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BEES AND SYSTEMIC INSECTICIDES (IMIDACLOPRID, FIPRONIL) IN POLLEN: SUBNANO-QUANTIFICATION BY HPLC/MS/MS AND GC/MS.

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ABSTRACT

Imidacloprid and fipronil are two insecticides acting on the central nervous system. They are used worldwide, from the mid nineties, especially for seed coating of crops. Concomitantly to their introduction and their increasing use in French fields, honeybee populations decreased. Bee problems are nowadays reported in other countries (also called *disappearing disease*, *despoblación de las colmenas*, *trouble des abeilles*, *deperimento degli apiari* or *colony collapse disorder*). Bee problems have certainly several origins. Contamination of pollen and nectar by such chemicals appeared as one of the most probable cause, since sublethal effects and chronic intoxications were observed on bees, at very low concentrations. We developed fully validated methods to measure contamination in pollen (sunflower and maize) for imidacloprid (HPLC/MS/MS) and for fipronil and 3 of its derivatives (GC/MS). For imidacloprid LOD and LOQ are 0.3 and 1 ng/g, respectively. For fipronil and each derivative (fiprole), LOD and LOQ are 0.07 and 0.2 ng/g, respectively. The averaged level of imidacloprid in pollen was 2-3 ng/g, which is 20-30 times higher than the concentration inducing significant mortality by chronic intoxication. Fiproles were detected in 48% of pollens issuing from treated crops. Fipronil and its sulfone derivative represented 77% and 17% of contaminants, respectively. The averaged fiprole sum Σf was 0.3 - 0.4 ng/g, which is 30-40 times higher than the concentration inducing significant mortality of bees by chronic intoxication.

KEY WORDS: imidacloprid, fipronil, toxicity, pollen, bee, colony collapse disorder.

INTRODUCTION

The systemic insecticides imidacloprid and fipronil are commercialised by Bayer AG (Gaucho®, Confidor®...) and BASF (Regent®, Schuss®...), respectively. Rapidly they were suspected of harmful effects on honeybees, particularly at sublethal doses or by chronic intoxication. Used worldwide for treatment of numerous crops, these powerful neurotoxins are insecticides acting on the central nervous system (CNS). Imidacloprid is a chlorinated neonicotinoid that binds acetylcholine receptors. Fipronil belongs to the phenylpyrazole class and binds GABA receptors. Both affect the CNS by interfering at the post synaptic level.

Far under lethal doses (LD50 is 4-6 ng/bee for both insecticides; CST 2003, CST 2004, Tingle 2003, Cox 2005, Agritox 2007), vital functions of bees are affected by very low concentrations inducing sublethal effects or chronic intoxications. These concentrations are in the range from 0.1 to 10 ng of imidacloprid per g of food supply (Suchail 2001, Colin 2004) and the situation appeared somewhat similar for fipronil. Here, recent studies (Decourtye 2005 and El-Hassani 2005) showed that the chronic exposure to fipronil during 11 days, with amounts extending from 0.075 to 0.3 ng/bee, has still lethal effects. Furthermore, a significant mortality was also observed, after 11 days, for 0.01 ng/g of fipronil in the food of bees (Belzunces 2003).

When applied as seed coating for crop protection (generally from 50 to 100 g/ha), the insecticide is first solubilised in the water of the soil, around seeds. The chemical is distributed between the two major components of the soil, namely: the soil water and the clay-humus complex. Thus, a quantity of chemical remains in a soluble state and is suitable to be absorbed by roots cells. In fact, imidacloprid or fipronil are absorbed by a simple process of passive diffusion. Then, it is transferred by the xylem pathway into the sap flow, this also depending on its relative solubility (imidacloprid: $pK_{ow} = 2.8$; fipronil: $pK_{ow} = 0.57$). Such systemic properties have been depicted by Bromilow in 1990. During the cycle of growth, high concentrations of the insecticide are observed into the first leaves, whereas higher parts display lower levels. At the time of flowering of the plant, a minor quantity of the product can be also transferred from the older leaves towards the inflorescence, by the flow of the elaborated sap (phloemic pathway). That are the reasons why, nectar and pollen can be significantly contaminated by the chemical.

