Bulletin of Insectology ***68*** *(2): 161-172, 2015*
ISSN 1721-8861

Application of predefined doses of neonicotinoid containing dusts in field trials and acute effects on honey bees

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Abstract

This study explored the effects of insecticidal dusts on honey bee colonies (*Apis mellifera* L.) after exposure to *a priori* defined dose under field conditions. For this purpose two different rates of abraded seed dust, containing active substance Clothianidin, were applied on flowering *Phacelia tanacetifolia* Bentham during bee-flight with a purpose-built dust applicator. We observed dose-related high acute effects on bee mortality at both application rates, 0.25 and 1.0 g a.s. Clothianidin in dust per ha, resulting in up to 4.3 and 17 fold higher mortality compared to pre-application level and an overall increase of mortality during the 7 days exposure period of 2.0 and 9.8 fold. In dead bees, residues detected between both rates applied were up to 2.6 fold higher in the 1.0 g a.s. Clothianidin dust exposure scenario. On day 7, residues up to 28 µg Clothianidin/kg were detected in bee bread of stored *Phacelia* pollen. The findings of high effects at chosen rates highlight the need to include specific dust drift field trials for seed treatment products with highly toxic insecticides in risk assessment used in crops with potential dust abrasion and emission from seeds. Further work is required to determine appropriate application rates in further semi-field and field testing that reflect field realistic drift exposure levels.

Key words: Clothianidin, *Apis mellifera*, drift, seed treatments, risk assessment, dust application, field test method.

Introduction

While in the scientific community numerous discussions and research are ongoing on potential sublethal and lethal effects on bees due to different routes of exposure, especially neonicotinoids are in focus of current discussions in science and policy (Godfray *et al*., 2014). Neonicotinoid uses include seed treatments on some of the most important crops including maize, cereals and oilseed rape and belong to the most widely used insecticides in crop protection (Elbert *et al*., 2008) as they provide effective control of a broad range of insect pests (Jeschke *et al*., 2011). The systemic nitroguanidine neonicotinoids Clothianidin, Thiamethoxam and Imidacloprid are highly toxic for bees both in oral and contact exposure (Iwasa *et al*., 2004; Laurino *et al*., 2011; EFSA, 2013a; 2013b; 2013c), while they have a comparably low mammalian and bird toxicity (Schmuck and Keppler, 2003).

In the past decade, several severe bee poisoning incidents during sowing maize seed treated with neonicotinoids, caused by emission of dust containing insecticides, with confirmed lethal effects on forager and hive bees occurred in Europe, Canada and the US (Pistorius *et al.*, 2009; Forster, 2009; Bortolotti *et al.*, 2009; ApeNet, 2009; 2010; 2011; Krupke *et al*., 2012; Cutler *et al.*, 2013). While insecticidal seed treatments in maize are generally seen critical, especially for neonicotinoids (Maini *et al.*, 2010), the incidents have highlighted the importance of insecticidal dust drift as a highly important route of exposure to be considered in risk assessment. The release of active substance from seed treatment during sowing and dust drift to off-crop areas has been identified first by Greatti *et al*., 2003, who demonstrated an emission of dusts from sowing machines and deposition in plants in the vicinity of fields sown with maize. Flying through dusts, the uptake of contaminated nectar and pollen and contact with dust during foraging activity have so far been identified as most important exposure routes for bees (Pistorius *et al*., 2009; Marzaro *et al.*, 2011; Girolami *et al.*, 2012; Tapparo *et al*., 2012; Krupke *et al*., 2012; EFSA, 2012). Potentially dust drift may occur during sowing of all seed treated crops or granular applications and may result in exposure for various non target organisms. Investigations on the dustiness of different seed treated crops and assessments of drift during sowing demonstrated that different exposure in off-crop areas may occur after sowing of different seed treated crops with different sowing machinery (Rautmann *et al*., 2009; Heimbach *et al*., 2010; 2014; Bahmer *et al.*, 2014, Devarrewaere *et al.*, 2014). In recent years efforts were undertaken to introduce assessment methods for dust abrasion as well as risk mitigation measures (Forster *et al*., 2012) to minimize pollinator dust exposure. However, up to date not all aspects of the process of dust drift are sufficiently understood (Nuyttens *et al.*, 2013). So far, available data on dust drift exposure and effects on bees were generated by field realistic sowing of a treated crop (Tremolada *et al.*, 2010; Pistorius *et al*., 2010; Girolami *et al*., 2012; Marzaro *et al.*, 2011; Georgiadis *et al.*, 2012a; 2012b; Tapparo *et al*., 2012; Heimbach *et al.*, 2014). Under such field conditions, a number of factors like wind strength and direction cannot be controlled and make field testing of dust drift challenging and almost impossible to reproduce if there is no high number of replications. As

field tests are affected by various environmental parameters (namely wind speed and direction and soil conditions) the results can have only limited value for risk assessment. So far, no methodology was available to apply defined doses of dust in field trials and the determination of defined application rates in exposed area adjacent to the drilling areas *a priori* was not possible (Sgolastra *et al.*, 2012) and yet no specific guidance is available in official regulatory test guidelines.

For risk assessment, laboratory studies and in addition for products with insecticidal action usually also semi-field and field studies are required in the registration process (EPPO, 2010; EC, 2009; EFSA, 2012). Both semi-field and field tests have a number of strengths and weaknesses (EFSA, 2012). Information from both test systems may be required for a full risk assessment; often field tests with honey bees are necessary, as only in field tests full size colonies can be used. Spray testing is usually conducted using the maximum intended application rate, in most cases on flowering, highly bee attractive crops like oilseed rape and *Phacelia* (Schick and Spürgin, 1997; EPPO, 2010; EFSA, 2012).

For risk assessment of exposure via dust, reproducible semi-field and field study tests with applications of defined doses of dust are needed, e.g. to determine NOED or LOED values. Due to solid state and the varying particle size it is challenging to develop standard ways of applying dust in situ and in vitro. In the field it is even more problematic to apply the low dust amounts required in a practical way uniformly to a larger area. In recent years suitable methods have been described to apply defined rates in semi-field trials by Sgolastra *et al.* (2012) and Georgiadis *et al*. (2012c). Both used abraded dust particles with known residue content and diluted with different materials like flour or standard soil of known particle size to achieve the low application rates of insecticides per ha needed.

