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Assessment of the Environmental Exposure of Honeybees to Particulate Matter Containing Neonicotinoid Insecticides Coming from Corn Coated Seeds

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ABSTRACT: Since seed coating with neonicotinoid insecticides was introduced in the late 1990s, European beekeepers have reported severe colony losses in the period of corn sowing (spring). As a consequence, seed-coating neonicotinoid insecticides that are used worldwide on corn crops have been blamed for honeybee decline. In view of the currently increasing crop production, and also of corn as a renewable energy source, the correct use of these insecticides within sustainable agriculture is a cause of concern. In this paper, a probable—but so far underestimated—route of environmental exposure of honeybees to and intoxication with neonicotinoid insecticides, namely, the atmospheric emission of particulate matter containing the insecticide by drilling machines, has been quantitatively studied. Using optimized analytical procedures, quantitative measurements of both the emitted particulate and the consequent direct contamination of single bees approaching the drilling machine during the foraging activity have been determined. Experimental results show that the environmental release of particles containing neonicotinoids can produce high exposure levels for bees, with lethal effects compatible with colony losses phenomena observed by beekeepers.

■ INTRODUCTION

In view of the evolution of farming systems associated with the increasing global food production expected to feed a growing global population, together with the greater and greater use of agricultural products as renewable energy sources,[1−5] particular attention should be given to effective strategies for the control of environmental pollutants released by crop activities. Several adverse effects have currently been associated with these emissions, such as the loss of biodiversity and ecosystem services due to an increasing use of agrochemical compounds, their effects on human health, or the contribution of greenhouse-gas emissions in agriculture to global warming (about 30%).[6]

In Europe, corn crops may represent an interesting case study for the assessment of the sustainability of future farming strategies. Corn is largely cultivated in Europe, especially in northern Italy, France, Germany, and the Balkan countries, and is largely used for both human consumption and livestock feeding. Moreover, the recent government subsidies to the "green energies" are transforming corn crops into profitable energy sources. Thus, severe drawbacks could be related to the consequent increase both in atmospheric emissions from biomass transformation processes, for instance the particulate matter emissions in highly critical areas such as the Po Valley in northern Italy, and the environmental releases of substances with recognized toxic and ecotoxic effects, such as neonicotinoid insecticides that have been associated with the worldwide crisis of honeybee colonies.[7,8]

In the past decade honeybee colonies throughout the world have been subject to rapid losses[7,9] in the order of 40%,[10,11] in particular in southern Europe. This phenomenon, also named colony collapse disorder, represents a worldwide crisis with adverse effects both on crop production and on ecosystems. In Italy and Europe, corn sowing—from mid-March to May—was often accompanied by a rapid disappearance of foraging bees.[12,13] These spring time deaths are chronologically distinguishable from those caused by *Varroa destructor*, and a close relationship was observed between the deaths of bees and the use of pneumatic drilling machines[14−17] for the sowing of corn seeds coated with neonicotinoid insecticides.[18,19] In pneumatic drilling machines, seeds are sucked in, causing the erosion of fragments of the insecticide shell that are forcefully expelled with a current of air. The widely accepted hypothesis is that bees die by collecting contaminated pollen and nectar, because solid fragments of the neonicotinoid seed coating fall

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on the vegetation surrounding the seeded areas.[13,14] But neonicotinoid concentrations in the vegetation at the margins of the seeded areas were shown to be about 50 ppb or lower,[15,20,21] which is not high enough to cause acute toxicity in foraging honeybees.[22−24]

More recently we have investigated other sources of contamination for bees present in the fields, which could justify such spring mortality,[25−27] and very recent results seem to confirm our hypothesis that the solid particles emitted by drilling machines, and containing a high insecticide concentration, can produce a direct powdering of foraging bees in free flight accidentally crossing the sowing fields.[15−17] This acute exposure may represent lethal doses for flying bees, coherent with the colony loss phenomena observed in spring when and where corn is sown.

The present paper reports on the accurate characterization of the particulate matter emitted by a drilling machine during corn sowing. A dimensional analysis of the coating particles emitted by seeds treated with different insecticides and a quantitative determination of the total concentration of insecticide present in the air at different distances from the drilling machine were carried out to assess both factor emissions during corn sowing activities and possible exposure to neonicotinoids for flying bees approaching the drilling machine. An analytical procedure was also optimized to quantify the effective contamination of single exposed bees in the field. Different geometries of the waste pipe of the drilling machine, proposed for the modification of relevant commercial models, have been tested and compared.