To evaluate properly the risk for bees foraging on treated crops (sunflower, maize), one of the first steps is to measure contamination of pollen in fields. Moreover, it is also necessary to take into account the first metabolites which sometimes are as much (or more) toxic than the parent compound. We have developed a HPLC/MS-MS methodology to measure and characterize the behaviour of imidacloprid in soils, plants and pollens. We also developed a GC/MS method to detect and to quantify fipronil and its 3 main metabolites in pollen. Pollens were sampled directly on flowers and also at the beehive entrance (trapped pollen effectively harvested by bees). Our analytical methods are particularly sensitive. They satisfied quality standards, European Directives (Directives 96/23/EC and 2002/657/EC) and specific criteria required by a French expert committee (*Scientific and Technical Committee of the multifactorial study of bee disorders*). Measures were performed according to the whole set of quality criteria and following GLPs.

MATERIALS AND METHODS

Samples

All samples were collected from 1998 to 2005 in the whole French territory and especially in intensive agricultural areas. Obviously, samples from treated crops have been collected before suspension of insecticides used in France (Gaucho®: 1999 for sunflower, 2004 for maize; Regent TS®: 2004 for sunflower and maize). Pollens from flowers (sunflower, maize, cistus, buckwheat...) were carefully sampled in the middle of the fields to circumvent from edge effects. Samples of trapped pollens were mostly collected from beehives located in -or very close to- the fields of interest. Sampling was also performed in crops growing under insect-proof tunnels in which beehives were setup. All pollens were sampled by a specialized company (TESTAPI, France) according to strict protocol adapted to this case study. Samples were bagged (double bag), kept safe from light and frozen at temperature $< -20^{\circ}\text{C}$.

Extraction and purification

Concerning imidacloprid, the preparative procedures are fully described in our previous publications (Bonmatin 2003, Bonmatin 2004 and Bonmatin 2005a). Briefly, pollen (10 g in ethanol/water) was mixed and extract was centrifuged and evaporated. A pH 7 buffer and dichloromethane were added and the organic phase was extracted and evaporated. The oily residue was diluted with hexane, ultra-sonicated and centrifuged

after adding acetonitrile/water. The upper phase was centrifuged and an aliquot of 25 μL was injected in HPLC.

Concerning fipronil, trapped pollens were dried whereas pollens directly sampled on flowers did not need this preliminary step. Then, pollen (10 g) was extracted twice with ACN. Extracted phases were evaporated and put in dichloromethane. This solution was purified, first on C_{18} , then on florisil. The recovered solution was evaporated and dissolved in ethyl acetate. 2 μL of the latter solution was injected in GC.

Analytical methods

For imidacloprid, the LC system was a Perkin Elmer (Framingham, USA). It was fitted with a C_{18} Supelcosil ABZ + (150 mm $\times$ 4.6 mm) from Supelco Park, PA, USA. The MS system was constituted of a standard atmospheric-pressure-ionisation source configured as APCI. The signal corresponding to imidacloprid (m/z : 256 $\rightarrow$ 209 and 209 $\rightarrow$ 175) in pollen is illustrated in Figure 1.

Figure 1: Structure of imidacloprid and chromatogram of a typical pollen containing imidacloprid at 2 ng/g ($m/z = 209$). The limit of detection (LOD) was 0.3 ng/g, whereas the limit of quantification (LOQ) was 1 ng/g.

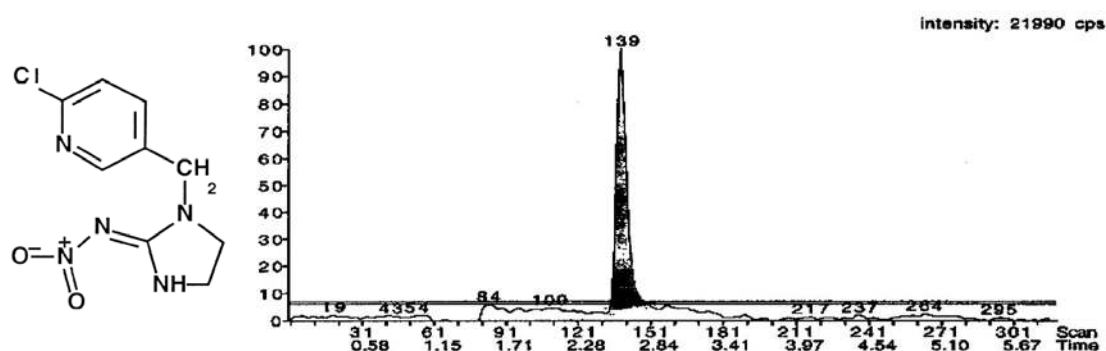
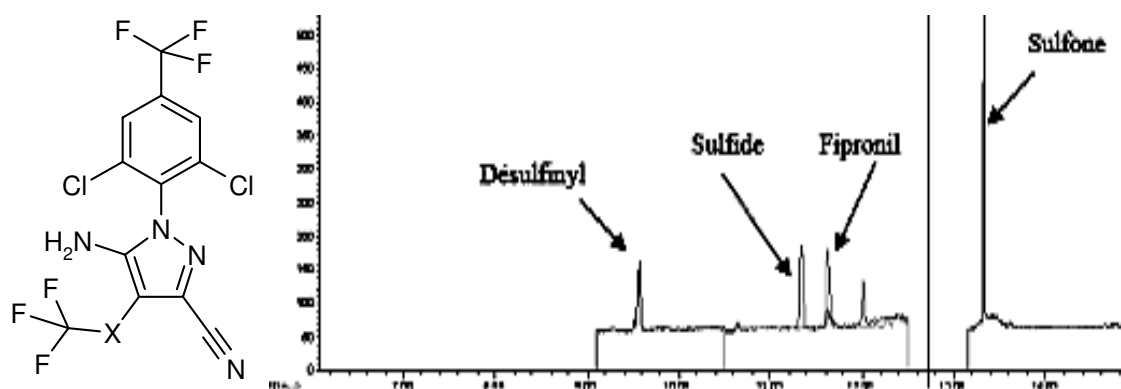