For the purpose, to test side effects on bees in realistic, but more reproducible field situations than in trials with dust drift during sowing a methodology was developed to apply target amounts of dusts in field conditions. A new machinery was developed that allows the application of defined doses of dust together with a dilution material. In order to determine the effects of a field application of dusts on honey bees (*Apis mellifera* L.), dust from maize seeds treated with the formulated product Poncho® were applied at rates of 0.25 g and 1 g a.s Clothiandin/ha in a collaborative trial of Eurofins and Julius Kühn-Institute. The dust was applied with a purpose-built dust applicator once during bee-flight to flowering *Phacelia tanacetifolia* Bentham in Germany.

Materials and methods

Obtaining dusts and diluting material

A larger amount of dust was obtained from a commercial seed treatment facility by aspiration of loose dusts from seeds during the seed treatment process and packaging of maize, treated with the Poncho® (FS 600 g/l Clothianidin). The obtained dust was separated to different particle size classes <80 µm, 80-160 µm, 160-250 µm, 250-500 µm, >500 µm), using an analytical sieve shaker (Retsch, AS 200). The different fractions were weighed and residue content analyzed separately (table 1). Of the total dust only limited amounts, 1.3% of the fractions <80 µm and 1.9% of the fraction 80-160 µm were obtained. Fractions <80 µm and 80-160 µm were mixed at 2:3 w/w (ratio in the whole dust lot), transported to the field, and carefully mixed with standard soil (LUFA 2.2, batch F 21593, only size fraction below 160 µm in the same proportion as the insecticidal dust). The mixture of soil and insecticidal dusts was transferred into the machinery at the field site.

Application of dusts

For application, a purpose build dust applicator was designed, made up of a commercial fan used in seeding machines, a dust applicator (based on the design of a micro-granulate applicator) and a seed distributor for pneumatic seeding using a 3 m boom with 24 nozzles. The application was performed with a target application rate of 600 g dust mixture/ha. Three treatment groups were set up: two test item treatment groups T1 (0.25 g a.s./ha) and T2 (1 g a.s./ha) and C, an untreated control. The dilution of the carrier (soil of > 160 µm fraction size) and dust mixture was approximately 424:1 in treatment group T1 and 103:1 in treatment group T2. In 2 preliminary semi-field trials no toxic effects nor increased mortality of bees following application of uncontaminated soil dusts (LUFA 2.2) were observed (unpublished data). Time needed for application of test fields with the machinery was 40 minutes in T1 and 48 minutes in T2.

Table 1. Proportions of different dust fractions and residue content.

Particle size (µm)	Proportion of Clothianidin (%)	Weight fraction (%)
$x \leq 80$	16.2	1.33
$80 < x \leq 160$	17.7	1.96
$160 < x \leq 250$	16.9	5.15
$250 < x \leq 355$	14.6	8.55
$355 < x \leq 450$	13.5	8.68
$450 < x \leq 500$	13.4	4.76
$x > 500$	12.4	69.57

Validation of the machinery

The validation of machinery (dust applicator) was done with dust traps, combining photo dishes filled with glycerol/water (50% v/v) and glycerol/water drenched gauze (100 Denier mosquito net, polyester multifilament fiber, 40 g/square meter, mesh 156, diamond shape hole, untreated, size 2 × 2 m) in a separate procedure outside of this project. For validation of the dust applicator seed dust of maize generated through abrasion was mixed with LUFA standard soil (50/50 2.3/2.4 sieved to ≤ 200 µm) as a dust carrier. The evenness of the distribution of dust particles on target areas and the recovery (mass balance) of the applied test item was evaluated by analytical verification of both photo dishes and gauze. Dust traps were set up on a table approximately 90 cm above the ground, about 20-30 cm below the boom. Before application, drenched gauze was fixed on a metal frame of 2 × 2m, approximately 10 cm above the photo dishes. Application was performed with the same target application rate of 600 g dust mixture/ha in three separate runs. For each run, 5 gauze samples and 10 photo dish samples were collected 30 minutes after application. The content of the 10 photo dishes (each 26.3 × 18.3 cm) was filled into storage containers and dishes rinsed twice with 80 ml ultrapure water. 5 Gauze pieces (each 30.5 × 49.2 cm) were cut and transferred to storage containers. Both gauze and photo dish samples were analyzed for residues of the a.s. and residues of a.s. calculated per ha. The average recovery of the applied a.s. in the three runs used was 85%. The highest value (88%) was analyzed for run 1, in run 2 a mean of 82% was found and in run 3 a mean of 85%.

Field test

The field study was conducted in Southern Germany near Niefern-Öschelbronn between 1st of August (8DBA, days before application) and 6th of September 2012 (28DAA, days after application), following EPPO 1/170 (4) guidelines (EPPO, 2010). Field sizes of the control field were 0.6374 ha, test item fields T1 and T2 each 0.4167 ha, all with a slope of 0%. Sowing rate of *P. tanacetifolia* was 12 kg/ha. The distance of treated fields T1 and T2 was 4.2 km, distance of the control to T1 7.8 km and 6.0 km to field T2. In total, 12 commercial bee colonies (4 colonies in the control, each 4 in treatments T1 and T2) of comparable size with at least 5 combs containing brood of all stages were set up eight days before the day of application at BBCH 65 on 9th of August. On each field site, 4 colonies per treatment were placed at a distance of about 3 m at the edge of the flowering *P. tanacetifolia* test fields. Mortality, foraging activity and behaviour of the bees were assessed over four days before and over seven days after the application. The condition of the colonies and the brood development of the colonies were checked once before and four times after application until 28DAA.

Flight activity and behaviour

During the crop exposure period the flight intensity and behaviour of honey bees were assessed daily from 4DBA to 7DAA. The number of bees either foraging on flowers or flying over the crop were counted for about 15 seconds in each of the 5 observation areas of 1 m^2 in the control and treated fields T1 and T2. On the day of application and 1DAA, additional activity assessments were made in the control due to the time needed for the application and relocation of the machinery. As the application in T1 started about 2 hours earlier than in T2. Therefore at time intervals corresponding to treatments in T1 and T2, data are presented as C for T1 and C for T2. During the assessments of flight intensity also the behaviour of the honey bees in the crop and around the hive was observed with special attention to aggressiveness towards the observer, guard honey bees attacking and/or preventing returning honey bees from entering the hive, intensive flying activity in front of the hives without entering the hive, intoxication symptoms (e.g. cramping, locomotion problems) and clustering of large numbers of honey bees at the hive entrance. Any other observations regarding unusually behaviour of the honey bees were also recorded.

