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Fipronil Metabolism and Dissipation in a Simplified Aquatic Ecosystem

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Several phenylpyrazole derivatives are selective inhibitors of chloride channel activities in insects. In this chemical family, fipronil is a powerful insecticide now widely used for several purposes. The dissipation of this molecule in a simplified aquatic ecosystem has been studied for 3 months, using ^{14}C-labeled fipronil. The main features of the complex process leading to fipronil transformation in this system were the following. The fipronil aqueous solution was submitted to two chemical transformations: the photodependent desulfuration of the side chain bound to the 4-position of the heterocyclic ring and the chemical hydrolysis of the nitrile function bound to the 3-position. Fipronil, rapidly transferred from the water solution to the organic matter, was protected from the previously mentioned chemical transformations but evolved to give two main metabolites, which were either reduced or oxidized in the side chain on the 4-position. These derivatives were powerful insecticides as shown by LC_{50} measurements on *Aedes aegypti* larvae (LC_{50} for CF_3-S-R and CF_3-SO_2-R = 8.8 nM). During the course of this experiment, nitrile hydrolysis took place slowly, originating either from the chemical hydrolysis in the aqueous solution or from enzymatic hydrolysis inside the microbial biomass. The fipronil-amide (3-NH_2-CO-R′) derivative, although much more polar than fipronil itself, was mostly bound to the organic matter. Other more polar derivatives were also detected but in very small amounts. No ^{14}CO emission was observed during the experiment.

KEYWORDS: Fipronil; aquatic ecosystem; *Aedes aegypti*; metabolism

INTRODUCTION

The fipronil insecticide group was recently invented by May and Baker and developed by Rhône-Poulenc and then by Aventis.

The chemical group of fipronil is characterized by a phenylpyrazole structure with a 4-sulfinyl diversely substituted lateral chain. The main commercial derivative is the 4-trifluoromethylsulfinyl-1*H*-pyrazole named fipronil.

The substance has a new biochemical mode of action as it acts upon the *δ*-aminobutyric acid-gated chloride channel involved in nervous influx transmission (*1*, *2*).

It is therefore active on insects presenting a resistance or tolerance toward the classical insecticide families such as pyrethroids or the organophosphorus, carbamate, or cyclodiene series, which present quite different biochemical modes of action.

On most insects, the LC_{50} of fipronil is noticeably low, reaching, for instance, 24.8 nM on mosquitoes (*3*).

Moreover, if fipronil has a broad action spectrum on insects (cockroaches, locusts, wire-worms, ticks, fleas, etc.), it is practically nontoxic on earthworms (*4*) and several aquatic shellfish species, such as daphnia (*5*).

All of these characteristics recently allowed increased use of this insecticide, both in household uses and in agriculture, mostly as a soil treatment or seed coating, or in a wider scale, for instance, against locusts.

From a practical point of view, it seems that fipronil and the parent compounds of the fipronil family are replacing the now prohibited lindane in most of its uses.

Although the effective amounts are clearly lower in the case of fipronil than for lindane, traces of this compound, its metabolites, or its transformation products are likely to be found in water. Dissipation processes and rates for these molecules have therefore to be carefully and precisely documented.

Hence, the purpose of this work was to study the fate of labeled fipronil in a simplified microecosystem constituted of an organic phase, consisting of fallen tree leaves, and an aqueous aerated phase for a 3-month period under light.

MATERIALS AND METHODS

Chemicals. [u-^{14}C-*phenyl*]Fipronil (4-CF_3-SO-R, 99.3% purity, 741 Bq/nmol), [^{14}C]fipronil-sulfone (4-CF_3-SO_2-R, 99.9% purity, 661 Bq/nmol), [^{14}C]fipronil-sulfure (4-CF_3-S-R, 99.2% purity, 731 Bq/nmol), desthiofipronil (4-CF_3-R), and the fipronil-amide (3-NH_2-CO-R′) were provided by Aventis Agriculture Ltd., Ongar, U.K. (for the formulas, see **Figure 5**).