■ EXPERIMENTAL SECTION

Seeds, Insecticides, and Bees. Seeds produced and marketed in 2008−2010 (hybrid employed X1180D 964890 and PR44G; Pioneer Hi-bred, Italy) were used for the emission tests. The seed coatings were Poncho (clothianidin 1.25 mg/seed, Bayer Cropscience AG., Leverkusen, Germany), Gaucho (imidacloprid, 0.5 mg/seed; Bayer Cropscience AG.), Cruiser (thiamethoxam 0.6 mg/seed, Syngenta, Basel, Switzerland), and Regent (fipronil 0.5 mg/seed, BASF SE). All seed batches exhibited dust abrasion levels under the limit of 3 g per 100 kg seeds (tested by Heubach test[28−30]).

Four hives were supplied by the Padova Beekeeping Association (APA Pad) for the exposure tests of flying bees (*Apis mellifera, L*).

Drilling Machines and the Sowing Area. All tests were carried out at the experimental farm of the University of Padova, located in Legnaro (Padova, Italy), in a 50 m wide by 70 m long sowing field (coordinates: 45°20′41.19″ N−11°57′16.22″ E).

A Ribouleau Monosem NG plus (four sowing rows, Largeasse, France) drilling machine was used, as a rule, in the emission tests. The air waste pipe of the fan, which drives the pneumatic system of seed distribution, is located on the right-hand side of the machine. During sowing it expels air (and dust) at ca. 230 m^3/h, at a height of 1.8 m and an angle of 45° to the horizontal. In a second series of experiments, a double pipe (i.d. 12 cm, length ca. 2 m) was fitted to the original outlet to funnel the air stream to the soil. All experiments reproduced standard sowing conditions: speed, 6 km/h (66 660 seeds/ha); seed distance, 75 cm between rows, 20 cm between seeds in the row; considering a seeding width of 3 m, the uninterrupted sowing time was about 33 min per 1 ha.

A Gaspardo model Monica drilling machine (six sowing rows, Gaspardo Seminatrici SPA, Italy), mounting a deflector at the outlet of the fan that should release the air stream directly toward the soil (without pipes), was also employed for comparison. This machine worked at 6 km/h (66 660 seeds/ha too with a distance of 75 cm between rows and 20 cm between seeds in the row). Considering a seeding width of 4.5 m, the sowing time was about 22 min per 1 ha.

Particulate Matter Emission Tests. Sowing tests were carried out in two ways. In standard sowing conditions, the drilling machine worked all along the field and the following samples were collected:

(a) The particulate matter that falls down to the ground (dry deposition) was sampled on a series of cellulose esters filters (diameter of 185 mm, Carl Schleicher et Schull, model Selecta) located at the field margin, along the wind direction. The filters, contained in a plastic vessel, were humidified by water to avoid the release of sampled particles by the wind.

(b) The total suspended particulate (TSP) present in the atmosphere at the field margin was sampled by the U.S. EPA standardized procedure using Zambelli pumps (model ZB1 timer, Milan, Italy) operating at 20 L/min and equipped with a standard 47 mm PTS filter holder and glass fiber filters (Whatman, 47 mm).

(c) PM_{10} was sampled at the field margin by a Zambelli model Explorer plus apparatus, operating under standardized conditions (EN 12341:1999 PM_{10} selector, flow rate 38.3 L/min, and 47 mm glass fiber filters).

Typical sampling times were 30 min for PTS and 1 h for PM_{10} samples. All filters were stored at −18 °C until the laboratory instrumental analysis.

A second experimental set was realized in order to perform more accurate analytical measurements and exposure tests: in this case the drilling machine worked in a static mode (motionless in the field) but with the same sowing parameters previously detailed, using the cardan joint of a second tractor to drive the seed distribution mechanism. Emission factors were computed by measuring the concentration of the total suspended particulate matter (TSP, sampling time 5 min) emitted by the drilling machine and collected under isokinetic conditions at the end of waste pipe of the fan. A standardized stainless steel isokinetic sampling line was used (EN 13284-1:2001), equipped with a Zambelli (model ZB1 timer) pump, 6 mm sampling inlet, 47 mm filter holder, and glass fiber filters (Whatman, 47 mm).

During the "static" sowing samples of TSP (at 5 and 10 m from the drilling machine, sampling time 30 min) and PM_{10} (at 10 m, sampling time 30 min) were collected using the same experimental condition as in standard sowing. Moreover, the size distribution of aerosol particulate matter released during the "static sowing" was measured by an optical particle counter (OPC, Grimm model 1.108) in the 0.23−32 μm diameter range. The instrument was placed 5 m from the pneumatic drilling machine in order to minimize the resuspension of dust from the soil. Both the rural background and the blank values (with the drilling machine operating without seeds) were registered and then subtracted from the experimental values measured during the emission tests.