Figure 2: Structure of fipronil ($X = \text{SO}$) and its 3 derivatives (sulfide: $X = \text{S}$; sulfone: $X = \text{SO}_2$; desulfinyl: $X = \text{none}$) and total ion chromatogram (TIC) of a pollen in which levels are at 0.2 ng/g. The LOD was 0.07 ng/g, whereas the LOQ was 0.2 ng/g.



For fipronil, the GC system was an Agilent 6890N with a DB-XLB column (30 m; 0.25 mm; 0.25 μm). The injector was an Agilent 7683 and the detector was a mass spectrometer quadrupole (Agilent 5973). For the source, the energy (Electronic Impact)

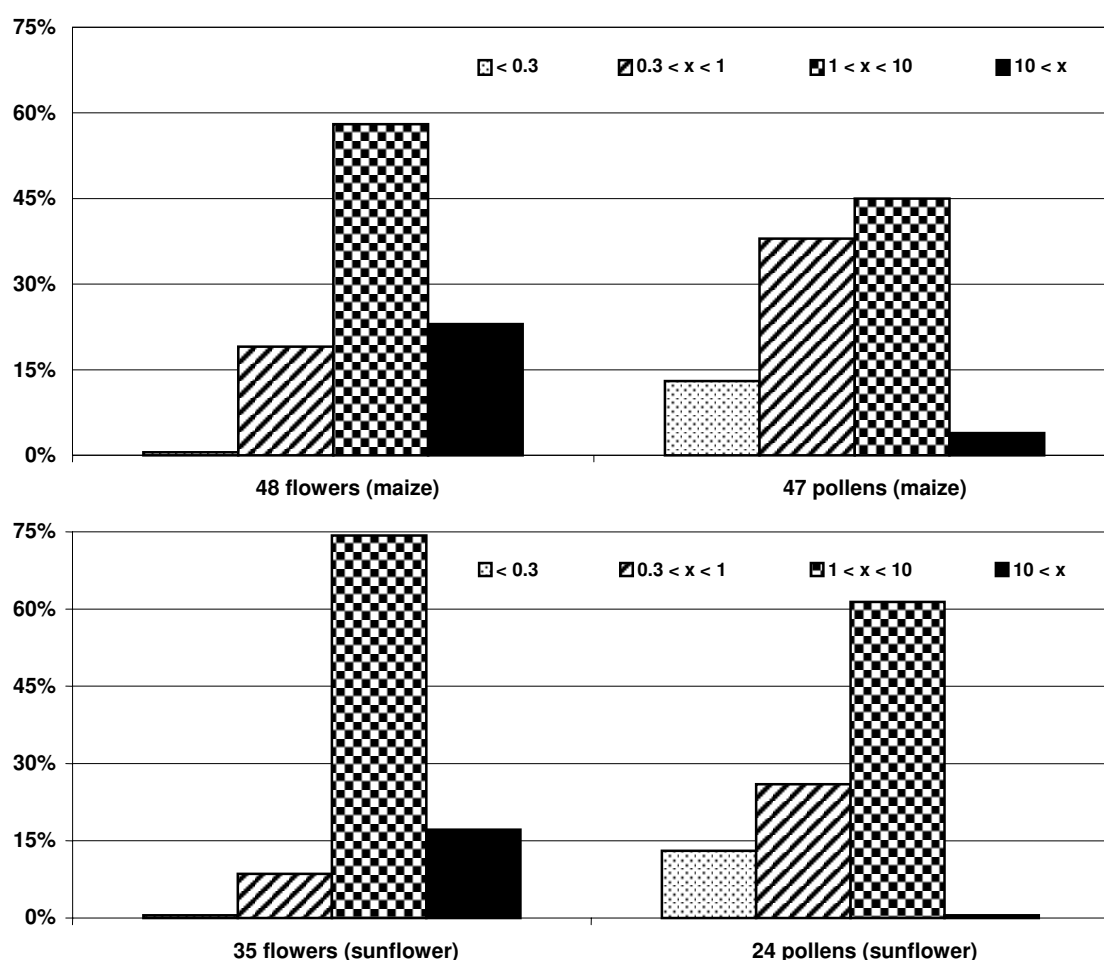
was 70 eV; temperature: 230°C. The 3 selected ions used for detection and calibration were at m/z 390-388-333 for fipronil desulfinyl, 420-353-351 for fipronil sulfide, 369-367-351 for fipronil and 452-385-383 for fipronil sulfone. An example of signal for fipronil and its metabolites (each at 0.2 ng/g) in pollen is shown in Figure 2.

