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Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior

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Abstract *Bombus terrestris* bumblebees are important pollinators of wild flowers, and in modern agriculture they are used to guarantee pollination of vegetables and fruits. In the field it is likely that worker bees are exposed to pesticides during foraging. To date, several tests exist to assess lethal and sublethal side-effects of pesticides on bee survival, growth/development and reproduction. Within the context of ecotoxicology and insect physiology, we report the development of a new bioassay to assess the impact of sublethal concentrations on the bumblebee foraging behavior under laboratory conditions. In brief, the experimental setup of this behavior test consists of two artificial nests connected with a tube of about 20 cm and use of queenless micro-colonies of 5 workers. In one nest the worker bees constructed brood, and in the other food (sugar and pollen) was provided. Before exposure, the worker bees were allowed a training to forage for untreated food; afterwards this was replaced by treated food. Using this setup we investigated the effects of sublethal concentrations of the neonicotinoid insecticide imidacloprid, known to negatively affect the foraging behavior of bees. For comparison within the family of neonicotinoid insecticides, we also tested different concentrations of two other neonicotinoids: thiamethoxam and thiacloprid, in the laboratory with the new bioassay. Finally to evaluate the new bioassay, we also tested sublethal concentrations of imidacloprid in the greenhouse with use of queenright colonies of *B. terrestris*, and here worker bees needed to forage/fly for food that was placed at a distance of 3 m from their hives. In general, the experiments showed that concentrations that may be considered safe for bumblebees can have a negative influence on their foraging behavior. Therefore it is recommended that behavior tests should be included in risk assessment tests for highly toxic pesticides because impairment of the foraging behavior can result in a decreased pollination, lower reproduction and finally in colony mortality due to a lack of food.

Keywords Bumblebee · *Bombus terrestris* · Behavior · Foraging · Laboratory test · Sugar water · Survival · Sublethal effects · Imidacloprid · Thiamethoxam · Thiacloprid

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Introduction

In the context of modern and safe crop protection strategies, environmental risk assessments are evident for all plant protection products (PPPs). During the last decades side-effects of pesticides on bees have gained great attention due to their value as pollinators. In Europe pesticides are tested following the EPPO (European and Mediterranean Plant Protection Organization) guidelines to exclude any harm to honeybees *Apis mellifera* (EPPO 2001). To date, most studies on bees mainly consider mortality (Suchail et al. 2000), while less attention is given to

sublethal effects which may be detrimental towards pollination and subsequent bee populations. Thompson et al. (2003) and Desneux et al. (2007) reported that sublethal effects must be considered when evaluating the impact of pesticides on pollinators. To date, multiple laboratory studies have focused on the impact of pesticides on the biochemistry of honeybees (Armengaud et al. 2000). However, interpretation of the negative effects on the neurophysiological capacities of insects is difficult, and in turn the effects for the insect or the whole colony are unknown. For honeybees the proboscis extension response (PER) assay has been used to determine the impact of pesticides on the learning ability (Lambin et al. 2001; Decourtye et al. 2004a; El Hassani et al. 2008). Further, Decourtye et al. (2004b) showed the reliability of this method to detect disturbances at the level of the learning behavior on free-flying foraging bees under more natural conditions. For honeybees such abilities are essential because they rely on their visual learning capacity to communicate the distance and the direction to the food source. Pesticide exposure to honeybees while foraging could affect their orientation behavior (Vandame et al. 1995; Bortolotti et al. 2003; Thompson 2003; Yang et al. 2008). In France, it was believed that honeybee mortalities in areas of sunflower crops were linked to the use of pesticides (Bonmatin et al. 2003). This assumption was not supported by Schmuck et al. (2001), who found that the residues in pollen and nectar of sunflowers were too low to be detrimental. But in the field, bees can be chronically exposed to pesticide residues via contaminated stored food (Schmuck et al. 2001; Bonmatin et al. 2003; Miranda et al. 2003; Chauzat et al. 2006). Numerous studies have reported that ingestion of small quantities can lead to sublethal effects in honeybees (Colin et al. 2001; Decourtye et al. 2003); in laboratory and semi-field tests it was reported that low concentrations of imidacloprid taken up via the pollen were responsible for changes in foraging and food collecting and for the loss of ability to communicate and learn (Colin et al. 2001; Decourtye et al. 2003). Similar sublethal effects on the learning ability were found after exposure to fipronil (Desneux et al. 2007), on the communication dance by carbamate insecticides (Thompson et al. 2003) and on the homing flight by the pyrethroid insecticide deltamethrin (Vandame et al. 1995; Ramirez-Romero et al. 2005). Until now, most studies have been performed on honeybees, while other bees like bumblebees may also be affected by pesticide poisoning.