Analysis of Single Bees Exposed to Neonicotinoids. For each bee the entire analytical procedure was carried out in separate containers. Single bees found dead in the field or close to the beehive during the sowing tests were collected in a 4 mL glass vial and stored at −80 °C. Before chemical analysis the samples were maintained some hours at −20 °C and lyophilized for 16 h in a vacuum box equipped with a high-vacuum pump (Speedvac Edwards model ED200A). Every bee was then ground up with a metal pestle, subsequently added with 500 μL of methanol, and treated in ultrasonic bath for 30 min at room temperature. The ultrasonic treatment was repeated after addition of 500 μL of water. The resulting extracts were transferred into 1.5 mL microcentrifuge tubes (VWR) and centrifuged for 60 min at 10 000 rpm (Hettich MIKRO 120). The upper clear solutions were collected by a syringe and transferred into 1.5 mL analytical vials after filtration on 0.2 μm syringe filters (Phenomenex, RC).

A UHPLC (ultra-high-performance liquid chromatography) analytical method was optimized for the determination of each seed-coating neonicotinoid insecticide. The method used a Shimadzu Prominence UFLC-XR chromatograph equipped with a Shimadzu SIL 20AC-XR autosampler, Shimadzu SPD-M20A UV−vis diode array detector (DAD), and a Shimadzu XR-ODS II (2.2 μm, 2 mm × 100 mm) analytical column with a Phenomenex (ODS 4 mm × 2 mm) guard column. The following instrumental parameters were adopted: eluent flow rate of 0.4 mL min^{-1}, water−acetonitrile gradient elution (0−2.65 min, linear gradient from 16% to 41% acetonitrile; 2.65−4.60 min, linear gradient to 100% acetonitrile; 4.60−5.25 min, 100% acetonitrile), 5 μL of injector volume, 45 °C of column temperature. Detector signals at λ = 215 nm for fipronil, λ = 252 nm for thiamethoxam, and λ = 269 nm for clothianidin and imidacloprid were adopted for analytes quantification. Although in Europe thiacloprid and acetamiprid are not used for corn seed coating, they can also be separated and quantified (λ = 244 nm) by the present procedure. Instrumental calibration (external) was performed by analysis of 0.05−10 mg L^{-1} standard solutions of each analyte in 50% water−methanol.

Chemicals for the preparation of the standard solutions of fipronil, thiamethoxam, clothianidin, imidacloprid, acetamiprid, and thiacloprid were purchased from Fluka (Pestanal, purity >99.7% for the five neonicotinoids and >97.5% for fipronil). Methanol (VWR) and acetonitrile (Riedel de Haen) were of HPLC grade. Water was purified by a Millipore Milli-Q equipment.

Analysis of the Sampled Particulate Matter. For the determination of neonicotinoid insecticides in the particulate samples, the filters (or fraction of filter) were introduced in 10 mL test tubes, added with 2.5 mL of methanol, and treated in ultrasonic bath for 30 min at room temperature. This treatment was repeated after addition of 2.5 mL of water. These solutions were directly analyzed by UHPLC, after filtration on 0.2 μm syringe filters (Phenomenex, RC), adopting the previously optimized rapid analytical procedure.[26]

RESULTS AND DISCUSSION

Particulates Emitted by the Drilling Machine. Since our first experiments, conducted in 2009 with corn seeds coated with clothianidin, the fundamental observations of Greatti et al.[14,20] have been fully confirmed: significant amounts of coating particles are effectively emitted by the drilling machine during corn sowing. Large fragments of the seed surface (ca. 1 mm, well visible around the fan outlet) were released in atmosphere through the outlet of the air flow generated in the pneumatic device of seeds distribution. Moreover, quantitative measurements carried out at the margin of the sowing field demonstrated that 1 h of normal activity of the drilling machine can generate the dry deposition of about 280 μg/m^2 of the insecticide (with clothianidin 2008 seed coating, about half when the 2009 seeds were used) and concentrations of clothianidin in the total suspended particulate (TSP at the field margin) of 0.24 and 0.10 μg/m^3 for the two different seed coatings (2008 and 2009, respectively). In addition, analysis of PM_{10} samples collected 10 m from the field margin (ca. 60 and 10 ng/m^3 of clothianidin for the 2008 and 2009 seed coatings, respectively) clearly indicated the presence of not negligible levels of micrometric particles containing the insecticide, which were emitted by the drilling machine together with the larger ones.