RESULTS

Imidacloprid

Flowers and pollens from organically farming crops were used as references. They did not display any imidacloprid signal and were the basis for calibration and comparisons. Note that untreated crops, issuing from a field which had received imidacloprid treated crops the year before, were not free from the chemical and represented improper references.

Figure 3: Distribution of the samples (flowers and pollens) as function of imidacloprid concentration ranges(x in ng/g). Top: sunflower; bottom: maize.



A set of 24 pollens from treated sunflower were analyzed (Figure 3). In only 17% of pollens, imidacloprid was not detected (LOD = 0.3 ng/g). 25% of samples were positive with the amount of imidacloprid not exceeding 1 ng/g. Other pollens (58%) contained imidacloprid with quantified concentrations, between 1 and 11 ng/g. The mean value on this data set is 3 ng/g.

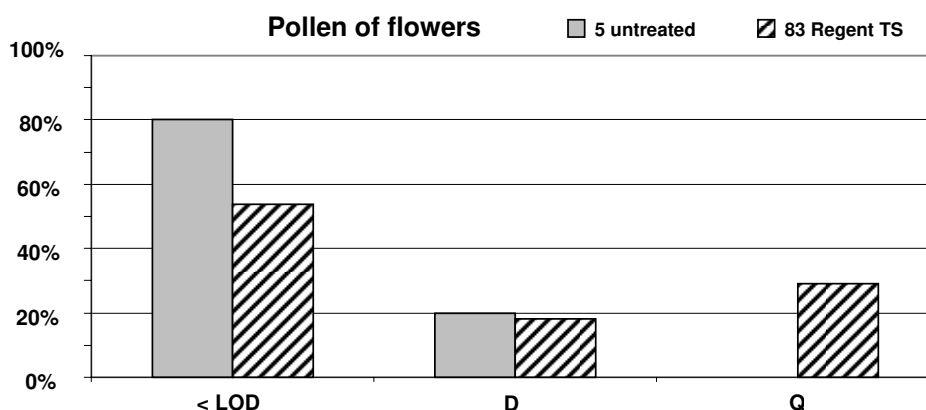
A similar situation was depicted from 47 maize pollens (Figure 3). Here, the mean value on the data set was 2 ng/g. It is noteworthy that imidacloprid was not accompanied by its main derivatives, whatever the crop (sunflower or maize). This suggests that flowers, thus pollens, display such levels of imidacloprid from the xylemic and/or the phloemic pathway without a fully efficient metabolism within the plant.

Fipronil

We report here results for 195 pollens. For each pollen sample, analysis gave 4 separate results corresponding to fipronil and its 3 derivatives (4 fiproles). In order to describe the fiprole sum (Σf) for each sample, these 4 data were added according to the following principles. When a level was found under the limit of detection (LOD = 0.7 ng/g), a value of zero was assumed. When a level was positive (between LOD and LOQ = 0.2 ng/g), a mean value of 0.14 ng/g was adopted. When a level was higher than the LOQ, the quantified result was kept. For instance, the fiprole sum is $\Sigma f = 0.64$ ng/g in the case of a sample having fipronil at 0.5 ng/g, a positive sulfone signal, and the two other derivatives not detected.