Bumblebees, such as *Bombus terrestris*, are of crucial importance for the pollination of wild flowers and economical important crops in modern agri/horticulture. Especially foraging worker bees are playing a key role as they are responsible for the amount of food brought to the colony. Therefore hazards of sublethal concentrations of pesticides on them are important. Moreover, as honeybees and bumblebees are very distinct in their behavior, the bioassays to assess sublethal effects of pesticides on honeybee behavior are not suitable for bumblebees. This lack of assessment, therefore, poses a serious problem and standardized laboratory tests for bumblebees are necessary to evaluate the impact of sublethal effects on their foraging behavior. In the past, several tests have been developed to evaluate the impact of insecticides on bumblebees (Van der Steen 2001; Tasei 2002). In this study we aimed to develop a bioassay evaluating the impact of sublethal pesticide concentrations on the foraging behavior of the pollinator *B. terrestris*. In essence, the experimental setup consists of two artificial nests connected with a tube of about 20 cm and use of queenless micro-colonies of 5 workers. As a model insecticide we employed the neonicotinoid imidacloprid that is known from literature to cause behavior changes on honeybees (Decourtye et al. 2003, 2004a, 2004b, 2005; Guez et al. 2003; Ramirez-Romero et al. 2005). For comparison within the family of neonicotinoid insecticides, we also tested different concentrations of two other neonicotinoids: thiamethoxam and thiacloprid, in the laboratory with the newly developed bioassay. Finally, to evaluate our newly developed bioassay under more field related conditions, we used queenright colonies of *B. terrestris* in the greenhouse. Here the foraging workers were exposed to sublethal imidacloprid concentrations and needed to fly/forage for food that was placed at 3 m from their hive.

Materials and methods

Products

The three different neonicotinoid insecticides that we tested in this study together with their respective type of formulation and MFRC (Maximum Field Recommended Concentration) and the producing company name are listed in Table 1. The products were stored in accordance with the manufacturers' guidelines. Unless otherwise stated, all other products were of analytical quality.

Insects

All experiments were performed with worker bumblebees obtained from a continuous mass rearing program (Biobest NV, Westerlo, Belgium) and conducted under standardized laboratory conditions of 28–30°C, 60–65% RH (Relative Humidity) and continuous darkness. The insects were provided ad libitum with commercial sugar water and pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain) as energy and protein source, respectively, as described by Mommaerts et al. (2006).

Table 1 List of the three different neonicotinoids tested, their commercial name, formulation type and percentage of active ingredient (AI), producing company, and their maximum field recommended concentration (MFRC) in % of formulation and corresponding amounts in ppm

Neonicotinoid	Commercial name	Formulation and % AI	Company	MFRC (%)	MFRC (ppm)
Imidacloprid	Confidor®	20% SC[a]	Bayer CropScience	0.1	200
Thiamethoxam	Actara®	25% WG[a]	Novartis	0.04	100
Thiacloprid	Calypso®	48% SC[a]	Bayer CropScience	0.025	120

[a] *SC* suspension concentrate, *WG* wettable granules

Insect bioassay with microhive colonies and worker bumblebees

Chronic toxicity assay not including foraging behavior

Newly emerged workers were collected from the bumblebee colony and five workers were placed in an artificial plastic nest box (15 cm × 15 cm × 10 cm). The experimental setup consisted of one artificial nest (box A) (Mommaerts et al. 2006). In brief, in each nest box a worker became dominant, developed her ovaries and laid eggs within a week, thus playing the role of a queen. The four other workers helped the false queen for brood care, which mainly consisted in feeding larvae, building and heating cells. As the false queen was not inseminated, its brood always resulted in a haploid male progeny.

Under these conditions, the adult workers were exposed orally to imidacloprid at 200 (MFRC), 20, 2 and 0.2 ppm and 20 and 10 ppb via treated sugar water in box A. In the control nests, workers were exposed to plain sugar water; here no worker mortality was observed after 11 weeks. Four artificial nests, each containing five worker bees, were exposed for each treatment, and each experiment was repeated twice. In the artificial nest boxes, worker survival was evaluated daily for the first 3 days post treatment and then on a weekly basis for a period of 11 weeks. The treatments were scored in accordance with the classification of IOBC (International Organization for Biological Control of Noxious Animals and Plants): 1 = less than 25% effect, non-toxic; 2 = 25–50% effect, weakly toxic; 3 = 50–75% effect, moderately toxic; and 4 = more than 75% effect, highly toxic (Mommaerts et al. 2006). Subsequently, the adverse sublethal effects on reproduction were monitored on a weekly basis for 11 weeks by scoring the numbers of drones produced per nest.

