Bulletin of Insectology **64** *(1): 107-113, 2011*
ISSN 1721-8861

Toxicity of neonicotinoid insecticides to honey bees: laboratory tests

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Abstract

Toxic effects on *Apis mellifera* L. of the neonicotinoid insecticides Thiametoxam, Clothianidin, Acetamiprid and Thiacloprid were tested in the laboratory. Commercial formulations, dispersed in sugar syrup and water, at the highest dose level marked on the label were used to carry out oral and indirect contact trials on each pesticide. Clothianidin and Thiametoxam caused higher mortality than the untreated controls and were also tested at decreasing concentrations until mortality was statistically insignificant in comparison with that of the control; the acute oral Lethal $Dose_{50}$, the acute indirect contact Lethal $Concentration_{50}$, and the related Hazard Quotient were calculated at 24, 48, and 72 hours from test initiation. On the contrary, Acetamiprid and Thiacloprid caused higher mortality than the untreated controls only in oral toxicity tests when honey bees, which had starved for two hours, were used. Honey bees that died during the trials were analyzed and the quantity of residues of insecticides determined. These quantities resulted much lower than the administered ones.

Key words: *Apis mellifera*, neonicotinoids, Acetamiprid, Thiacloprid, Thiametoxam, Clothianidin, mortality, toxicity.

Introduction

Neonicotinoids, a class of neurotoxic insecticides designed in the '80s, are highly systemic with long-term persistence. They permanently bind to nicotinic receptors of acetylcholine, blocking them and consequently the passage of nerve impulses (Tomizawa and Casida, 2005). This mode of action allows control of the insects that attack the roots and the neck as well as feeding on the aerial part of the plant. Acting on contact, the neonicotinoids are particularly suited for controlling many insects with biting and sucking mouth parts especially if swallowed. They are also used in seed dressing for protection from soil insects; they are absorbed by the radical apparatus and are then distributed evenly, maintaining an effective concentration of active substance in young plants. Their intended use is very broad: pome fruits, stone fruits, citrus, grape, horticultural and industrial crops, flower and ornamental plants. Aphids, whiteflies, planthoppers, scale insects, Lepidoptera, soil insects, Colorado potato beetle are included among the target pests (Muccinelli, 2008).

The neonicotinoids have higher selectivity factors for insects versus mammals than most insecticides apart from pyrethroids (Tomizawa and Casida, 2005).

Several neonicotinoids, however, show very strong toxicity to pollinating insects and in particular to the honey bee (*Apis mellifera* L.), causing also other effects which are seldom easily identifiable, such as behavioural disturbances, orientation difficulties and impairment of social activities (e.g. Guez *et al.*, 2001; Bortolotti *et al.*, 2003; Medrzycki *et al.*, 2003; Decourtye *et al.*, 2004a; 2004b; Desneux *et al.*, 2007; El Hassani *et al.*, 2008; Maini *et al.*, 2010).

Although potential problems could be reduced by treating seeds and not spraying flowering crops (Tomlin, 2003), alarming bee mortalities, clearly due to the use of neonicotinoids either for seed dressing or crop spraying, were recorded in many countries during the past few years, and various limitation in their use were enforced (Greatti *et al.*, 2003; 2006; Colin *et al.*, 2004; Janke *et al.*, 2009; Pistorius *et al.*, 2009; Forster *et al.*, 2009; Maini *et al.*, 2010; Marzaro *et al*., 2011).

Before registration, formulated pesticides currently undergo various tests to assess the risk posed by these molecules to honey bees. In the European Union, the European and Mediterranean Plant Protection Organization guidelines No. 170 (OEPP/EPPO, 2001) and the relative risk assessment procedure (OEPP/EPPO, 2003) are usually followed, but their efficacy for systemic insecticide has recently been challenged with special reference to neonicotinoids (Halm *et al.*, 2006). Although semi-field and field tests and/or in deep evaluation of chronic, sub-lethal, and behavioural effects are generally called upon for a thorough understanding of neonicotinoid side effects on honey bees (cfr. Maini *et al.*, 2010 and the literature cited therein), it seems likely that some new information might also arise from acute toxicity tests, if they were carried out following different procedures from the OEPP/EPPO guidelines. Thus, methods which had formerly been designed to test the action of insecticides towards honey bees by Arzone and Vidano (1980) were used. These methods differ from OEPP/EPPO (2003) provisions, above all, since honey bee behaviour and mortality are repeatedly checked during ingestion tests and because indirect contact tests are preferred to topic contact tests as they better simulate the situation in the field.