Although larger particles undergo rapid sedimentation (very close to the waste pipe) and in 2009−2010 new types of seed coatings (with thicker films) were introduced in Europe, as they are supposed to be more resistant to abrasion, German—before the ban on neonicotinoids—and Austrian and Slovenian beekeepers continued to report extended losses of bee colonies in spring in conjunction with corn sowing. On the contrary, no colony losses were observed in Italy, after the neonicotinoids ban. Thus, taking into account the hypothesis of a possible acute toxic effect of the emitted particles on honeybees, a series of experiments were carried out in order to better characterize these atmospheric emissions and to assess the possible exposure of honeybees to the insecticides contained in these particles in open fields.

The size distribution analysis of the emitted particles, measured by an OPC instrument during "static sowing" of corn seeds coated with clothianidin (Poncho 2009 and 2010), revealed a typical coarse distribution ascribable to the erosion processes occurring on the seed surface. At 5 m from the working drilling machine, a significant increase in the particles concentration was registered (with respect to the blank values, Figure 1) only for particles with a diameter larger than 2 μm.

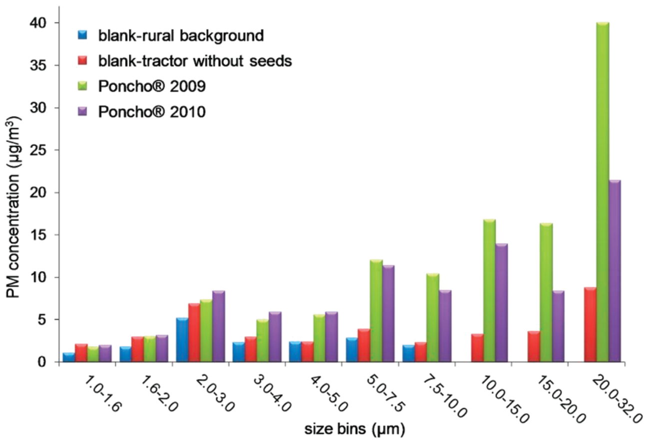

Figure 1. Dimensional distribution of particles emitted by the drilling machine during the sowing of coated seeds, measured by OPC instrumentation 5 m from the outlet of the air fan.

The mass concentration of the coating particles (estimated by the OPC at 5 m from the waste pipe, using the 2−32 μm diameter range) was 79.4 μg/m^3 for the 2009 seed coating and 49.8 μg/m^3 for the 2010 seed coating. However, in the latter case, sedimentation of very large particles (0.5−2 mm) was also observed close to the waste pipe. These results show that significant concentrations of the coating particles can surround

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Table 1. Concentration of Insecticides Measured at the Waste Pipe of the Monosem Drilling Machine during the Sowing of Corn Coated Seeds and Relevant Emission Factors Estimated Using Normal Sowing Parameters[a]

Corn seed, insecticide	Insecticide concentration at the outlet of the air fan (mg/m^3)	Emission factors g/h	Emission factors g/ha	Fraction of released insecticide (%)
Poncho 2008, Clothianidin 1.25 mg/seed	3.6[b]	0.83	0.46	0.55
Poncho 2009, Clothianidin 1.25 mg/seed	3.39 ± 0.47	0.78	0.43	0.52
Poncho 2010, Clothianidin 1.25 mg/seed	12.0 ± 1.2	2.76	1.53	1.84
Cruiser 2010, Thiamethoxam 0.6 mg/seed	5.8 ± 1.5	1.33	0.74	1.85
Regent 2010, Fipronil 0.5 mg/seed	3.57 ± 0.46	0.82	0.46	1.37

[a]Data obtained from the analysis of three independent samples (isokinetic TSP) collected during "static sowing" experiments using the Monosem drilling machine. Sowing conditions: speed, 6 km/h; four rows of seed distribution; distance between rows, 75 cm; seeds distance, 20 cm (66 660 seeds/ha); air flow, 230 m^3/h. [b]Value obtained from a single sample collected during the preliminary tests.