In an additional set of experiments two other neonicotinoids were tested: thiomethoxam at 100 (MFRC), 10, 1, 0.5, 0.2, 0.1 ppm and 10 ppb, and thiacloprid at 120 (MFRC), 60, 12, 1.2, 0.12 ppm and 12 ppb. Worker bumblebees were exposed to each neonicotinoid insecticide via the drinking of treated sugar water. Four artificial nests each with 5 bumblebee workers per treatment were evaluated for survival and nest development and reproduction.

Chronic toxicity assay including foraging behavior

As above, newly emerged workers were collected from the bumblebee colony and five workers were placed in an artificial plastic nest box (15 cm × 15 cm × 10 cm). The experimental setup of the foraging behavior test consisted of two artificial nest boxes (A and B) connected with a tube of about 20 cm in length and 2 cm in diameter. In one box (A) the workers constructed their nest. Then, after 2 weeks, when third and fourth-instar larvae appeared in the nests, food was removed from box A and placed in box B.

Before exposure to insecticide (imidacloprid), the workers were allowed a training period of 2 days to forage in box B for untreated food, i.e. untreated sugar water and pollen. To attract the bees, box B was placed under light, while box A was not in the direct light. Then, after 2 days when the bumblebee workers had found their way to box B, plain sugar water in box B was replaced by sugar water treated with imidacloprid; imidacloprid was dosed at 200 (MFRC), 20, 2 and 0.2 ppm, and 20 and 10 ppb. Per treatment, four replicates were performed each consisting of five worker bees, and each experiment was repeated twice. Worker survival and drone production were observed on a weekly basis, in a manner similar as described above, over a period of 11 weeks. Simultaneously, the overall behavior of the worker bumblebees was followed during the entire test period.

In addition, the amounts of sugar water consumed per worker were determined on a daily basis to assess the dose of imidacloprid consumed per day and per worker bee. This was followed by measuring the net loss of weight of sugar water due to worker consumption on a weekly basis over the whole experiment. The impact of evaporation was also subtracted from the weight loss; by assessing the weight of sugar containers without bumblebee artificial nest boxes that were kept in parallel with the insect bioassay under the same conditions.

As above, we tested in a separate series of experiments the effects of two other neonicotinoids for potential effects on foraging behavior: thiamethoxam at 0.1 ppm (1/1000 MFRC) and thiacloprid at 12 ppm (1/10 MFRC). Worker bumblebees were exposed to each of the two neonicotinoids via the drinking of treated sugar water as described

above with four nest boxes each consisting of 5 bumblebee workers at each dose.

Chronic test in the greenhouse, including foraging behavior

The aim of this experiment was to assess whether colony performance was affected by exposure to sublethal concentrations of imidacloprid as determined in the previous laboratory assays, when bees needed to forage for food and this in a greenhouse condition. We used here queenright hives of *B. terrestris* from the mass rearing program of Biobest, containing one queen, 25 workers and brood. The workers in each hive present at the start of the experiment were individually labeled with opalith plates under red light. The experiment was conducted in a greenhouse, and a separated area of 3 × 7 × 2 m with gauze was prepared for each treatment. We tested in parallel imidacloprid at three concentrations (20, 10 and 2 ppb) in the sugar water and compared with a blank control with untreated sugar water; the sugar water was provided in containers of 2 l and placed at a distance of 3 m from the hives. Next to sugar water as carbohydrate source, we provided pollen (same as with the rearing program) as protein source but these were not treated and also placed at 3 m from the hives. The pollen were refreshed every 2 days to avoid unattractive reactions of the worker bees towards pollen. For each treatment three hives were used, and the experiment was two times repeated. In the hives, worker survival was evaluated at the beginning of the experiment and then on a weekly basis for a period of 2 weeks. The treatments were scored in accordance with the classification of IOBC for field and semi-field testing: N = harmless or slightly harmful, 0–50%; M = moderately harmful, 51–75%; and T = harmful, > 75%.