Mortality

Mortality of the honey bees was recorded by counting the number of dead honey bees in the dead bee traps (type "Gary" traps, 435 × 400 × 300 mm) in front of the colonies and on linen sheets. Before start of the exposure phase, three linen sheets (0.5 × 10 m each; totally covered area: 15 m^2) were spread out on three impartially selected places in the field. One linen sheet (approximately 5 × 1 m) was spread out in front of the honey bee hives. Mortality was assessed once daily from 4DBA to 7DAA; on the day of application, mortality was assessed once before and 1, 4 and 6 hours after application. Dead bees were differentiated between adult worker bees, males, freshly emerged bees, pupae and larvae during each assessment. Dead male bees and male brood were also recorded but were excluded from evaluation of mortality, as it was assumed that bees flying outside hives and food processing worker bees are most exposed.

Colony development and brood development

The condition of the colonies and the development of the bee brood were checked once on 7DBA and four times afterwards: once at last day honey bee exposure at the *P. tanacetifolia* fields on 7DAA, and 14, 21 and 28 days after application at a remote monitoring site where all colonies were moved to on 7DAA. Colony strength (number of bees), comb area containing cells with eggs, larvae and capped cells, pollen storage area and area with nectar or honey were assessed with the Liebefelder method for each frame and comb side (Imdorf *et al.*, 1987). The total number of honey bees and the area containing brood stages, pollen and nectar per hive were calculated. Afterwards the mean values were calculated for each assessment date. The calculation of the area containing brood or food stages was based on a comb size of 800 cm^2 (per comb side) and assuming 400 cells per 100 cm^2. For the calculation of colony strength 125 honey bees per 100 cm^2 were assumed as full coverage (Imdorf *et al.*, 2008). At each assessment, colonies were visually inspected for bee diseases. Accordingly, any unusual occurrences (e.g. presence of dead bees, dark "bald" bees, "crawlers" or flightless bees, unusual brood patterns or

brood age structure) and clear symptoms of disease (e.g. chalk brood, sacbrood, *Nosema* spp., American or European foulbrood) or pests (e.g. *Varroa* sp., *Aethina tumida* Murray, *Tropilaelaps* spp.) were recorded.

Residues in bees and bee bread

Samples of dead bees were taken daily from bee traps after each mortality assessment. Dead bees from the 4 colonies were combined in order to have one pooled sample for each treatment group: C, T1 and T2. For each treatment group, one sample was collected before application on 0DBA and 10 samples after application, one sample each 1, 2 and 4 hours after the application and one per day from 1DBA to 7DAA. Bees were immediately frozen after collection using dry ice. For pollen analysis, sampling of *Phacelia* pollen, stored as bee bread in combs was done once 7 days after application by cutting 5 × 5 cm comb pieces with only purple pollen in cells. Two samples, A and B of each colony were sampled and analyzed separately. In total 24 samples, 8 of each C, T1 and T2 were obtained. All samples were immediately stored on the field in a box with ice and transported after the daily assessments to the lab; samples were immediately stored at −20 °C until bee bread was separated from wax and residues in bee bread were analyzed.

Residue analysis

A representative portion of each matrix (20 bees, approximately 2 g bees, or 2 g to 3 g bee bread) was spiked with surrogate Acetamiprid D3 D3 (N-methyl D3) in a extraction tube and left for half an hour to equilibrate. After extraction with acetone/water 2:1 (v:v), using an Ultra-Turrax® for 3 minutes, an aliquot of extract was transferred to a Chem Elut® cartridge and reextracted with dichloromethane for the cleanup step. Isotopically labelled internal standard (IS) Clothianidin D3 (N´-methyl D3) was added after evaporation of the extract to dryness, and the extract was brought to a final volume. Clothianidin and its metabolites were analyzed by liquid chromatography-mass spectrometer-system (LC-MS/MS: Triple quadrupole mass spectrometer API 4000 QTRAP (Applied Biosystems MDS Sciex) coupled to a Shimadzu HPLC-system). Matrix-matched calibration with IS was necessary for quantification. The method was validated by recovery experiments with bees and bee bread material. The limit of quantification (LOQ) + limit of detection (LOD) were 2.0 µg/kg + 1.0 µg/kg for Clothianidin in bees and 1.0 µg/kg + 0.5 µg/kg in bee bread matrix. The mean recoveries in bees at the LOQ level were 93%, with a relative standard deviation of 15.8%, and in bee bread 108%, with a relative standard deviation of 4.7%. The LOQ for the metabolites thiazolylmethylurea (TZMU) and thiazolylnitroguanidine (TZNG) was 10 µg/kg, with LOD´s of 1.0 µg/kg and 5.0 µg/kg and recovery values from 84% to 125%.

Statistical analyses

Statistical analyses were performed with SAS. Analyses were performed following recommendations of OECD Guideline 54 (OECD, 2006) for statistical analyses of ecotoxicity tests. Analyses comprised Shapiro-Wilks test in order to verify the normal distribution of the data. For single comparisons folded F-test was used to verify homoscedasticity followed by student t-test. For multiple comparisons in cases of well proven normality (p-value >0.2 in Shapiro-Wilks test) Bartlett test was used for analysis of homogeneity of variances. Else homoscedasticity was proven by means of Levene's test. Multiple comparisons were analyzed using Dunnett's t-test. Data for mortality and foraging activity were log10(x+0.5) transformed. Effect of treatment on colony strength and brood were analyzed by comparing the changes in number of adult bees and total number brood cells calculated by subtraction of the values assessed for each colony before application from the values assessed at each assessment after application. These changes in numbers of bees and total number of brood cells were used for statistical analysis in the way described above. All differences in numbers of adult bees and total sum of brood cells were normally distributed and homogenous regarding the variances of their distribution. All data were analyzed using Dunnett's t-test. For all statistical tests a significance level of a = 0.05 was used.

Results

Weather conditions

Weather conditions were normal during the trial and favourable for foraging activity, 1 to 4 mm precipitation was recorded from 4DBA to 2DBA and 5 to 11 mm on 7DAA. Environmental conditions did not have any adverse effect on the outcome of the trial.

Application

The application was performed with a target dust-soil mixture and an application rate of 600 g/ha during bee flight. The remaining dust in the machine was emptied and weighed; deviation of the applied dust/soil mixture rate was +8.7% (T1) and 0.0% (T2) of the target rate resulting in actually applied amount of 0.27 g a.s./ha in T1 and 1.0 g a.s./ha in T2.

Foraging activity

The mean foraging activity (figure 1) during the pre-application period, 4DBA to 0DBA on the treated area was high and not significantly different between C and T1 (C: 15.3 bees; T1: 14.0 bees; p = 0.083) and C and T2 (C: 16.6 bees; T2: 15.4 bees; p = 0.313). Notably on 3DBA no or only few bees were observed in all treatment groups, due to unfavourable weather conditions.