Table 2. Concentration of Neonicotinoid Insecticides in the Particulate Matter Sampled near the Drilling Machine during the Sowing of Corn Coated Seeds[a]

Corn seed, insecticide	Drilling machine and air fan outlet modification	5 m: Insecticide in TSP ($\mu g/m^3$)	10 m: Insecticide in TSP ($\mu g/m^3$)	10 m: Insecticide in PM_{10} ($\mu g/m^3$)
Poncho 2009, Clothianidin 1.25 mg/seed	Monosem, unmodified	28.4	13.1	nd
Poncho 2009, Clothianidin 1.25 mg/seed	Monosem, dual pipe outlet	4.7	1.2	0.2
Poncho 2010, Clothianidin 1.25 mg/seed	Monosem, unmodified	15.0	5.3	nd
Poncho 2010, Clothianidin 1.25 mg/seed	Monosem, dual pipe outlet	6.1	1.5	1.2
Poncho 2010, Clothianidin 1.25 mg/seed	Gaspardo, mod. Monica	4.5	0.8	0.2
Cruiser 2010, Thiamethoxam 0.6 mg/seed	Monosem, unmodified	4.2	1.0	0.6
Cruiser 2010, Thiamethoxam 0.6 mg/seed	Monosem, dual pipe outlet	7.2	2.8	1.6
Regent 2010, Fipronil 0.5 mg/seed	Monosem, unmodified	12.0	1.9	0.5

[a]Average values of three independent samples and determinations. Uncertainty (standard deviation) ca. 5%. nd: not determined.

the drilling machine during corn sowing. Moreover, they seem to indicate that the coating proposed in 2010 emits more particles, but with a larger diameter and a reduced capability to be carried by the wind (i.e., they fall to the ground near the drilling machine) compared to particles coming from the 2009 seed-coating batches.

In any event, besides the larger particles emitted by the drilling machine, the presence of a significant tail of the dimensional distribution of these erosion (coarse) particles approaching the range of fine particles (few micrometers) is well evidenced for both coatings. Low-vacuum scanning electron microscopy−energy-dispersive spectrometry (SEM−EDS) analysis of the sampled TSP (collected on polycarbonate filters) confirmed the presence of fine particles containing the insecticides. Of course, the environmental spreading of these fine particles is expected to be higher than that associated with the coarse ones, and as a consequence, increased toxic effects on bees could be expected.

The effective total amount of insecticide emitted by the seed-coating particles released by the drilling machine has been assessed by the analysis of TSP isokinetically sampled at the waste pipe of the fan. Our results are reported in Table 1 together with emission factors of the drilling machine estimated considering the usual sowing parameters (see the Experimental Section). These data suggest that high quantities of insecticide are emitted during corn sowing. For instance, about 0.5% of the clothianidin employed in Poncho 2008 and 2009 seeds (that means more than 0.4 g/ha) is effectively released in the atmosphere as coarse particles. More recent seed coatings (2010) show higher emission factors (1.53 and 0.74 g/ha for clothianidin and thiamethoxam, respectively), but as discussed above, they are probably determined by the larger emitted particles (0.5−2 mm) that deposit quickly (very close to the air outlet) and are not carried in the atmosphere by moderate wind. Nevertheless, both OPC observation and analytical measurements in the field (see below) reveal that all kinds of seed coating release significant amounts of particles approaching the range of the fine ones and with relevant atmospheric mobility.

Analyses of the particulate matter (TSP and PM_{10}) sampled 5 and 10 m from the drilling machine (operating in static mode with different seed coatings) have also shown elevated values of the insecticide concentration in the air surrounding the working machine (Table 2). Of course, higher values are measured close to the emission source (5 m), but it is worth noticing that significant concentrations of insecticide can be observed also at a distance of 10 m from the drilling machine. Although strictly depending on wind direction and speed, these figures fully agree with the data drawn from OPC size distribution analysis: significant amounts of insecticide are emitted as few micrometer particles (sampled and better quantified in PM_{10}), together with the coarse ones. These particles are characterized by high atmospheric mobility and can be efficiently intercepted by the flying bees.[15−17]

Data in Table 2 also show that, during sowing, the Poncho 2009 corn seed coating seems to produce more particles than its 2010 version, although a higher factor emission was found for the latter. This discrepancy could be explained considering that a significant fraction of the 2010 coating is released as very large particles that cannot be easily transported to the sampling TSP apparatus (5 or 10 m). In conclusion, the two kinds of coatings show a different behavior toward surface erosion, and during sowing, the 2009 version produces a more concentrated cloud of fine−coarse particles surrounding the drilling machine.