The currently marketed neonicotinoids can be divided into two subclasses, that is chloronicotinyles and thianicotinyles, based on the chemical group characterizing them which is a chlorpyridinyl in the former and a chlorthiazolyl in the latter. The active ingredients (a.i.) currently on sale in Italy are: Acetamiprid, Clothianidin, Imidacloprid, Thiacloprid, and Thiametoxam (Muccinelli, 2008).

The laboratory experiment was conducted to assess the danger of Acetamiprid, Clothianidin Thiacloprid,

and Thiametoxam for the honey bee. Imidacloprid was not investigated because the scientific literature already has a large store of information (e.g. Bortolotti *et al.*, 2003; Doucet-Personeni *et al.*, 2003, Marletto *et al.*, 2003; Maus *et al.*, 2003; Ramirez-Romero *et al.*, 2005; Maini *et al.*, 2010).

Materials and methods

Commercial formulations available in Italy were used (table 1). Each a.i. was tested both by ingestion and indirect contact at the highest concentration recommended on the label for crop treatment (field concentration). If 100% mortality was observed, a ten fold lower concentration was tested and the process was repeated until the concentration that had caused mortality not significantly different from that of the untreated controls was reached. Intermediate concentrations in the range between 100% and untreated control mortality were also tested in order to better highlight honey bee response to chemicals.

The honey bees were considered "dead" when they remained absolutely still during a 10 second observation period, a rather conservative criterion if compared with that adopted by other authors (i.e. Iwasa *et al.*, 2004).

Ingestion tests

Acute oral toxicity tests were conducted using materials and procedures elsewhere detailed (Laurino *et al.*, 2010).

Tested a.i. concentrations were: Acetamiprid: 100 ppm; Clothianidin: 75 ppm, 7.5 ppm, 3.75 ppm, 1.5 ppm, 0.75 ppm, 0.375 ppm, 0.075 ppm, 0.0375 ppm, and 0.0075 ppm; Thiacloprid: 144 ppm; Thiametoxam: 100 ppm, 10 ppm, 5 ppm, 2 ppm, 1 ppm, 0.5 ppm, 0.2 ppm, 0.1 ppm, 0.05 ppm, and 0.01 ppm.

Indirect contact tests

Spanish chestnut (*Castanea sativa* Mill.) leaves were collected in a wood far from possible pollution sources, sprayed to drip with a high-volume pneumatic hand sprayer, and left to dry in the shade for at least three hours. Water suspensions of the products to be tested and pure water for untreated controls were used. The leaves were then introduced into cages similar to those used for ingestion tests so as to completely cover the floor (figure 1).

The honey bees, introduced into the cages (10 bees/cage), could walk freely on the bottom covered with leaves, on the four walls, and on the cover for three hours, then the leaves were removed.

During the trial, the honey bees were fed sugar candy from a feeder obtained by opposing two hour glasses so as to obtain a 1 mm slot from which the honey bees could feed without touching the sugar candy, except with their proboscis (figure 1).

Tests started at 12.00 h and mortality was checked at 15.00 h and 18.00 h on the first day of the trial and at 9.00 h, 12.00 h, 15.00 h, and 18.00 h during the following days.

Tested a.i. concentrations were: Acetamiprid: 100 ppm; Clothianidin: 75 ppm, 37.5 ppm, 15 ppm, 7.5 ppm, 3.75 ppm, and 1.5 ppm; Thiacloprid: 144 ppm; Thiametoxam: 100 ppm, 10 ppm, 5 ppm, 2 ppm, and 1 ppm.

Ingestion tests after starvation

Since Acetamiprid and Thiacloprid showed no harm to the honey bees both in ingestion and indirect contact tests further ingestion tests with starved honey bees were carried out.

In order to perform these trials, besides being kept cool (11-13 °C) and in the dark the honey bees were starved for two hours after capture. Preliminary tests had shown that such condition did not impair honey bee survival chances while crop content was completely consumed. After this starvation period the normal procedure for the ingestion test was followed.

Tested a.i. concentrations were: Acetamiprid: 100 ppm, 50 ppm, and 20 ppm; Thiacloprid: 144 ppm, 72 ppm, 36 ppm and 18 ppm.

Table 1. Characteristics of Acetamiprid, Clothianidin, Thiacloprid, and Thiametoxam commercial formulations used in the tests.