Toxicity Bioassay. The fourth-instar larvae of *Aedes aegypti* Bora-Bora strain were used for bioassays. The tolerance of these larvae to fipronil, to its derivatives, and to the extracts obtained from the aquatic ecosystem was analyzed by standard bioassay techniques for mosquito larvae (*6*). The bioassays were conducted in triplicate. Forty larvae were

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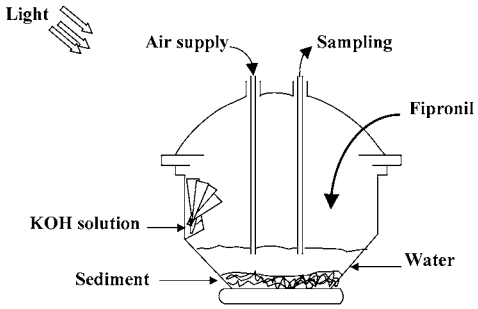

Figure 1. Structure of the simplified ecosystem.

placed into plastic containers containing 20 mL of aqueous solutions. Mortality was recorded after 24 h of exposure.

In all cases, after correction by Abbot's formula (*7*), dose−mortality data were subjected to log-probit analysis according to the method of Raymond (*8*).

Aquatic Ecosystem and Sampling Treatments. The principle leading to the construction of our device was to mix 150 g of a natural organic phase with 470 mL of distilled water (**Figure 1**). This organic phase was made of dead tree leaves, fallen in a lake and allowed to rot for 1 month. These leaves came from *Alnus incana*, *Salix cinerea*, *Tilia sylvatica*, *Carpinus betula*, *Quercus pedunculata*, and *Castanea vulgaris* and had been broken into small pieces by water movement before they were collected. They were further fragmented in a blender and sieved at 500 μm.

During the course of the experiment (3 months) saprophytic aerobic microorganisms and photosynthetic, aquatic organisms, mostly algae, could grow. pH was maintained between 7 and 7.2 during the 3 months. [^{14}C]Fipronil was added at the beginning of the experiment, as a true water solution, with the addition of 1‰ ethanol; the final concentration of fipronil was 0.43 μM. Small amounts of water and organic matter were periodically sampled and analyzed. A constant amount of water was maintained in the ecosystem. Air bubbling and $^{14}CO_2$ trap were maintained for 3 months.

Organic matter and water were separated in the samples by centrifugation. Water was successively extracted by Et_2O and AcOEt. The solid pellet was extracted by Me_2CO and then partitioned with petroleum ether (bp 40−60 °C). The remaining hydrophilic phase was evaporated to discard Me_2CO, giving an aqueous phase that was then partitioned with Et_2O and AcOEt.

Thin-Layer Chromatographic (TLC) and Gas−Liquid Chromatographic (GLC) Analyses. All of the fractions were analyzed by TLC on silica and chromatographed on 2 cm in dichloroethane/acetic acid (19:1, v/v) as a first solvent and then in petroleum ether/dichloroethane/acetic acid (20:19:1, v/v/v) as a second solvent. The quantitative estimation of the different compounds was established by using a ^{14}C label thin-layer chromatoscanner (automatic TLC linear analyzer, Berthold LB 283).

GLC was also used: splitless injection mode (injection with surge, 2.5 min); injection temperature, 230 °C; column, [(50% phenyl)-methylpolysiloxane] BPL 94 ARR, DB-XLB, 30 m, 0.32 mm, 0.5 μm film; initial oven temperature, 50 °C for 1.5 min; injection volume, 100 μL.

For single ion monitoring (SIM) five groups of ions were chosen: fipronil, fipronil-sulfone (4-CF_3-SO_2-R), fipronil-sulfure (4-CF_3-S-R), desthiofipronil (4-CF_3-R), and fipronil-amide (3-NH_2-CO-R′).

RESULTS

Evolution of the Radiolabeled Content of the Simplified Ecosystem. No significant amount of $^{14}CO_2$ was obtained on the KOH trap, and the ^{14}C content of the biphasic system (H_2O + solid organic matter) remained constant for 3 months: 90 ± 6% (**Figure 2**). However, the ^{14}C partition between solid and water changed clearly, and three different steps were noted (**Figure 2**), with a final amount of label reaching 90 ± 6% of the initial amount.

A rapid increase in the partition coefficient solid/water was observed during the first week, partly explained by a diffusion of the product inside the solid particles, until the partition equilibrium was reached.

During a second step, for more than 1 month, the partition coefficient solid/water increased continuously and slowly, reaching a value 3 times higher than the one measured at the beginning of the experiment. This might be due either to the formation of new, highly lipophilic organic matter, contained in living organisms (algae and microorganisms) or to the formation of fipronil derivatives more tightly bound to the organic solid than the parent compound. Known fipronil metabolites such as fipronil-sulfone (4-CF_3-SO_2-R), fipronil-sulfure (4-CF_3-S-R), or desthiofipronil (4-CF_3-R) are more lipophilic than fipronil itself and can actually be in this situation.