Mean foraging activity in the post-application period from 0DAA to 7DAA increased in C but significantly decreased (C: 25.9 bees, T1: 12.1 bees; p < 0.001) after the treatment in T1. No significant differences were detected between C and treatment T2 (C: 25.6 bees; T2: 22.4 bees, p = 0.115). Comparing pre- and post data, significant differences for foraging activity were observed in the C for T1 (p < 0.001) and in C for T2 (p = 0.001) and in T2 (p = 0.001). In T1 (p = 0.094) difference between pre- and post data did not prove

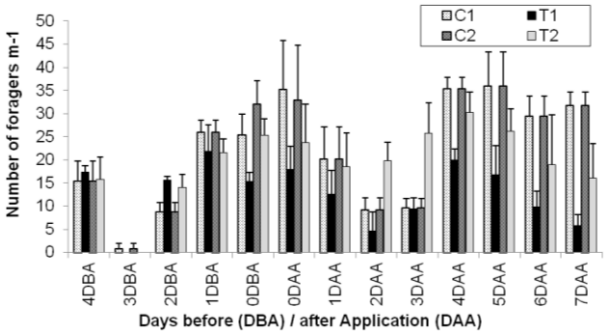

Figure 1. Mean number of bees foraging per m² in flowering *Phacelia* before and after application. Means and standard deviations (bars) were calculated with non-rounded values.

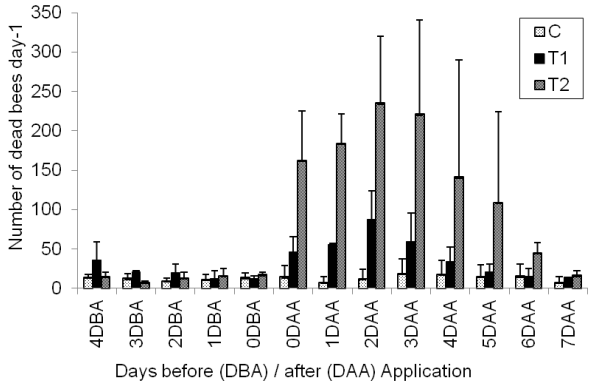

Figure 2. Mean number of dead worker bees per day before and after application. Means and standard deviations (bars) were calculated with non-rounded values; to calculate total mortality per hive, the total number of dead bees on the linen sheet in front of 4 hives was divided by 4 and added to dead bees in the traps of each of the 4 colonies.

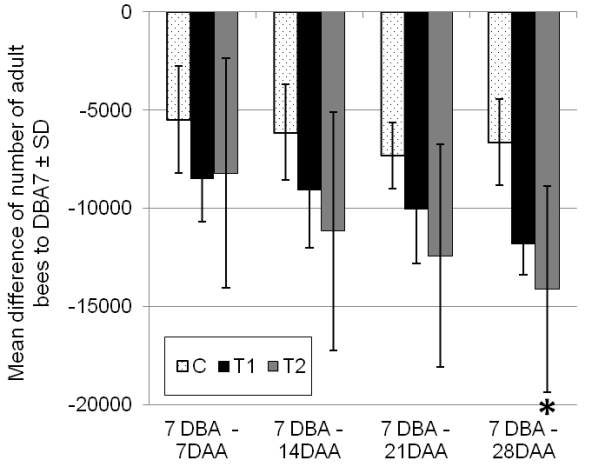

Figure 3. Cumulative loss of bee numbers and SD between colony assessments of hives in control (n = 4), test item treatment T1 (n = 4) and T2 (n = 4) at first assessment at start of experiment, 7 days before application (DBA) and the 2nd, 3rd, 4th and 5th assessment, 7, 14, 21 and 28 days after application (DAA). *Statistically significant differences between first assessment on 7DBA and 2nd-5th assessment ($p < 0.05$).

statistically significant differences. During assessments of foraging behaviour no unusual foraging pattern or behavioural effects were observed.

Mortality

In the pre-application period (4DBA to 0DBA) mean honey bee mortality per day and colony (figure 2) was not significantly different between C and T1 and between C and T2 (C: 11.4 bees, T1: 18.9 bees; T2: 11.8 bees; p (T1) = 0.224; p (T2) = 0.999).

Foraging bees in treatment groups T1 and T2 were exposed to aerial contamination during application and to dust particles deposited on foraging bees, on flowers and leaves and potentially on nectaries and anthers. Application of the test item rates resulted in clearly adverse effects in both treatment groups. Compared to control, a significant increase of mortality was detected in the post application period for T1 (C: 13.5 bees; T1: 39.9 bees; $p = 0.007$) and highly significant increase for T2 (T2: 131.7 bees; $p < 0.001$). For mean mortality during post- compared to pre- application period (0DAA to 7DAA) within a treatment group significant difference were found for T1 ($p = 0.0293$) and highly significant differences in T2 ($p = 0.001$). No statistically significant differences were found for C ($p = 0.518$). The cumulated mortality before application was 60.8 in the C, 102.6 in T1 and 69.1 dead bees in T2 from 4DBA to 00BA; in the post application period from 0DAA to 7DAA cumulated mean per hive was 108.5 bees in C, 333.9 in T1 and 1113.7 in T2.

Effects were observed in all colonies of both T1 and T2. Individual colonies showed maximum mortality between 2DAA to 4DAA, while maximum mean mortality was observed in both T1 and T2 on 2DAA. Mortality decreased to control level at 5DAA in T1 and at 7DAA in T2. During the post-application period most observed behavioural abnormalities in dead bee traps and linen sheets in front of hives were 95% and 98% cramping and bees with locomotive problems, of in total 83 bees in T1 and 242 in T2. 87% and 95% of these effects were observed from 0DAA to 2DAA. In the C two male bees with locomotive problems and one hive showing aggressive behaviour were observed in the post application period. No larval mortality was observed in the bee traps. On linen sheets in the field only low numbers of bees were detected, with mean pre-application daily mortality from 4DBA to 0DBA of 0.4 in C, 4.8 in T1 and 1.0 in T2 and means from 0DAA to 7DAA of 1.5 in C, 1.5 in T1 and 1.9 in T2 after application.