As for the modification of the air fan outlet in the attempt to reduce the environmental release of the particles containing the insecticide, we must underline that the strategies so far proposed often consist in the mere application of a pipe (or a deflector in the Gaspardo model) that funnels the air flow toward the ground.[31] Of course, taking into account the size and the aerodynamic properties of the particles described above, it is easy to foresee the limited efficiency of this apparatus. In any case, we modified the waste pipe of the Monosem drilling machine as proposed by the French Agency for Food, Environmental, and Occupational Health and Safety (AFSSA)[28,32] using a dual pipe that splits the air flow into two components, both downward directed and released at 20 cm from the soil. Experimental results (Table 2) confirm a reduction of the clothianidin concentration measured at the modified drilling machines (for both the modified Monosem and Gaspardo) compared to the unmodified Monosem. On the other hand, improvement has not been observed using the seeds coated with thiamethoxam. Anyway, it seems clear that the modified drilling machines also emit large amounts of micrometric particles of ecotoxicological relevance, whose acute effects on flying bees have been recently well illustrated.[15−17,33]

Regarding other relevant properties of these particle clouds (i.e., their spatial and temporal dimension), although preliminary information have been acquired by toxicity data (ca. 15 m around the drilling machine; a few minutes after sowing was completed),[15,17] we are aware that more detailed experiments are needed.

Analytical Method for Single-Bee Analyses after Field Exposure. Since the first sowing tests with both static and normal operating drilling machine we observed the death of a significant number of bees whose beehives were ca. 100 m far from the sowing field. Short-term mortality and the characteristic symptoms of neonicotinoid neurotoxicity[25,34,35] gave rise to the hypothesis of a direct acute exposure of the flying bees to the emitted particles as they approached the drilling machine, rather than an indirect contamination via the vegetation (pollen, nectar, dew) surrounding the sown area. Therefore, a series of specific exposure experiments were carried out using both caged bees positioned at various distances from the air outlet[15,17] and foraging bees conditioned to fly over the sowing field to visit a dispenser of sugar solution.[16]

In this connection, an analytical method for the determination of the insecticide content in a single bee has been optimized and validated, taking into account the advantage of the rapid UHPLC procedure recently proposed for the analysis of corn guttation drops.[26] In the present procedure, the lyophilized sample (a single bee) was ground, extracted with methanol, and analyzed by a UHPLC-DAD instrumental method that allows the complete elution of the neonicotinoid insecticides of interest, and of fipronil, in about 6 min. The method shows excellent precision: repeatability, from replicate analyses of real samples, was better than 4% for concentration levels higher than 200 ng/bee of each insecticide (4−8% at 50 ng/bee). Although an instrumental limit of detection (LOD) of ca. 2 μg/L has been computed for each neonicotinoid insecticide from the parameters of the analytical calibration function (by the procedure suggested by IUPAC[36]), experimental uncertainties measured in the analysis of real samples indicate a reasonable LOD of ca. 10 ng/bee for the complete analytical procedure. Very limited chromatographic interferences for the UHPLC-DAD method were observed in the analysis of spring−summer sampled bees, and recovery tests, using spiked samples (blank bees added with 50−200 ng/bee of thiamethoxam, clothianidin, and imidacloprid), showed satisfactory recovery factors in the range 78−104%. A slightly worse chromatographic resolution (that gave higher uncertainties and lower recovery factors) was observed in the analysis of winter samples and in the quantification of fipronil.

Compared with the performance of HPLC−MS methodologies,[37,38] the LOD of the UHPLC-DAD method appears to be quite elevated. Nevertheless, the optimized procedure is rapid enough, uses a simpler instrumentation, and both accuracy and LOD are adequate for the purpose, i.e., the analysis of single bees after the acute exposure to particulates containing neonicotinoid insecticides.

Insecticide Content in Exposed Bees. Application of the analytical method to the analyses of single bees directly exposed in the field to the emitted particles has always evidenced elevated levels of the insecticide content. Although the assessment of a reliable correlation between the insecticide amounts emitted by the drilling machine and the bee uptake requires a more rigorous experimental approach than that adoptable in the field (i.e., a dedicated exposure chamber, a wind tunnel, or an isolated laboratory for emission tests as that set up by Biocca et al.[31]), the analyses of single bees sampled during the field sowing experiments revealed important information on both the effective bee exposure and the insecticide uptake mechanism.