During the last step, from 40 to 90 days, the partition coefficient slowly decreased and reached the same value as the one found after 1 week, probably showing the slow formation of more hydrophilic fipronil derivatives or the disappearance of a highly adsorbing component from the solid matrix (**Figure 2**).

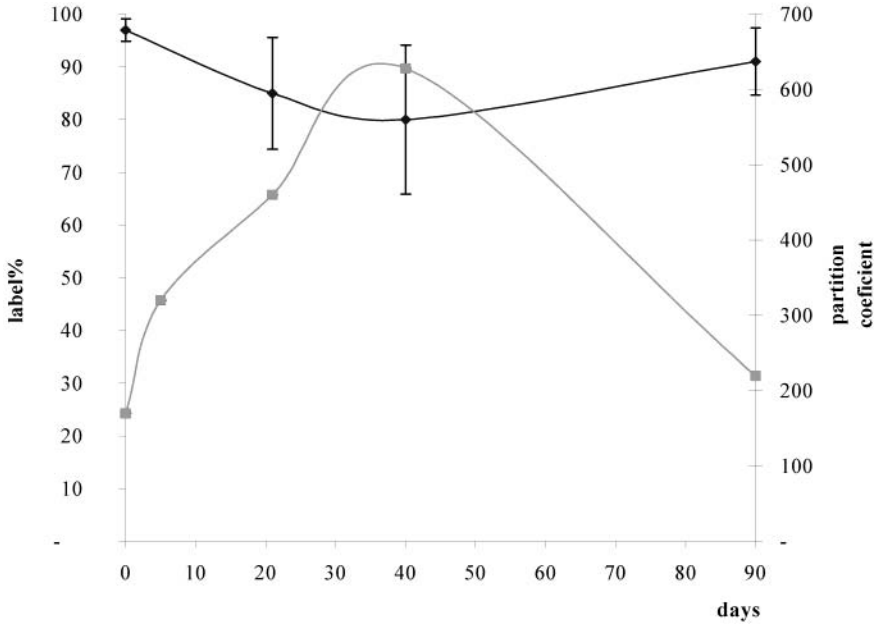

Figure 2. Evolution of ^{14}C and of its partition in the ecosystem: (■) partition coefficient (solid/water); (◆) ^{14}C label. Error bars: mean of two experiments.

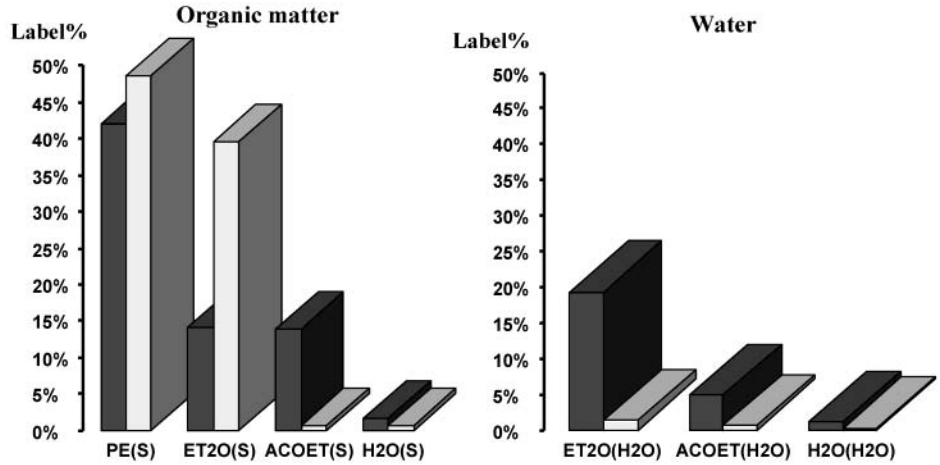

Figure 3. Changes in the label contents of the different extracts separated from the ecosystem: (dark bars) 0 days; (light bars) 90 days. PE, petroleum ether; Et_2O, diethyl ether; AcOEt, ethyl acetate.