Colony strength

The estimated mean colony strength (estimated number of adult bees inside the hive) (figure 3) before set-up on the 2nd of August on 7DBA was 19001, 22766 and 23766 honey bees in the control, the test item treatment T1 and T2, respectively. The mean strength of the colonies decreased by 5485 bees in C, 8531 in T1 and 8219 in T2, from the 1st colony assessment on 7DBA to the 2nd colony assessment on 7DAA (13516 bees in C; T1: 14235; T2: 15547). Thus, T1 colonies lost 3046, T2 colonies 2734 more bees compared to colonies of the C. On the third assessment a decrease of bee numbers by

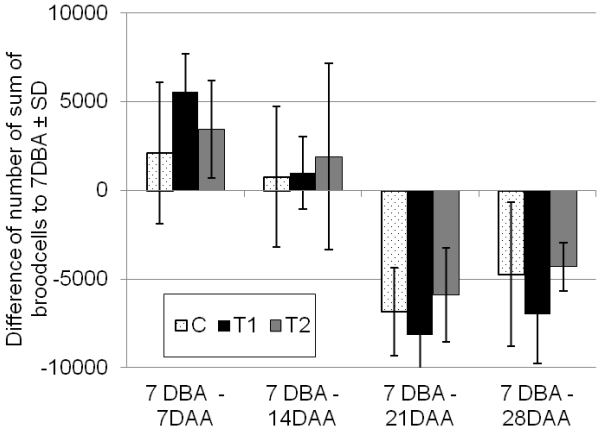

Figure 4. Mean difference and SD of sum of brood cells (eggs, larvae and capped brood) in hives of control, test item treatment T1 and T2 at first assessment at start of experiment, 7DBA and the 2nd, 3rd, 4th and 5th assessment, 7, 14, 21 and 28 days after application (DAA). No statistical differences ($p > 0.05$) were observed between treatments.

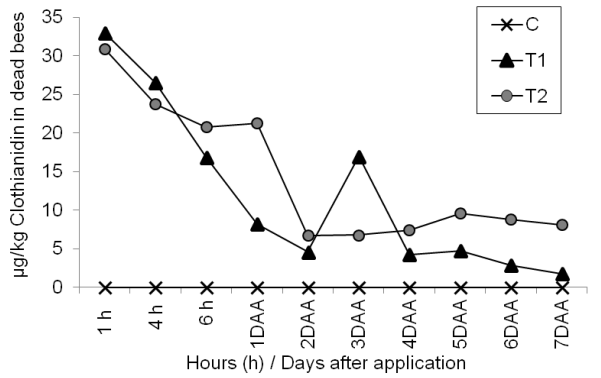

Figure 5. Residues in dead bees (n = 30) from traps in control, test item treatment T1 and T2 after application.

656 in C, 578 in T1 and 2953 in T2 was observed. In the test item treatment groups T1 and T2 the colony strength decreased from the 2nd to the 5th colony assessment on 28DAA (C: 12360; T1: 10938; T2: 9641). Compared to number of adult bees in the hive on 7DAA, no significant differences were found between treatment groups and control for the 2nd, 3rd, 4th assessment dates; on the 5th assessment date, significant differences were found in T2 (left-sided; $p = 0.0219$) but no significant differences for T1. However, the total reduction 7DBA to 28DAA was 6641 bees in C, 11828 in T1 and 14125 in T2; 5187 more bees in were lost T1, 7484 more in T2 compared to controls indicating a greater loss of bees in treatment groups T1 and T2.

The brood in the C and the test item treatment group (figure 4) showed nearly the same development in the entire observation period for eggs, larvae and pupae. From the 1st (C: 19250, T1: 18500, T2: 16850), 2nd (C: 21350, T1: 24000, T2: 20300), to the 3rd colony assessment (C: 20000; T1: 19700; T2: 18750) the mean area of combs containing brood (eggs, larvae and pupae) remained at nearly the same level in the C, T1 and T2, while on the 2nd assessment all groups had slightly more brood cells (C: 21350; T1: 24000; T2: 20300). During the 4th colony assessment a decrease in the amount of cells containing eggs, larvae and pupae was observed in all treatment groups (C: 12400; T1: 10550; T2: 10950), no larvae were recorded in one colony of C and T2. At the last assessment on 28DAA number of cells were increased in all treatment groups (C: 14500; T1: 11700; T2: 12250). Compared to pre-application brood strength on 7DAA, no significant differences were found between treatment groups and control for the 2nd, 3rd, 4th and 5th assessment. The amount of pollen cells stored in combs increased in all treatment groups from 7DBA (C: 3750; T1: 5250; T2: 4550) during exposure phase at the field site until next assessment on 7DAA (C: 6550; T1: 7200; T2: 8200). A high portion of homing pollen with purple colour was observed during but not quantified here. From 14DAA (C: 7550; T1: 7450; T2: 6900) onwards a constant decrease of pollen stores at all colonies was observed at following assessments on 21DAA (C: 6100; T1: 5150; T2: 5600) and on 28DAA (C: 4950; T1: 3500; T2: 3850) with lower stores in both T1 and T2.

During the trial, no evidence of disease related symptoms (e.g. chalk brood, sacbrood, *Nosema* spp., American or European foulbrood) or pests (e.g. *Varroa* spp., *A. tumida*, *Tropilaelaps* spp.) was detected in the colonies, general colony health was considered good. No abnormalities of larval development and unusual brood patterns as well as no dead pupae were observed during the colony assessments.

Residue analyses

No residues were detected in dead bees before application in C, T1 and T2. After application in C, no residues were detected in dead bees in any of the 10 samples from 0DBA to 7DAA but in all bee samples of both treatment groups T1 and T2.

Residues in bees were highest at the day of application up to 24 hours after the application (figure 5). After 1DAA, residues within a treatment group stayed on a similar and not significantly different ($p = 0.49$) level up to 7DAA with only slight variation and decrease, except for 3DAA in T1, where residues were even higher than 1DAA. Interestingly, in spite of the consistently higher mortality per day in T2, higher residues in dead bees were detected on 1, 4, 5, 6 and 7DAA in T2 (figure 5) but not on 3DAA. The residue levels in dead bees on 0DAA were on the same level in both treatment groups, with a maximum of 33.0 and 30.9 µg/kg for T1 and T2, 1 hour after application, which, assuming a weight of 100 mg/bee represents up to 3.3 and 3.1 ng/bee. Median in T1 was 6.5 and 9.2 µg/kg in T2.

No residues were detected in any of the 8 bee bread samples in the control. In T1, maximum residues were 28.0 µg/kg, with 62,5% of T1 samples contaminated and a median of 2.6 µg/kg compared to 87,5% positive samples in T2 with a maximum of 18.4 µg/kg and a median of 7.2 µg/kg (figure 6). Comparing mean values of sample A an B of each treatment group there were no statistically significant differences between residues in bee bread in T1 and T2 (T1: 7.8 µg/kg; T2: 7.7 µg/kg; t-test, two-tailed, $p = 0.986$).