Pollens from flowers: On 5 samples of pollens from untreated flowers (sunflower, corn and buckwheat), only one is positive (Figure 3) leading to an averaged $\Sigma f = 0.03$ ng/g. On the 83 samples of pollen of flower from seeds treated with Regent TS, 47 % were positive. Among them, 28 % appeared contaminated higher than LOQ (Figure 4). Here the maximum value was 8.3 ng/g. For this data set, the averaged Σf was 0.42 ng/g.

Figure 4: Distribution of pollens sampled on flowers, according to the level of fiproles. LOD = 0.07 ng/g and LOQ = 0.2 ng/g. D: at least one fiprole was detected; Q: at least one fiprole was quantified.

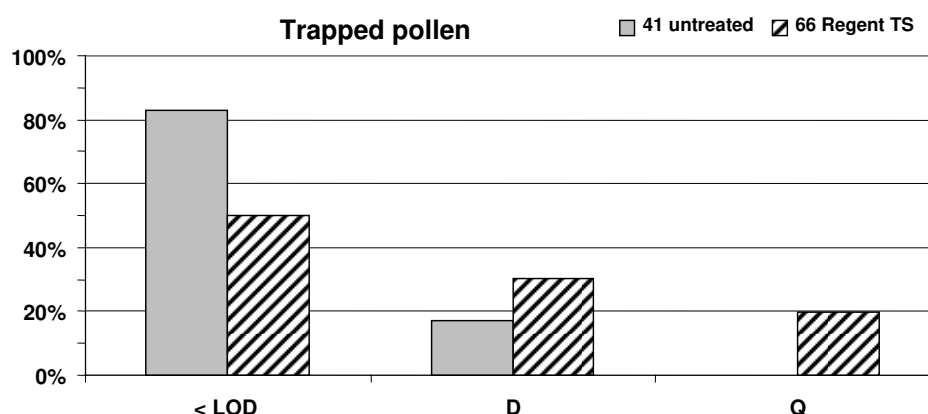


Trapped pollens: We sampled 41 trapped pollens from untreated crops (sunflower, corn and buckwheat). On this data set, 17% of the samples are positive (Figure 5). The averaged Σf was 0.06 ng/g. When seeds were treated, 50% of the 66 pollen data set was positive. Among them, 20% were higher than LOQ. The maximum value was 4.3 ng/g. For this data set, the averaged Σf was 0.29 ng/g.

Figure 4 and 5 show that about 20% of both types of pollen from untreated fields are positive for at least one fiprole. This probably originates from diffuse pollution. This result is in agreement with recent data from Kadar et al. (2006) who have also analyzed untreated pollens collected in 2005. Such contamination of untreated pollen could have

a first effect on pollinators at all. Further, when comparing pollens from untreated crops to that from treated crops, it is clear that a novel class of contaminated pollens appears in treated crops. Here, levels are higher than $LOQ = 0.2 \text{ ng/g}$. Such pollens represent 20 to 30% of each data set. Obviously, pollinators should be as much affected by the latter pollens. In other terms, we demonstrated i) a slight contamination of 20% of pollens by diffuse pollution in untreated fields and ii) the direct (and higher) additional contamination of 20 – 30% of pollens because of the systemic properties of fipronil, consecutively to seed dressing. As a matter of fact, fiproles were detected in 48% of all pollens from treated seeds. Here, our results confirm and precise data from Chauzat et al. (2006) who have shown that 3 pollen samples contained fipronil.

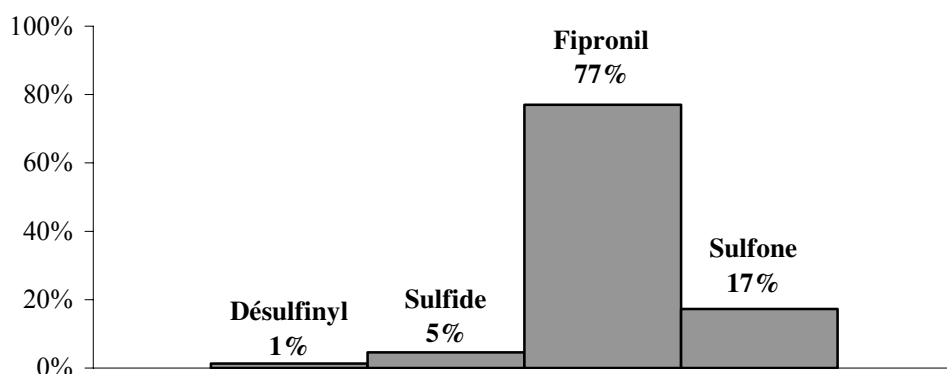
Figure 5: Distribution of pollens collected by bees, according to the level of fiproles. $LOD = 0.07 \text{ ng/g}$ and $LOQ = 0.2 \text{ ng/g}$. D: at least one fiprole was detected; Q at least one fiprole was quantified.