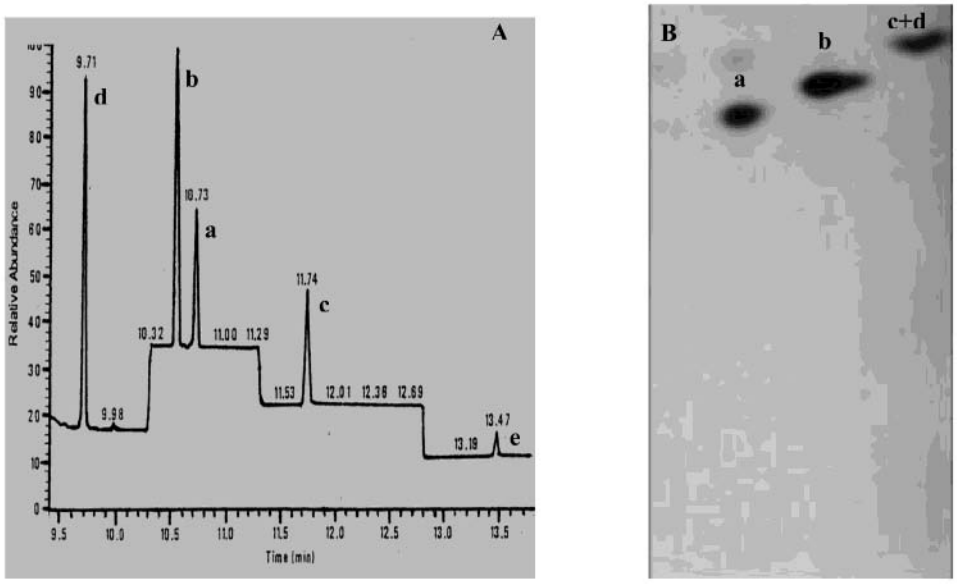

Figure 4. Presence of fipronil metabolites in the ecosystem shown either through TLC and autoradiography (B) or through GLC–mass (A): a, fipronil; b, fipronil-sulfone; c, fipronil-sulfure; d, desthiofipronil; e, fipronil-amide.

Another similar ecosystem was evolving in parallel for 3 months. At the end of this period, it was demonstrated that its general composition was the same: there was no $^{14}CO_2$ emission, the fipronil-sulfure (4-CF_3-S-R) was the main metabolite, and the insecticidal activity in the different fractions was the same as for the studied ecosystem.

Extractibility of the Labeled Compounds from Organic Matter or Water. When the results of the labeled compound partitions between the first and the last week of the experiment were compared, it could be verified that most of the label was associated with the solid phase but that the label content in the petroleum ether or in Et_2O extracts slowly increased with time (**Figure 3**).

A radiolabeled fraction remained with the pellet after standard extraction. It was demonstrated that it was not corresponding to "bound residues", because it was possible to make soluble the label by repeating the extraction with the same solvent.

The rapid transfer of label from water to solid during the first week was observed, as shown in **Figure 2**, and seemed to be simply the result of the partition equilibrium being reached progressively.

Analysis of the Different Metabolites Formed during the Experiment. The different extracts obtained either from water or from the solid organic matter were analyzed through TLC or GLC as shown in **Figure 4**.

As demonstrated by TLC and GLC−mass, the main metabolites that can be formed with fipronil as substrate are obtained through a transformation of the side chains on the 4- and 3-positions of the pyrazole heterocycle (*9*).

Figure 5 shows the structures of fipronil and these derivatives.

As shown by TLC and GLC, the disappearance of the S atom in the 4-side chain under the action of light is a fast phenomenon occurring mostly when the insecticide is dissolved in water, giving the desthiofipronil (4-CF_3-R) derivative. The main true metabolite, fipronil-sulfure (4-CF_3-S-R), formed in the system and accumulated over all of the experiment is the derivative with the CF_3-S- chain on the 4-position.

During the first month, the accumulation of desthiofipronil (4-CF_3-R) and fipronil-sulfure (4-CF_3-S-R), which are more lipophilic than fipronil, can explain the increase in the partition coefficient as seen in **Figure 2**. The decrease in this coefficient during the last month is at least partly explained by the transformation of the 3-position side chain, giving the fipronil-amide (NH_2-CO-R′), which is more polar than the parent compound. The relative lipophilicity of fipronil derivatives studied here was established through R_f measurements on TLC or high-