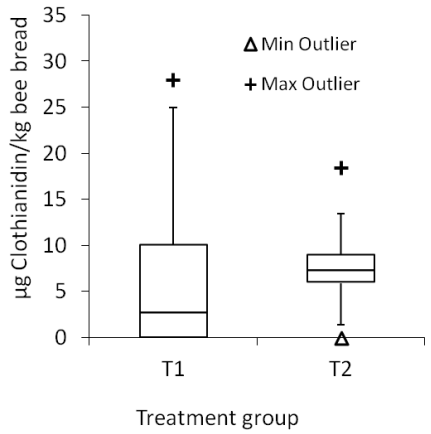

Figure 6. Median, upper and lower quartiles and outliers of residues in beebread from combs, control (n = 4), test item treatment colonies T1 (n = 4) and T2 (n = 4), 7DAA.

Discussion

The aim of the study presented here was to investigate effects of low application rates of dust on honey bee forage plants during bee flight and the feasibility of an experimental procedure of dust application in field conditions. To our knowledge, this is the first published study with an application of defined doses of insecticidal dust in field conditions. Our results suggest that even relatively low amounts of exposure to a.s. in dusts may result in high mortality and clearly unacceptable effects on bees.

Clear high acute effects on bee mortality due to exposure to the test item Clothianidin were demonstrated at both application rates, 0.25 and 1.0 g a.s./ha. A clear and significant dose-related difference in mortality between the two rates compared to the control was observed while control mortality remained on a low pre-application level. However, contamination detected in dead bees did not show a clear relation between the applied dose and the contamination, while median residues in bee bread collected from the hives 7 days after application were approximately 2.8 fold higher in the higher dosed treatment group.

The control site was separated by at least 6 km to each of the test item treatment groups. This distance made it highly unlikely that bees would forage on test item fields (Winston, 1987; Couvillon *et al.*, 2015). To our knowledge, no other apiaries were present in the vicinity within at least 500 m surrounding, however the presence of foraging bees on the test crop from other colonies than test colonies cannot be fully excluded. Phacelia is also likely to attract bees from larger distances. In any case it is assumed that foragers of test colonies were highly attracted by the crop which was closest available forage. No residues of Clothianidin were detected before and after application in any of the control samples and control mortality was not increased after application of the test items. During and after application flight and foraging activity were high thus a good nectar and pollen flow during and after over dusting ensured a high contact exposure may be assumed. Observed contrary fluctuations in flight activity on the treated fields showing decrease in T1 but increase in T2 indicate there is no repellent effect and reduction was not directly treatment related. Dying bees, showing severe intoxication symptoms like cramping, disorientation, locomotion problems and abdominal spasms were frequently observed in bee traps in front of hives of treatment 1 and 2 during the mortality assessments. Those bees are likely to have been counted as dead bees in traps on assessments next day. Also some transient aggressiveness was observed, which has also been noted in dust drift trials evaluated by EFSA (2013a). During foraging activity, no behavioural impairments could be observed in our study.

As the trial was started rather late in the season, also a natural decrease of adult bee numbers in hive and brood nest size with higher variability between colonies was observed. A natural, reasonable mortality and reduction of the number of bees in-hive, preparing the colony for the winter is a normal phenomenon in our climatic conditions in August and September as colonies prepare for overwintering (Liebig, 2002; Imdorf *et al*., 2008) which may potentially confound interpretability of the data on colony strength here. The highest decrease of bee numbers was observed in colony assessments 7 days before to 7 days after application with 5485 bees lost in control, 8531 in T1 and 8219 in T2. While T2 lost more bees compared to controls on every assessment, higher losses were observed in T1 only from 7DBA to 7DAA and 21 to 28DAA. In the entire observation period from first to last colony assessment, a mean reduction of 6641 bees in control, 11828 in T1 and 14125 in T2 was found. Although no significant decrease was found from the assessment 7 days before to 7 days after treatment, a higher decrease of bee numbers seems apparent for treatment groups T1 and T2 compared to control. However, statistical methods used revealed significant differences of bee numbers only in T2 on 28DAA, indicating a weakening of the colony and in addition to the acute effects also a long term effect. In this study, some existing effects may also not have been detected due of the colony numbers and the variability of colonies used in this trial. This implies that higher colony numbers should be used in such trials increase statistical power and studies should be started earlier in the season to reduce the seasonal influence and variability between colonies. On the other hand, such late applications may be considered useful in some cases to create an exposure of developing bees in autumn and studies that aim at future investigation effects on longevity of winter bees and overwintering.

As a conclusion on effects on adult bees, a highly significant increase of mortality for several days has demonstrated clear unacceptable effects of the application on bee mortality in bee traps, indicating the principal suitability of this tool, in spite of the known weakness of bee traps (Illies *et al*., 2002; EFSA, 2012), as a part of a larger toolbox of different methods to investigate effects on bees. Rueppell *et al*. (2010) demonstrated that ill feeling or otherwise compromised bees actively abandon their social role as foragers and remove them-

selves from their colony. In normal conditions, only a very small fraction of dead bees are found in traps compared to natural daily turnover. However, depending on the level and route of exposure and the substance-specific properties effects of insecticides seem to make it impossible to leave the hive in time and to overcome the bee traps for a portion of the bees. As a personal observation, often bees showing severe sublethal effects were seen tumbling out of the flight entrance but were in consequence unable to leave the traps and may be considered as bees which were confronted with higher doses. As the exposure is unlikely to be uniform for bees, the extent of effects on individual bees will vary, and numbers of sublethal affected foragers may still be able to leave the colonies, and detract themselves from being found in traps. Thus, also extended colony strength and brood assessments are another essential tool to be considered, and preferably with a setup of better statistical power. Our data on colony strength demonstrated or gave indication of higher bee losses after the acute exposure phase but were not as clear as acute mortality data for the exposure scenario and active substance used here. Given the significance of mortality in this trial, it seems also likely that effects at lower rates than in our trial would result in detectable effects on bee mortality.