It is noteworthy that Figure 4 and 5 demonstrate a similar situation whatever the type of sample (pollens directly sampled on flowers or trapped pollens which are collected by bees). We also observed that results are very similar when comparing the 2 major crops of interest: sunflower and maize. This has two consequences. The first is that the systemic impact of fipronil is now well established up to pollen. The second is that we can cumulate data issuing from these 2 crops, in order to describe more accurately the contamination of pollens, especially in terms of metabolism.

Metabolism in pollen: Examination of the metabolite content was done in contaminated pollens from treated crops (48% of pollens). This examination was performed in term of occurrence and in term of quantity. In term of occurrence, fipronil was detected in 95% of these pollen samples. Occurrence is 53% for sulfone fipronil, 24% for sulfide fipronil and 13% for desulfinyl fipronil. This shows that fipronil and its sulfone derivative are the 2 compounds that appeared the more often in pollens. Other derivatives were detected more rarely and were generally associated to the previous ones. In term of quantity, the mean level of fiproles is $\Sigma f = 0.76 \text{ ng/g}$ in these pollens. The quantitative repartition is 0.59 ng/g for fipronil (77%) and 0.13 ng/g for its sulfone derivative (17%), as shown in Figure 6. This indicated that fipronil largely dominates (77%) whereas its oxidized derivative occurs for 17%, its reduced derivative for 5% and its photo-degradation product for only 1%.

Figure 6 : Mean quantitative distribution of fiproles in contaminated pollens from treated crops. 100% corresponds to the mean fiprole level: $\Sigma f = 0.76$ ng/g.



CONCLUSION

Imidacloprid was measured with a sensitive HPLC/MS-MS methodology with LOD = 0.3 ng/g and LOQ = 1 ng/g (Bonmatin 2003). For a comprehensive approach, this technique allowed to follow imidacloprid from seed to soil, in the plant and up to pollen (Bonmatin 2005a, Bonmatin 2005b). As a matter of fact, pollen contained principally imidacloprid with an averaged level at 2 ng/g (maize) and 3 ng/g (sunflower). When compared to the level inducing significant bee mortality on 11 days (by repeated doses at 0.1 ng/g), the risk appeared worrying (CST 2003, Rortais 2005).

For fipronil, we developed and validated a new method by GC/MS in order to detect and to quantify this compound and 3 of its derivatives in pollen. Two types of pollen were extensively studied: pollen directly sampled on crops (pollens from flower) and pollen collected by foraging bees and sampled at the entrance of beehives (trapped pollens). From treated crops, half of pollens (48%) were found positive (i.e. > LOD = 0.07 ng/g). The averaged fiprole levels were $\Sigma f = 0.4$ ng/g (pollens from flowers) and $\Sigma f = 0.3$ ng/g (pollens from traps). Considering only contaminated pollens from treated crops, the averaged fiprole level was $\Sigma f = 0.76$ ng/g. Here, fipronil was the major component (77%) followed by its sulfone derivative (17%).

Globally, pollens appeared less contaminated (10 fold lower) by fipronil than by imidacloprid. This is in agreement with their respective pKow (0.57 and 2.8, respectively). The case of fipronil differs from that of imidacloprid because half pollens were not contaminated (this ratio is 17% for imidacloprid). However fipronil and its derivatives are about 10 times more toxic for bees, especially in terms of chronic intoxication leading to mortality (imidacloprid: 0.1 ng/g; fipronil: 0.01 ng/g). Both insecticides appeared bio-available in fields. For each insecticide, there is no significant difference, in term of spatial or temporal distribution, with respect to sampling on treated crops. For both insecticides, mean concentration in pollen was, at least, an order of magnitude higher than the lowest level which still induces mortality on bees. According to the PEC/PNEC method of risk assessment in fields (CST 2003 and CST 2004, Halm 2006), the risk appears as much worrying for both systemic insecticides.

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