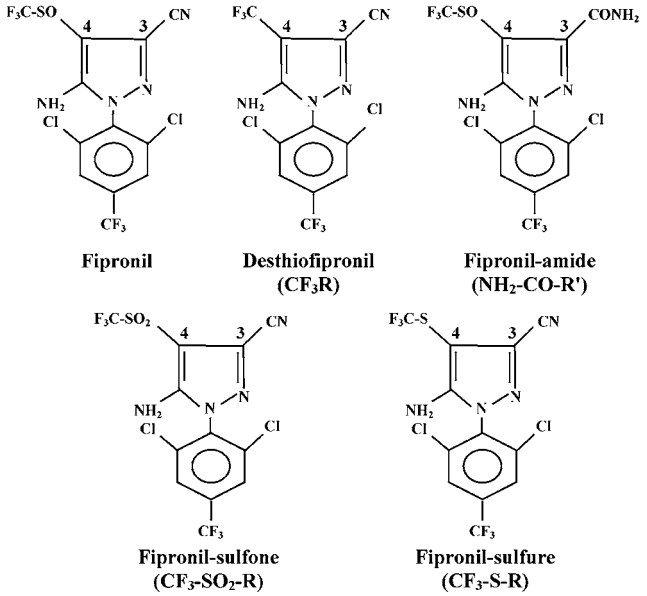

Figure 5. Structures of the main metabolites of fipronil, known to be formed either in living beings or in soil.

performance liquid chromatography (HPLC) on silica gel or C_{18} silica gel with the appropriate solvents (result not shown).

Moreover, the TLC analysis shows the presence of polar derivatives present in the solid and extracted by Et_2O or AcOEt. However, the amounts of these compounds remain low (**Figure 3**).

Evolution of the Insecticidal Potential Contained in the Ecosystem during the Experiment. The different extracts obtained from the solid matrix or from water (petroleum ether, Et_2O, AcOEt, or H_2O extracts) were tested, at the appropriate dilution in water with 1‰ EtOH, for their insecticidal potential against the aquatic larvae of *A. aegypti* under controlled conditions (*6*).

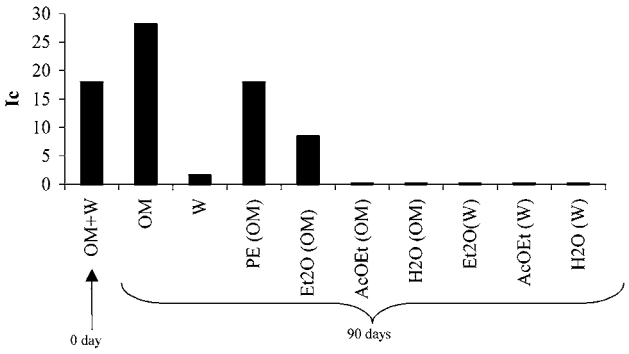

Figure 6. Insecticidal potential of the studied fractions the first day and after 90 days. OM, solid phase; W, water; PE, petroleum ether fraction; AcOEt, ethyl acetate fraction; Et_2O, diethyl ether fraction; Ic, arbitrary units of insecticidal activity. Within the brace is shown the nature of the extracted phase, either organic matter (OM) or water (W).

In this test, log probit analysis gave an LC_{50} of 24.8 nM after 24 h and and LC_{50} of 15.1 nM after 48 h for fipronil (*3*). These values were 5 times higher than that found by Ali et al. (*10*) in the presence of food.

The amount of fipronil added to the medium, at the beginning of the experiment carried out here, gave a concentration (454 nM) that was 18 times higher than the LC_{50}. It was therefore postulated that the studied ecosystem contained 18 insecticidal arbitrary units. The insecticidal potential was then measured periodically in the different samples. **Figure 6** shows the corresponding results after 90 days.

Surprisingly, after 3 months, the insecticidal potential had changed from 17.3 units to 28.7 + 1.6 units, showing therefore a 175% increase. Most of this potential was associated with the solid matrix, and 84% could be extracted by petroleum ether or Et_2O: the insecticidal components were therefore lipophilic.