For instance, foraging bees induced to fly over the sowing field to reach a sugar dispenser, here captured at the end of the sowing experiment (Poncho 2010, sowing time 1 h) and maintained in laboratory under high-humidity condition until death,[16,17] showed a concentration of clothianidin in the range of 78−1240 ng/bee (n = 5, mean 570 ng/bee). A wide spread of values was also observed using Cruiser 2010 seed coating: 128−302 ng/bee of thiamethoxam (n = 4, mean 189). Taking into account the satisfactory precision of the optimized analytical procedure, this high variability is probably due mainly both to the different number of flights over the field (or different paths approaching drilling machine) that each bee has completed before being sampled and to the effect of probable cleaning processes (dust off) occurring in flight or inside the hive. For this reason, strong dependence of the insecticide concentration on the sampling time (during sowing) has never been observed. On the other hand, in partial confirmation of the cleaning processes, non-negligible differences in insecticide concentration were observed in bees captured at the dispenser and maintained, until death, under different humidity conditions.[16] Thus, after 30 min from the start of the Cruiser 2010 sowing, thiamethoxam concentration was 267 ± 59 ng/bee (n = 5, humidity >95%) and 104 ± 87 ng/bee (n = 5, humidity <70%); using Regent 2010 seeds, fipronil concentration was 850 ± 330 ng/bee (n = 4, humidity >95%) and 210 ± 160 ng/bee (n = 6, humidity <70%). Despite their high (but justified) variability, these concentrations well support both the bee mortality data obtained by Girolami and co-workers, in which a strong dependence on the air humidity was reported,[15−17] and the hypothesis of a contact uptake in flight of the insecticide through the bee tegument, facilitated by the humidity.

The effective and lethal powdering of the flying bees has also been confirmed by quantitative measures of the insecticide "lying" on the bee surface. At the end of a sowing with Poncho 2009 (1 h), several dead bees were found at the sugar dispenser and immediately frozen. Before the analysis, seven bees were externally washed with methanol (15 min, in an ultrasonic bath) and then analyzed by the optimized procedure. The results revealed an external concentration of clothianidin of 396 ng/bees and a total concentration of 674 ng/bee. Dead bees sampled at the hive subsequent to the end of sowing (3 h, n = 7; 24 h, n = 14) showed a significantly lower content of insecticide: the external concentration was always below the LOD while total levels of 155 and 119 ng/bee were measured on the bees sampled after 3 and 24 h, respectively. A similar decreasing trend was also observed after exposure of the flying bees to other neonicotinoid particulates. For instance, using Gaucho 2009, external concentrations of imidacloprid up to 3000 ng/bee have been detected in the bees collected at the end of the sowing (240 ng/bee after 2 h, <LOD after 24 h); the total concentrations were 3650 and 325 ng/bee for bees sampled at the end of sowing and after 2 h, respectively (<LOD after 24 h). These results appear to be very informative: they confirm (i) the elevated capability of the flying bees approaching the drilling machine to intercept the suspended coating particles, (ii) the effective lethal contamination of bees with the insecticide that can be taken up by contact, and (iii) the possible partial removal of the particles during the foraging activity or in the hive.

The presence of coating particles on the abdomen of the flying bees, and the related uptake mechanism of the insecticide, has also been confirmed by electronic microscopy (low-vacuum SEM analysis, Figure 2) observing modified coating particles that adhere to the bee tegument. This modification, that is reasonably influenced by air humidity, could explain the different toxic effects observed on powered bees maintained at different conditions of humidity.[16] Moreover, it supports the experimental data that indicate a major self-capability of bees to remove coating particles (finding lower concentrations) when maintained, after exposure, under normal humidity condition.

Short time exposure of single caged bees to the air flow emitted by the fan of the drilling machine (about 30 s, simulating 1−2 flight across the sowing field at different distance from the drilling machine) always induced acute lethal effects toward the bees, more evident if the exposed bees are maintained, until death, under high-humidity conditions.[15−17] According to the observed toxic effects,[17] elevated levels of insecticide were always measured. For instance, caged bees exposed at different distances from the air outlet of the Monosem drilling machine (1−9 m, using Poncho 2010, in absence of a dominant wind) evidenced concentrations of clothianidin significantly higher for bees exposed on the right side (in front of the waste pipe) with respect to those exposed on the left side of the machine (Table 3). As expected for the latter ones, the dependence of the concentration on the distance from the drilling machine is not clear, as an effect of the turbulence of the air surrounding the working drilling machine. At the same time, this turbulence can also explain the variable values measured by using the modified Monosem drilling machine (with a dual-pipe outlet releasing particles, downward to the soil, from both sides): actually, only a concentration range of clothianidin (71−434 ng/bee, n = 10, mean 197 ± 129 ng/bee, humidity <70%; 70−446 ng/bee, n = 9, mean 216 ± 141 ng/bee, humidity >95%) can be reasonably furnished as representative of the caged bees exposed in the 1−9 m range from the back of the machine (toward the wind direction, 1−2 m/s), without correlation with the distance.