Active ingredient (a.i.)	Acetamiprid	Clothianidin	Thiacloprid	Thiametoxam
Trade name	Epik®	Dantop® 50 WG	Calypso®	Actara® 25 WG
Formulation	soluble powder in water-soluble bags	hydro dispersible granules	concentrated suspension	hydro dispersible granules
a.i. %	5% w/w	50% w/w	40.4% w/w (480 g/l)	25% w/w
Field concentration*				
- commercial formulation	150-200 g/hl	15 g/hl	30 ml/hl	30-40 g/hl
- a.i.	7.5-10 g/hl	7.5 g/hl	14.4 g/hl	7.5-10 g/hl
Crops	Ornamentals	Apple, Pear	Courgette, Cucumber, Muskmelon, Watermelon, Ornamentals	Apple, Pear
Pests	Whiteflies Thrips	Aphids	Whiteflies	Aphids, Psyllids, Leaf Miners, Sawflies
Highest tested concentration	100 ppm	75 ppm	144 ppm	100 ppm

* The highest concentration recommended on the label for crop treatment.

Figure 1. Cage prepared for indirect contact tests (left); detail of the feeder, which is made so that honey bees can feed on sugar candy without touching it except with the proboscis (right).
(In colour at www.bulletinofinsectology.org)

Research of the tested a.i. in the dead honey bees

Whenever mortality was checked, the dead honey bees were removed from the cages and immediately frozen at −18 °C. At the end of the trials, they were sent in refrigerated containers to the Floramo Corp. S.r.l. laboratory for chemical analysis with the aim to research the presence and quantity of a.i. used.

A LC-MS/MS analytical procedure adapted from A.O.A.C. (2007) methods was adopted.

Statistical analysis

For each a.i. at each concentration and for the controls, 30 honey bees (three cages) were used. The number of dead and live honey bees was compared with that of the relative control group by the Fisher exact test. If statistically significant differences were not detected, 30 other honey bees underwent trial and the resulting mortality pooled with the previous one. The chi-square test was performed on the resulting 60 honey bees and relative controls. Only the counts done after 1 h (for ingestion test only), 3 h, 6 h, 24 h, 48 h, and 72 h from the beginning of the trials were statistically checked.

The Lethal Concentration (LC_{50}) both by ingestion and indirect contact for Clothianidin and Thiametoxam was calculated by means of logit analysis on two repetitions of 30 honey bees for each concentration from the lowest concentration, which caused 100% mortality, to the highest concentration which caused mortality not significantly different from that of the untreated controls.

The amount of 25% (w/v) sucrose syrup ingested by each honey bee during acute oral toxicity tests had previously been determined by weighing the feeder at the beginning and at the end of the allowed one hour feeding period as well as taking into account syrup density (Laurino *et al*., 2010). Since it resulted 35 µl on average, the ingestion Lethal Dose (LD_{50}) was obtained from the relative LC_{50}. LD_{50} were used to calculate the Hazard Quotients: HQ = field application rate (g/ha)/(oral LD_{50} (µg/bee)) (OEPP/EPPO, 2003) relative to the field application adopted for field concentration determination (table 2). LD_{50} and HQ could not be calculated for the indirect contact tests because the absorbed amount of the various a.i. could not be determined.

Results

During the trials, the honey bees showed obvious symptoms of poisoning, such as shaking and tremors, uncoordinated and uncontrolled movements, staggering, inability to take up a correct position of the body, and prolonged frenetic movement of the legs and rotation when

Table 2. LC_{50}, LD_{50}, and HQ of Clothianidin and Thiametoxam. In brackets LC_{50} and LD_{50} upper and lower limits at 95%.

	24 h	48 h	72 h
Clothianidin - ingestion			
Cl_{50} (ppm = ng/µl)	0.081 (0.050-0.116)	0.077 (0.050-0.105)	0.075 (0.055-0.094)
Dl_{50} (ng/honey bee)	2.844 (1.733-4.045)	2.689 (1.749-3.679)	2.608 (1.938-3.293)
HQ	26375	27890	28762
Clothianidin - indirect contact			
Cl_{50} (ppm = ng/µl)	4.485 (3.820-5.167)	2.967 (2.398-3.467)	2.667 (2.121-3.156)
Thiametoxam - ingestion			
Cl_{50} (ppm = ng/µl)	0.134 (0.110-0.159)	0.126 (3.612-0.150)	0.123 (0.100-0.147)
Dl_{50} (ng/honey bee)	4.679 (3.862-5.552)	4.411 (3.612-5.252)	4.316 (3.517-5.154)
HQ	21369	22670	23167
Thiametoxam - indirect contact			
Cl_{50} (ppm = ng/µl)	5.200 (4.302-6.227)	3.313 (2.786-3.806)	2.462 (2.156-2.903)