The insecticidal activity of the known pure metabolites was then measured, in comparison with fipronil. **Figure 7** shows

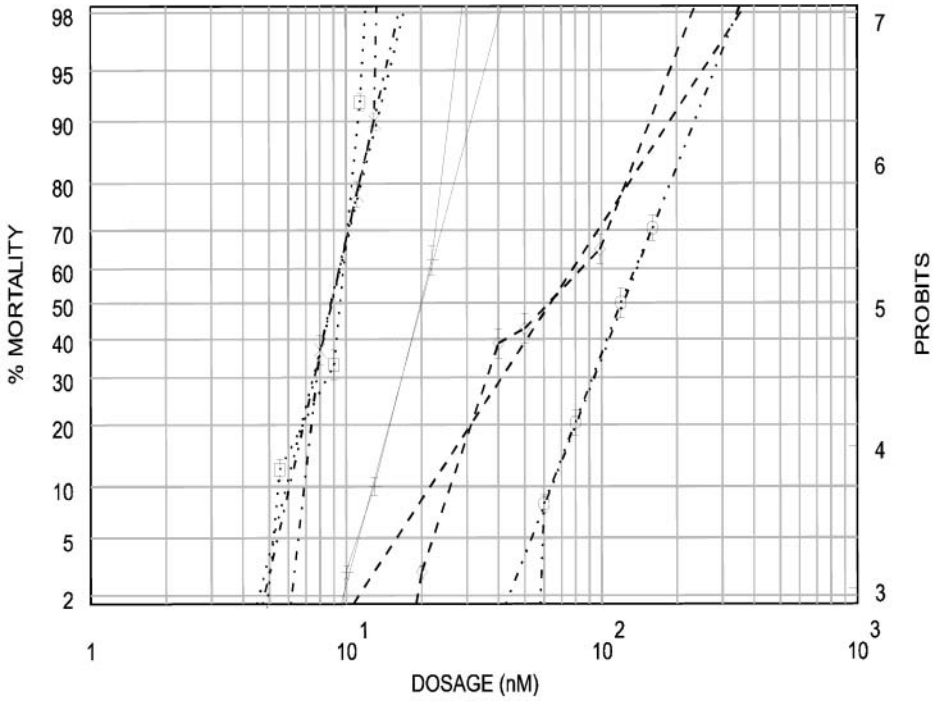

Figure 7. Insecticidal effects of pure fipronil metabolites on *A. aegypti* larvae: (—+) fipronil; (– – ◇) desthiofipronil; (•••□) fipronil-sulfure; (• – •×) fipronil-sulfone; (••–••○) fipronil-amide.

the LC_{50} after 24 h of the different pure compounds involved in this study, measured through a probit analysis on *A. aegypti* larvae under standard conditions.

For the fipronil-sulfone (4-CF_3-SO_2-R) and the fipronil-sulfure (4-CF_3-S-R) the LC_{50} reached 8.8 nM against 19.9 nM for fipronil. The two other derivatives had higher LC_{50} values: 62.7 nM for the desthiofipronil (4-CF_3-R) and 121.6 nM for the fipronil-amide (3-NH_2-CO-R′).

It was therefore suggested that the increase in the insecticidal potential of the studied ecosystem depended on the presence of the fipronil-sulfure (4-CF_3-S-R) derivative, maintained at a high level after 1 month of culture, and, to a lesser extent, on the presence of the fipronil-sulfone (4-CF_3-SO_2-R).

The light-dependent formation of the desthiofipronil (4-CF_3-R) compound and the biological formation of the fipronil-amide metabolite (3-NH_2-CO-R′) lowered the insecticidal activity.

DISCUSSION

Although the system submitted to our study was highly simplified, as compared to what occurs, for instance, inside a lake, it reaches a high level of complexity, especially concerning its composition and its evolution.

The major process taking place in this system in the first step was partitioning. Fipronil is poorly soluble in water (1.8−2 mg/L) and can easily bind to lipophilic organic matrices such as membrane lipids or oils, suberin, lignin, and lipophilic proteins. Moreover, the sulfurated 4-position chain may be responsible for other binding mechanisms to organic matter as is the well-known case for proteins.

The partitioning rate in our system is fast in the first step, probably involving adsorption on the free surface of the fragments, and much lower in the second step of 1 week's duration, suggesting an equilibrium of fipronil concentration inside the whole volume of the small leaf fragments.

Concurrently, the light-dependent desulfuration of fipronil took place in water, fairly rapidly, if we take into account the fact that the system was exposed to a moderately intense light (5000 lx during the day), without any wavelengths shorter than 3000 Å. The derivative obtained was more lipophilic than fipronil and could therefore easily bind to organic matter.

After full partition with the organic matter, it seemed that the rate of the photoreaction became negligible. The biochemical processes affecting fipronil during the experiment affected the 4- and 3-position lateral chains.