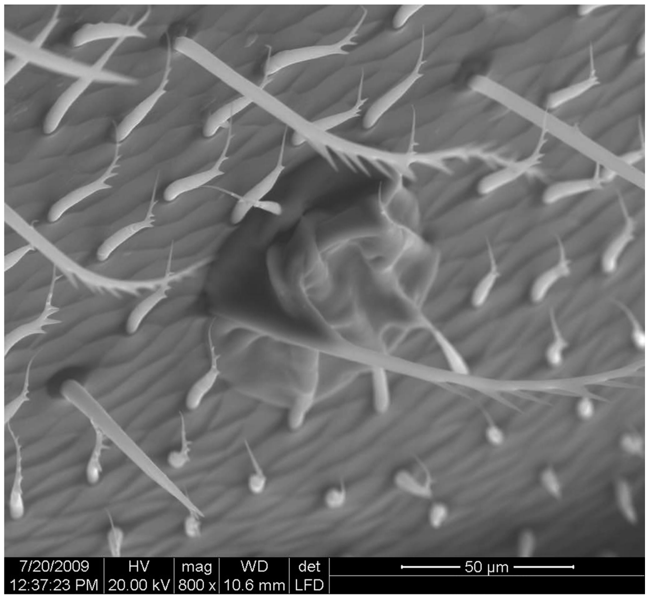

Figure 2. Low-vacuum SEM image of a seed-coating particle (Poncho 2009) that, partially modified by the air humidity, adheres to the abdomen tegument of a bee exposed to the drilling machine emissions.

Table 3. Clothianidin Concentration in Caged Bees Exposed, for 30 s at Different Distances (Both Right- and Left-Hand Side), to the Air Flow Emitted by the Monosem Drilling Machine during the Sowing of Poncho 2010 Seeds

distance from the air outlet (m)	concn detected in bees exposed on the right side (ng/bee)[a]	concn detected in bees exposed on the left side (ng/bee)[a]
1.00	1393.6 ± 0.6	115.3 ± 0.6
2.25	808 ± 2	80.7 ± 0.6
4.50	64 ± 4	110 ± 1
6.75	164 ± 4	598.7 ± 0.6
9.00	100.5 ± 0.7	25 ± 1

[a] Average values and standard deviation of the instrumental measurements (n = 3) on single-bee samples.

According to the high insecticide levels measured in air around the drilling machine (Table 2), huge contents of insecticide have been measured in the dead bees collected at the beehive after the sowing experiment also using the modified drilling machine. For instance, the sowing (1.5 h) of Poncho 2010 corn seeds by the Gaspardo drilling machine (with the outlet air flow directed downward by an external deflector) induced the rapid death of more than 200 foraging bees flying across the sowing area, revealing a clothianidin content in the range of 0.5−11 μg/bee. It is worth noticing that a significant decrease in the insecticide content seems to be evidenced when the sampling of the bees is delayed after death. In the hypothesis that the metabolic degradation of the insecticide (probably effective also postmortem) may affect the concentration experimentally found in real samples, to such an extent that very low levels could be found also after significant exposure, specific research is in progress in our laboratory.

In conclusion, particulate matter released by the drilling machine during the sowing of corn seeds coated with neonicotinoid insecticides represents a significant mechanism of environmental diffusion of these insecticides. Bees flying over the sowing field and approaching the emission cloud of the drilling machine can efficiently intercept the suspended particles being directly contaminated with elevated dose of insecticide, significantly higher than the LD50 values estimated for contact, with the cuticle, administration (18, 22, and 30 ng/bee for imidacloprid, clothianidin, and thiamethoxam, respectively[39]). The consequent acute lethal effect evidenced in all the field sowing experiment can be well compared with the colony loss phenomena widely reported by beekeepers in spring and often associated to corn sowing. Analytical results regarding factor emissions, air concentration of insecticide around the drilling machine and consequent bee contamination, reveal that all kinds of the tested seed coatings (also those more recently proposed) do not prevent the dispersion of large amounts of micrometric particles containing the insecticide, producing lethal exposure of flying bees. Moreover, the modifications of the air outlet of drilling machines so far adopted seem to have a limited effect on both the factor emission and the effective bee contamination.

This emission source of particles with acute toxic effects on bees (and on other insects too) is of concern for both apiculture and crop productions based on bee pollination. But it is also a widespread ecological problem that, in view of the worldwide increase in corn production partly promoted by government subsidies to renewable energy sources, and the consequent predictable exacerbation of the problem, should require a deeper analysis of the related agricultural policies. In this connection, immediate contributions for the reduction of atmospheric factor emissions of neonicotinoid insecticides should come from studies oriented to the realization of suitable devices for an efficient reduction of toxic particles inside the seed distribution mechanism of drilling machines and supported by quantitative data both on particulate emissions and biological effects on honeybees.

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Notes

The authors declare no competing financial interest.

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