To assess the potential risks to bees following dust drift, so far drift trials in the field were conducted with realistic sowing of treated seeds and a flowering crop in the downwind direction (Tremolada *et al.*, 2010; Marzaro *et al.,* 2011; Girolami *et al.*, 2012; Georgiadis *et al.*, 2012a; 2012b; Heimbach *et al.*, 2014). Fewer studies have yet been performed in semi-field trials with *a priori* determined doses. In the study of Sgolastra *et al.* (2012), about 10 fold increase in mortality index of treatment compared to control as well as ratio post/pre-treatment was demonstrated at a rate 0.0512 g Clothianidin/ha, while the difference in the absolute mortality between control and treatment was 20 bees on the day after application. Maximum mortality in our trial was approximately 4 fold compared to in T1 and about 10 fold higher in T2 in our study, with mean differences of 75 more dead bees per colony in T1 and 223 more dead bees in T2 compared to the control on 2DAA. The higher absolute mortality in our study compared to the study of Sgolastra *et al*. (2012) is likely to be due to the 5 and 20 fold higher application rates used here. The pattern of increase of mortality in our study from before to after application was comparable to studies with dust manually applied in semi-field studies on much smaller areas. At a rate of 0.25 g Clothianidin/ha Georgiadis *et al.* 2014 also observed increased mortality, but clearly lower absolute mortality compared to our field data. The higher absolute mortality compared to semi-field tests may be explained by the larger size of colonies used in the field trial, which have a higher absolute number of forager bees (Danka *et al*., 1986). This is likely to result in higher numbers exposed to particles during foraging activity. Residue levels in dead bees in our trial were in the range of levels detected in reported incident samples. Bortolotti *et al.* (2009) found Clothianidin in dead honey bees from 4 to 39 µg/kg up to 241 µg/kg and 25-138 µg/kg Thiamethoxam. In Germany, 71 of 77 bee samples from the region with dust drift incidents in 2008 had detectable residues of Clothianidin. In about 4 % of these samples up to 5 µg/kg, in 64 % up to 15 µg/kg and in 25 % more than 15 µg/kg with a maximum of 212 µg/kg were detected (Pistorius *et al.*, 2009). Krupke *et al.* (2012) reported 4 - 13 µg Clothianidin/kg dead bees, Cutler *et al.* (2013) up to 72 µg/kg Clothianidin and 168 µg/kg Thiamethoxam in single samples. In our trial, a clear dose-dependent treatment related mortality of unacceptable and biologically relevant magnitude of acute mortality was demonstrated which experimentally reconfirms the causality conclusions of reported bee incidents.

However, it is acknowledged that further investigations are necessary to establish the link between residues in dead bees and the real dust exposure dose as well as the impact of aerial dust contamination, contact exposure during foraging activity on flowers and contamination via oral collection and consumption of nectar and pollen for methods with application of defined dust amounts in the field.

In our study, a constant rise in mortality of all treatment colonies was observed from 0DAA to 3DAA, but acute mortality returned to similar levels to control and pre–application mortality on 5DAA in T1 and 7DAA in T2. While it has been demonstrated that highest effects are likely to occur within the first days after initial dust exposure (Georgiadis *et al*., 2012a; 2012c), and beekeepers reported from incidents caused by dust drift during sowing of maize a prolonged slightly increased mortality over several weeks after the sowing was completed. This indicated that also contaminated food stores may have caused increased prolonged mortality (Pistorius *et al.*, 2009), and dietary exposure to contaminated pollen is confirmed by data presented here.

Bees may be exposed to dusts from single or combined routes of exposure, by direct contact (e.g. bees flying through the toxic cloud in the sown field), by indirect contact (e.g. bees walking on contaminated leaves of the vegetation surrounding the sown field) or by ingestion (e.g. bees collecting nectar, pollen or dew from the vegetation contaminated with the dispersed dusts) (EFSA, 2012). While in this study highest residues in dead bees were detected within the first 24 hours after application in both treatment groups, residues in dead bees were detected in all dead bee samples until 7DAA. Findings of Marzaro *et al*. (2011) and Girolami *et al*. (2012) demonstrated exposure of bees from aerial contamination during sowing may result in residues in bees up to several mg/kg. While also in our trials highest contamination took place immediately during flying and foraging activity, further exposure persists when aerial contamination is assumed to have ceased. Thus aerial contamination is undoubtfully an important route of exposure and should in future work also be quantified,, but residue findings in bee bread in our study also demonstrate that also lasting dietary oral exposure occurs even after 7DAA, potentially resulting in delayed effects. On 7DAA residues of Clothianidin in bee bread from combs in levels of the same range were found as those in confirmed incidents with colonies poisoned from dust

exposure. Analyses of 117 samples of bee bread samples from colonies damaged during the dust incidents in 2008 (Trenkle, 2008) revealed no residues in 66 % of cases, 6 % had up to 5 µg/kg, 24 % 5-20 µg/kg, 11% 20 -50 µg/kg and 4 % more than 50 µg/kg Clothianidin. Krupke *et al.* (2012) found 11 µg/kg Clothianidin and 20 µg/kg Thiamethoxam in bee bread collected in hives from "sick" appearing colonies. Thus, the residues detected here at 7DAA are in the upper range of detections compared to published incident data. This might be partly also due to the season the experiment was performed since there are clearly much less flowering alternative plants available for the bees to forage on compared to springtime when incident samples were obtained. Assuming a pollen uptake of 65 mg of nurse bees (Rortais *et al*., 2005) during 10 days, this would result in total doses between 1,2 ng to 1,8 ng/bee for oral exposure to Pollen, values below the oral LD_{50}. However, it needs to be considered that the LD50 is not a NOEC and thus at the given rates, increased mortality of exposed to such oral doses bees is likely. Bees are also able to metabolise Clothianidin to some extent (Cresswell *et al*., 2014). Assuming that in general the consumption of total dose in 1 day may also not reflect reality, it is clear the exposure of individual bees may be more variable and different for dust exposure than for e.g. residues in nectar and pollen contaminated from systemically translocated residues, which may be assumed to create more uniform and homogeneous exposure that dusts. As dust particles may contaminate single cells, which may be underestimated by considering the residues found in bee bread which represent a mean value, individual bees could be exposed to higher doses than the mean. Thus the direct comparison of residues in bee bread with LD50 values and dietary consumption has flaws and needs to be interpreted carefully in order not to underestimate the exposure of individual bees as well as effects on bees for dusts.

