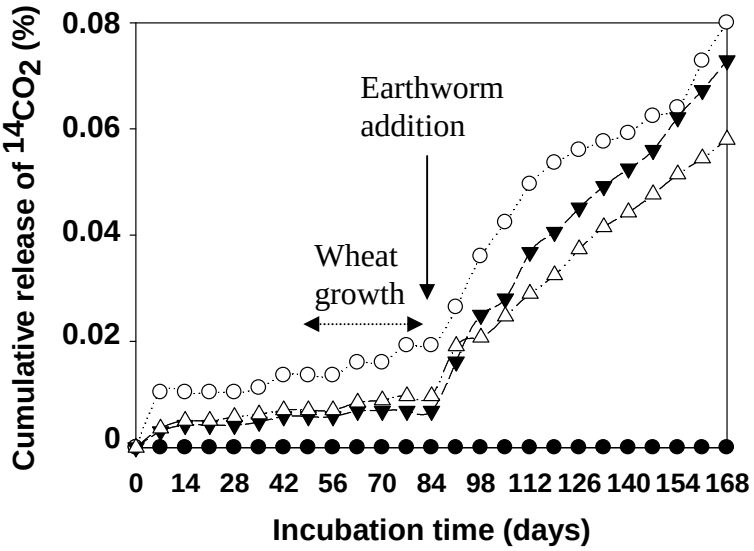

Figure 1. Mineralization of [2-14C]-ciprofloxacin in control soil (□), soil amended with slurry spiked with the low (O) and high (▼) amounts of antibiotic, and soil spread with the high amount of antibiotic (Δ) during the 168 days of incubation. Wheat seedlings were allowed to growth between 56 and 84 days of incubation. Earthworms have been introduced in the soils after 84 days of incubation.

Earthworm bioturbation can affect the fate of contaminants by changing their mobility and sorption to the soil in modifying the quality and dynamics of soil organic matter. It can also provide hot spots of microbial activity. Finally, earthworms can be efficient degraders directly by their own enzymatic equipment, or indirectly because of the microflora present in their intestine. However, ciprofloxacin mineralization remained low. Repeated application of the antibiotic (i.e. by slurry amendment) is exceeding its degradation rates, ensuring the accumulation of the antibiotic on certain soil layers. Our results are consistent with previous studies [5].

Distribution and transfer of labelled carbon among the compartments of the system

The mass-balance analysis of labelled carbon was performed in the two microcosms treated with the highest amount of ciprofloxacin at the beginning of the experiment, and then after 84 and 168 days of incubation (Table 1). Just after soil spiking, labelled carbon in the two microcosms was mainly associated with the solid phase of the soil (77-78 % of initial labelled carbon applied), and only 21% of ciprofloxacin could be extracted.

Table 1. Mass-balance analysis at the beginning of the experiment, and after 84 and 168 daysa of incubation, in the compartments of our soil-plant-water systems filled with soil amended with slurry spiked with the high amounts of antibiotic, or soil spread with the high amount of antibiotic.

Compartment	% of initial labelled carbon applied					
	At the beginning		After 84 days		After 168 days	
	spiked	spread	spiked	spread	spiked	spread
Mineralized	0	0	0.007	0.010	0.073	0.058
Upper soil layer						
-non-extracted	78.021	77.378	79.060	85.312	51.709	47.988
- extracted	21.527	21.488	0.560	0.570	n.d.	n.d.
Lower soil layer						
-non extracted	n.m.	n.m.	8.602	13.556	38.399	43.178
- extracted	n.m.	n.m.	n.d.	n.d.	n.d.	n.d.

[a] if not indicated otherwise; n.d. not detected; n.m. not measured

After 84 days of incubation including the simulated rainfall event, a more detailed analysis showed that the main fraction of radioactive carbon was stabilized in the upper soils. A part of the radioactivity

was measured in the lower layers of soil, showing a significant mobility of the antibiotic after the rainfall. Radioactive material was not detected in the extracts of that layer of soil.
After 168 days of incubation, earthworm activity resulted in a strong mixing of the upper and lower layers in the microcosms. 51.7% and 38.4% of the radioactivity were detected in the soil fractions corresponding to the previous upper and lower layers of the soil amended with the spiked slurry. These values were 48.0 and 43.2% in the case of the soil spread with the antibiotic. Radioactive carbon was never detected in the organic extracts obtained after 168 days of incubation of soils.
When expressed as concentrations of equivalent ciprofloxacin, the values were 0.46 and 0.16 $\mu g.L^{-1}$ corresponding to 1.39 and 0.48 nM in leachates, after rainfall simulation at 70 days of incubation. Ciprofloxacin was also absorbed by wheat seedlings during the 56-84-day growing period. Expressed as % of the applied dose or calculated as concentrations ($\mu g.kg^{-1}$ dry biomass), values of absorbed radioactivity were very low, suggesting a weak transfer to that higher plant.
Finally, total amounts of labelled carbon measured ranged from 88 to 100% of initial amounts applied. They were around 90% after 161 days.

Effects of ciprofloxacin on soil microbial communities
In our integrated microcosms spiked with contaminated pig slurry, hydrolase enzymes merely responded to the time of incubation. In contrast, oxydo-reductases were influenced by antibiotic concentration rather than time of incubation. Ciprofloxacin at 250 $ng.kg^{-1}$ dry soil decreased the activity of soil dehydrogenase, when compared to a green slurry treatment, over 28-day incubations. Laccase activity was similarly decreased in the presence of the highest concentration of antibiotics. We determined bacterial and fungal biomasses using q-PCR. Bacterial biomass was slightly increased in the presence of ciprofloxacin at 250 $ng.kg^{-1}$, but only at day 28. In contrast, whatever its concentration, ciprofloxacin did not modify fungal biomass in contaminated soil.

Conclusion and perspectives
We conclude that the antibiotic introduced in a French Luvisol through spiked pig slurry or direct spreading undergoes a very weak microbial mineralization under aerobic conditions, and may stabilize in the soil as non-extractable residues. Nevertheless, its fate and transformation were affected by the action of earthworms through their engineering activity. Additional experiments are needed to evidence possible effects of antibiotics residues on soil microorganisms, as well as to clarity the real action of earthworms on the breakdown of the antibiotic.

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