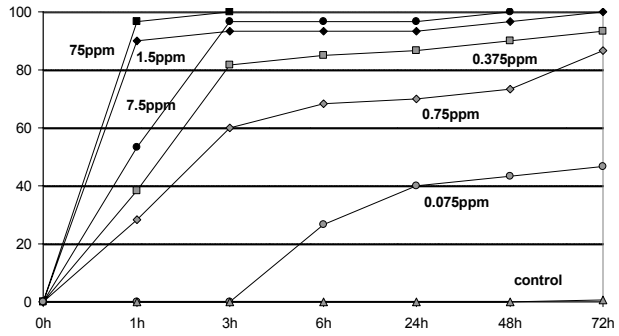

Figure 2. Mortality of foraging honey bees free to feed, during 1 h, sugar solutions containing 6 decreasing concentrations of Clothianidin.

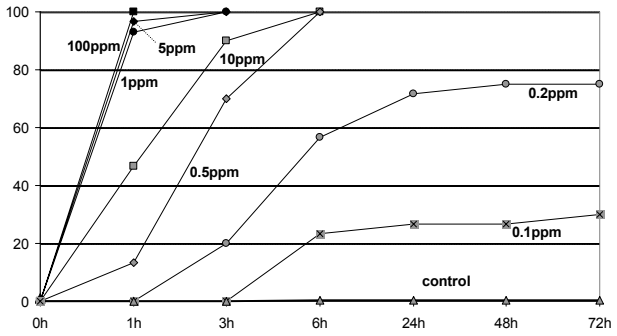

Figure 3. Mortality of foraging honey bees free to feed, during 1 h, sugar solutions containing 8 decreasing concentrations of Thiametoxam.

in the supine position. Direct observation of the behaviour of the honey bees in cages proved that it was transitory for Acetamiprid and Thiacloprid at field concentration and for Clothianidin and Thiametoxam at a lower concentration. Moreover, in ingestion trials, the highest concentrations of Clothianidin and Thiametoxam caused extensive vomiting in the honey bees.

Ingestion tests

Acetamiprid and Thiacloprid showed no mortality in the ingestion tests even 72 h from test initiation. Clothianidin caused the death of all the tested honey bees within 3 h from the start of the trial at the field concentration of 75 ppm, and within 72 h at the concentration of 1.5 ppm, 50 times lower. The mortality at the concentration of 1.5 ppm at 1 h from the beginning of the test was greater than that at the 7.5 ppm concentration and the 0.75 ppm concentration caused lower mortality than the 0.375 ppm concentration. The product caused statistically significant mortality up to 0.075 ppm, a concentration 1000 times lower than the field one (figure 2).

Thiametoxam caused the death of all the tested honey bees up to the concentration of 0.5 ppm, 200 times less than the field concentration, within 6 h from test initiation. The product caused statistically significant mortality up to 0.1 ppm, a concentration 1000 times lower than the field one. At the concentration of 10 ppm the mortality grew more slowly than at the concentrations of 5 ppm, 2 ppm, and 1 ppm (figure 3).

Clothianidin and Thiametoxam ingestion LC_{50}, LD_{50}, and HQ are reported (table 3).

Indirect contact test

Acetamiprid and Thiacloprid showed no mortality in the indirect contact tests even 72 h from test initiation.

Clothianidin caused total mortality within 24 h at the concentration of 37.5 ppm (half of field concentration) and within 48 h at the concentration of 15 ppm. The product caused statistically significant mortality up to 3.75 ppm, a concentration 20 times lower than the field one (figure 4).

Thiametoxam caused total mortality within 6 hours at the field concentration of 100 ppm and within 72 h at the concentration of 10 ppm. The product caused statistically significant mortality up to 2 ppm, a concentration 50 times lower than the field one (figure 5).

Clothianidin and Thiametoxam indirect contact LC_{50} are reported (table 3).

Table 3. Amounts of Clothianidin and Thiametoxam present in dead honey bees as a consequence of ingestion tests.