Transformation of the 4-position chain occurred through two opposite pathways, either oxidation or reduction.

A sulfoxidation process was previously described in plants for pesticide transformation in several cases (*11*−*13*), possibly leading to GST interactions (*14*).

Reduction, although infrequent in the plant kingdom, has been established, for instance, in the case of metribuzin deamination (*15*).

It seems to play a major role in our system and probably takes place in a reducing compartment of the living biomass. The identification of such a compartment needs further studies. However, it seems unlikely it could be located in photosynthetic organisms or free aerobic microorganisms. We can suggest that it might occur in partly anaerobic microorganisms developing in the depth of organic particles.

Nitrile hydrolysis can be purely chemical at alkaline pH (*16*). However, the action of a microbial nitrilase might be suspected. Such a hydrolysis of a CN group is well documented in the case of several pesticides (*17*, *18*).

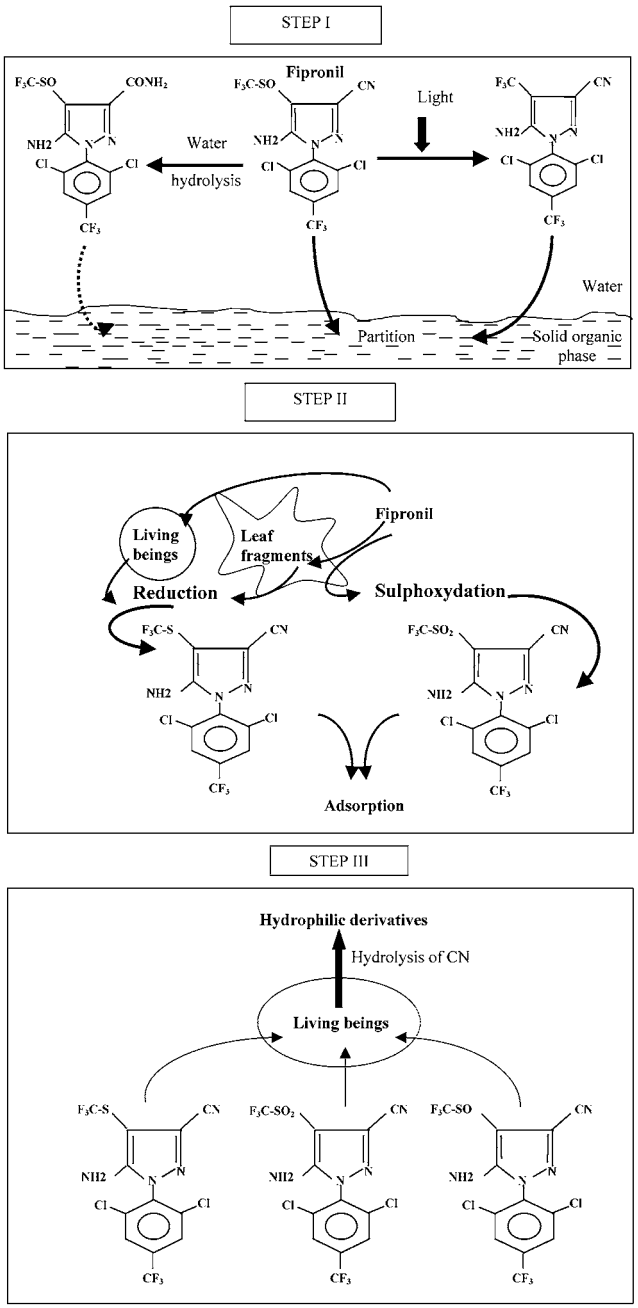

Figure 8. Three main steps of fipronil bioavailability changes in a system of leaf organic matter/water.

The fipronil-amide derivative (3-NH_2-CO-R′) is likely to lead to 3-COOH-R′, which might be one of the very hydrophilic derivatives shown on TLC.

From an environmental point of view, it is interesting to notice that the binding of fipronil and of its main lipophilic metabolites to organic matter prevents a possible increase of the insecticide concentration in water and allows the insensitive microbial biomass to further transform these products.

Figure 8 tentatively summarizes the events occurring in our simplified ecosystem at three stages in the evolution process.

ABBREVIATIONS USED

CF_3-SO_2-R, fipronil-sulfone; CF_3-S-R, fipronil-sulfure; CF_3-R, desthiofipronil; NH_2-CO-R′, fipronil-amide.

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