Application

The dust applicator has proven to be a viable method to apply small quantities of solid material over a larger area, deviation during application was below 10%. One limitation is the amount that can be applied i.e. there is a minimum of material needed to run the machine and ensure even distribution in the field. Dust from natural soils seem to be a good dilution material because similar to dust from the seeds during drilling soil dust is transported by wind to adjacent areas together with seed dust. Dust particles can have a wide range of different irregular shapes and densities (Nuyttens *et al*., 2012; Foqué *et al*., 2014) and size (Heimbach, unpublished data; Pistorius *et al*., 2009). Also the shape of dust particles from treated seeds varies greatly according to the crop (Foqué *et al*., 2014), and will be influenced also by the seed treatment process (e.g. adding of materials which are intended to reduce friction and ensure smooth flow of seeds such as talcum). In addition, different drilling technique might have an influence on the fraction size by different mechanical stress. In our trials, only dust smaller than 160 µm was used as earlier work indicated that smaller fractions may cause greater effects (Georgiadis *et al.*, 2012c), have a higher residue content (Heimbach, unpublished data; Pistorius *et al*., 2009), are more likely to drift and be relevant for honey bees. As the dust fraction of < 160 µm used contains particles in the size of pollen, bees may unwillingly collect dusts on body hairs (Girolami *et al*., 2012) or mistake particles for pollen and collect them deliberately e.g. from leaves or flowers.

Application rate

The application rates of 0.25 and 1 g were chosen considering residue data obtained after field realistic sowing of treated seeds, considering residue deposition data in petri dishes and adjacent flowering crops and 3-D dust samplers. So far, no clear guidance on application rates for such tests reflecting a realistic worst-case exposure scenario is available.

Greatti *et al*. (2003; 2006) revealed deposition of a.s. used for seed treatments in the vicinity of sowing, in grass and flowers which were sampled after sowing was completed in the adjacent field. On average 0.021 and 0.032 mg a.s./kg plant or flower mass were detected, but a direct extrapolation from plant mass to the application rate of the a.s. on seeds is impossible. In ApeNet (2010) residues in petri dishes of up to 0.0512 g a.s./ha were found in a distance of 5 m to the field edge. Tapparo *et al*. (2012) found in studies with maize seeds 2008-2010 an emission of 0.43 to 1.53 g a.s./ha with a fraction of released insecticide of 0.52-1.84%. In trials of the JKI 2008-2012 (Heimbach *et al*., 2014) mean deposition at distances of 1 to 5 m was 0,022 to 0,41 g a.s. after 4 trials with sowing of maize and 0.026 g a.s. to 0.3 g a.s. in petri dishes in 3 trials with sowing of barley. In the trials with sowing of maize, deflectors were used, as the use of drift reducing devices (air deflectors) may result in decreasing emissions (Biocca *et al.*, 2011) and in Germany, only certified machinery with deflectors achieving at least 90% drift reduction are allowed for sowing of insecticide treated maize (Rautmann *et al.*, 2009; Forster *et al*., 2012). The contents of a.s. (g a.s./ha) in adjacent flowering plants of several JKI field experiments were up to about 4.5 times higher at 1 m distance (average of nine experiments 2.42 times higher) compared to values of petri dishes on bare soil in adjacent non crop areas (EFSA, 2012; Heimbach *et al*., 2014). For uses in Europe, application rates of 50 and 125 g a.s./ha have been used for seed treatments with Clothianidin in maize. EFSA (2013d) proposed as worst-case estimation to use deposition rates off-crop of 5.6% of the application rate per hectar for maize sown without deflectors, which would result at rates of 1.12 g to 2.8 g a.s. deposited per hectare. With use of deflector, a rate of 0.56% is proposed, thus the rates of 0.25 g and 1 g.a.s were chosen here to reflect rates in a range which might occur following dust drift during sowing of maize.

Compared to spray drift, dust particles may drift longer distances than spray droplets; the aerial drift of both dust and spray drift largely depend on the particle size (Ganzelmeier, 1986). In 20 m distance mean drift deposition was 70% less compared to 1-5 m distance in nine field experiments with different crops (Heimbach

et al., 2014), and bigger particles sediment more rapidly than smaller ones (Bahmer *et al.*, 2014). Accordingly, in realistic conditions, the residues in petri dishes and flowers as well as exposure of bees decline with the distance to the drilled field. In reality field edges are the highest contaminated areas (ApeNet, 2009; Forster, 2009); if single bees forage there, effects on these foragers may occur whereas at some meters distance the residues may be reduced to less critical levels. In our study, we chose relatively high rates of dust reflecting highest exposure rates at the field edges, and applied these uniformly on the whole area of the flowering crop. Potentially, dust applicator modifications could realize a more field realistic exposure by applying different amounts at different strips of the crops in future trials. Due to potentially flowering small field margins, other flowers and hedges also conservative exposure estimates need to be considered in testing and risk assessment; especially as situations with high amounts of flowers in the field margins may occur in practice naturally or if flower strips are present at the field borders, which is increasingly done to improve the nutritional situation for pollinators and foster biodiversity. However, more data are needed for more clear conclusions on the application rates to be tested in trials reflecting worst-case sowing exposure scenarios.

Defined rates of dusts can be applied with machinery in field trials according to our findings. Yet, further investigations are needed to which extent a comparability of the dust applicator and realistic sowing procedure is given, in order to appropriately reflect exposure and dosing of realistic dust drift scenarios, with further consideration of particle size emissions and of exposure generated for bees via different routes. As the focus of our study were acute effects on bees, further work is needed to assess also specific sublethal effects, which are an important component to consider in risk assessment (Thompson and Maus, 2007); ignoring sublethal effects might result in an underestimation of adverse effects of insecticides as the overall impact of neonicotinoids includes sublethal as well as lethal effects (Desneux *et al*., 2007). Further work is needed to investigate longer-term effects of dusts on colonies and to generate threshold values in semi-field and field conditions, which could not be derived from our study. Semi-field and field data comparing effects of dust and spray exposure seem essential to understand differences of the impact of these exposure routes. Our proposal for a field test design may serve as an additional tool to further improve bee and pollinator testing for risk assessment purposes.

Conclusion

This work demonstrated the possibility and practicability of an application technique to apply target rates of dusts in field studies as a fundamental component for further risk assessment studies and to investigate the effects of dust particles from treated seeds on bees and also other organisms. Already low application rates of dusts containing insecticides may cause strong effects on bees. Hence, further data on potential NOEC/LOEC values for highly bee toxic substances used for seeds treatment and data for modelling potential exposure scenarios are needed for risk assessment. Further work is considered necessary to quantify the exposure and effects of different exposure routes for both applications of defined amounts of dusts in semi-field and field studies as well as field realistic dust drift during sowing of insecticide seed-treated crops. Regardless of the specific issue of neonicotinoids, also for other highly bee toxic insecticidal seed treatments and granule applications strict implementation of risk mitigation measures are needed to avoid the occurrence and further dispersal of dusts during the sowing process to ensure bee and pollinator safe use of seed treatments in agriculture.

Acknowledgements

The helpful input of three anonymous reviewers, whose comments helped greatly to improve the manuscript.

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Received January 22, 2015. Accepted June 17, 2015.