Concentration (ppm)	Mortality 24 h (%)	Mortality 48 h (%)	Ingested a.i. dose (ID) (ng/honey bee)	Detected a.i. amount (DA) (ng/honey bee)	DA/ID•100
Clothianidin					
75.00	100.00	-	2625.00	26.6	1.01
7.50	96.67	100.00	262.25	5.4	2.06
0.75	70.00	73.33	26.25	2.9	11.05
0.375	86.67	90.00	13.12	1.2	9.14
0.09375	83.33	86.67	3.28	0.8	24.38
Thiametoxam					
100.00	100.00	-	3500.00	19.0	0.54
10.00	100.00	-	350.00	6.2	1.77
5.00	100.00	-	175.00	2.3	1.31
2.00	100.00	-	70.00	1.4	2.00

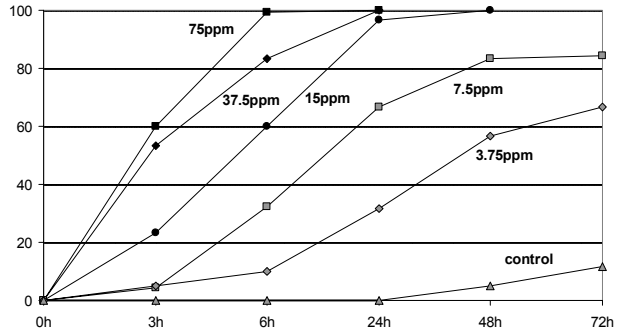

Figure 4. Mortality of foraging honey bees free to enter in contact for 3 h with chestnut leaves treated with decreasing concentrations of Clothianidin.

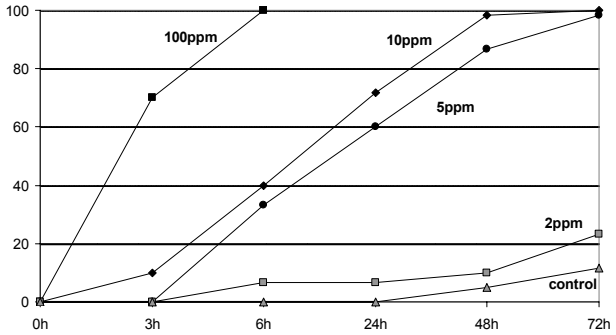

Figure 5. Mortality of foraging honey bees free to enter in contact for 3 h with chestnut leaves treated with decreasing concentrations of Thiametoxam.

Ingestion tests after starvation

With this procedure the honey bees eagerly fed on the sugar solution containing both a.i. at all tested concentrations, allowing any toxic effect to be highlighted.

The mortality caused by Acetamiprid was 50.85% at the field concentration of 100 ppm. Statistically significant mortality was observed at 50 ppm 72 h from test initiation (figure 6).

The mortality caused by Thiacloprid was not total even 72 h from test initiation, but resulted statistically significant up to the concentration of 36 ppm, one fourth of the field concentration (figure 7).

Research of the tested a.i. in the dead honey bees

Only the honey bees that died as a result of Clothianidin and Thiametoxam action were analyzed. Higher amounts of the two a.i. were detected in the honey bees that had been subjected to higher concentrations both in ingestion (table 3) and indirect contact (table 4) tests. In the ingestion test the ratio between detected amount and ingested dose increased at decreasing concentrations.

Discussion and conclusions

Poisoning symptoms similar to those observed in the trials had already been reported for various neonicotinoid insecticides (Bortolotti *et al.*, 2003; Medrzicky *et al.*, 2003; Maccagnani *et al.*, 2008; Decourtye and Devillers, 2010). The highlighted disabling behaviour, although transitory for some a.i. like Acetamiprid, could irreversibly affect honey bee survival in the field, taking into account external dangers that may occur, such as cold and predation. Moreover, even if the poisoned honey bees were able to return to the colony, their memory and communication abilities could be impaired (Desneux *et al.*, 2007; Maccagnani *et al.*, 2008; Decourtye and Devillers, 2010).

The graphs that show ingestion trial results are somehow irregular with some lines overlapping. Conceivably that was due to the observed vomiting phenomena. The latter very likely reduced a.i. absorption by honey bees, thus slightly extending their life. In the indirect contact trials there was a greater regularity in the results since the bees had no opportunity of getting get rid of the insecticide through vomiting.

Test results presented in this paper are in line with those reported in the literature even if most concern Imidacloprid (Bailey *et al.*, 2005; Muccinelli, 2008).

Clothianidin and Thiametoxam are highly toxic both via ingestion and indirect contact even if the latter is somehow less dangerous at reduced concentrations. In the indirect contact test Thiametoxam was lethal to a concentration 20 times lower than the field one, showing a degree of danger long after administration. The calculated acute ingestion toxicity LD_{50} are in accordance with those reported in the literature:

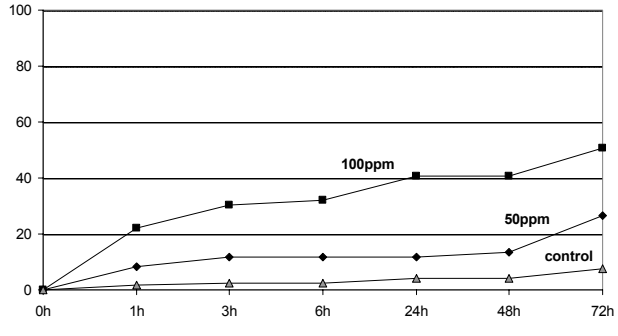

Figure 6. Mortality of foraging honey bees starved for 2 h and then let free to feed, during 1 h, sugar solutions containing 2 decreasing concentrations of Acetamiprid.

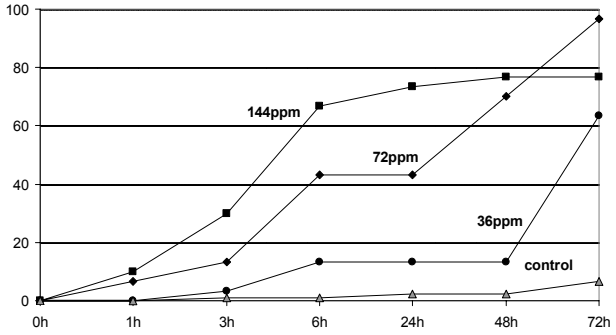

Figure 7. Mortality of foraging honey bees starved for 2 h and then let free to feed, during 1 h, sugar solutions containing 3 decreasing concentrations of Thiacloprid.

Table 4. Amounts of Clothianidin and Thiametoxam present in dead honey bees as a consequence of indirect contact tests.

Concentration (ppm)	Mortality 24 h (%)	Mortality 48 h (%)	Detected a.i. amount (DA) (ng/honey bee)
Clothianidin			
75.00	100.00	-	59.0
37.50	100.00	-	28.0
15.00	96.67	100.00	5.8
7.50	66.67	83.33	2.4
3.75	31.67	56.67	0.3
Thiametoxam			
100.00	100.00	-	27.0
10.00	71.67	98.33	2.9
5.00	60.00	86.67	2.0

1.8-3.8 ng/honey bee at 24 h for Clothianidin and 5.0 ng / honey bee at 48 h for Thiametoxam (Tomlin, 2003). HQs of both a.i. are exceedingly high when compared with those of other insecticides and their order of magnitude is similar to the Imidacloprid HQ regarding bumble bees (Marletto *et al.*, 2003).

Acetamiprid and Thiacloprid, as also evidenced in other acute toxicity trials (Iwasa *et al.*, 2004; Maccagnani *et al.*, 2008), were apparently not dangerous to the honey bees unless they were starved. This result suggests that there is a repellent effect of both a.i. as also reported for Imidacloprid (Ramirez-Romero *et al.*, 2005) and a food preference test would prove such an effect. If so, and disregarding sub-lethal effects, some hazards can arise when colonies are severely short of stores or after prolonged seclusion.

The low amount of a.i. recovered from the dead honey bees compared to the ingested doses could be due to the low stability of the molecules and/or to metabolite formation. Both phenomena are well documented (cfr Tomizawa and Casida, 2005 and the literature cited in it) and therefore special sampling, sample storage, and handling procedures are recommended in environmental fate and risk assessment investigations (Doucet-Personen *et al.*, 2003).

The residues present in honey bees after dosing them with one LD_{50} of various insecticides was determined years ago and the relative subsequent residue levels (SRL) make allowance for residue losses before and during the analysis procedures, although they do not consider extra losses before discovery of dead bees at colonies (Greig-Smith *et al.*, 1994). Our data on Clothianidin and Thiametoxam are similar to the SRLs of other neurotoxic insecticides and therefore the same criteria proposed by Greig-Smith *et al.* (1994) should be adopted during investigations on possible poisoning incidents in which neonicotinoids might be involved.

Acknowledgements

We thank Floramo Corp. S.r.l. for chemical analysis of dead honey bees and the Assessorato all'Agricoltura of the Regione Piemonte (Italy) for financial support.

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Received July 27, 2010. Accepted March 24, 2011.