Next to mortality, we also evaluated sublethal effects against colony and brood growth. Hereto the net increase in fresh weight of the hives, the net consumption of sugar water, and also the numbers of filled sugar water cups and dead larvae were scored at weekly intervals. In addition, we followed the foraging activity and this at 3 h after opening of the nests by visual counting bumblebee workers entering/leaving the hive during 30 min. The latter countings were always started at 4:00 pm. At the end of the experiment, all the hives were killed by freezing at −20°C and the numbers of filled sugar water cups, newborn workers and mean total amount of brood (sum of egg masses, larvae and pupae present in the hive) were used as endpoints of colony performance.

Statistical analysis

Unless otherwise stated, data were analyzed by one-way analysis of variance (ANOVA). Means ± SEM were separated using a *post-hoc* Tukey–Kramer test ($p = 0.05$) in SPSS v15.0 (SPSS Inc., Chicago, IL). Medium response concentrations (LC_{50}s and corresponding 95% confidence interval (CI)) were calculated using GraphPad Prism v4 (GraphPad Software, San Diego, CA); the goodness that the data fit to the curve model was evaluated based on R^2 values, and LC_{50} values are significantly different when their respective 95% CIs are not overlapping. The data from the chronic test in the greenhouse were analysed with an independent sample t-test ($p = 0.05$) in SPSS v15.0.

Results

Chronic toxicity assay not including foraging behavior

For imidacloprid at 200, 20, 2 and 0.2 ppm, 100% mortality (IOBC class 4 of highly toxic) was observed in the nests and this was after a few hours, 14, 28 and 49 days, respectively. In contrast, at 20 and 10 ppb worker mortality was much lower with 15% and 0%, respectively. Probit analyses of the data resulted in a LC_{50} value for imidacloprid of 59 ppb (95% CI: 52–68 ppb; $R^2 = 0.99$) which corresponds to 1/3390 of the MFRC (Table 2).

Sublethal effects were evaluated and in the nests exposed to concentrations of imidacloprid up to 0.2 ppm the production of drones was significantly ($p < 0.05$) lower (ANOVA: $F = 171.9$, $df = 39$, $p < 0.001$). In these nests, zero to only a few drones were observed due to the high worker mortality after 11 weeks. In contrast, imidacloprid

Table 2 Effects of imidacloprid on worker survival and nest production after oral exposure in treated sugar water via a chronic toxicity assay without and including foraging

	Lethal effect		Sublethal effect	
	LC_{50} (95% CI); ratio of MFRC	NOEC for survival (ppb)	EC_{50} (95% CI); ratio of MFRC	NOEC for reproduction (ppb)
Without foraging	59 ppb (52–68); 1/3390	10	37 ppb (26–51); 1/5410	20
With foraging	20 ppb (19–21); 1/9850	10	3.7 ppb (2.5–5.5); 1/54100	<2.5

Data are expressed as median lethal (LC_{50}) and sublethal (EC_{50}) concentrations with corresponding 95% confidence interval (95% CI), the ratio of MFRC, and the NOEC for survival and reproduction

at 20 and 10 ppb did not pose sublethal effects on the nest reproduction as the respective numbers of drones were not significantly ($p > 0.05$) lower, 27.5 ± 2.5 and 27.8 ± 4.9, as compared to 36.8 ± 6.9 in the control nests ($F = 5.0$, $df = 23$, $p = 0.05$). Based on these results the EC_{50} value was calculated to be 37 ppb (95% CI: 26–51 ppb; $R^2 = 0.99$), which corresponds to 1/5410 of the MFRC. For imidacloprid, the NOEC was 20 ppb (=1/10000 MFRC) (Table 2).

When testing the two other neonicotinoid insecticides, thiamethoxam was the most toxic and thiacloprid the least toxic. With 0.5 and 1 ppm thiamethoxam there was zero worker survival after 1 and 3 weeks of exposure, respectively, ranking this neonicotinoid in the IOBC class 4. With lower concentrations, the mortality declined: 0.2, 0.1 ppm and 10 ppb killed 83, 25 and 6% of the workers, respectively. After probit analysis the LC_{50} value for thiamethoxam was 0.12 ppm (95% CI: 0.04–0.38 ppm; $R^2 = 0.89$), corresponding to 1/833 of the MFRC. In contrast to thiamethoxam, the LC_{50} value for thiacloprid was significantly higher with 18 ppm (95% CI: 3.8–85 ppm; $R^2 = 0.89$); this corresponds to 1/7 of the MFRC and 100% toxicity was only seen in those nests that were exposed to 120 ppm thiacloprid for 11 weeks. Exposure to 60, 12, 1.2 and 0.12 ppm and 12 ppb thiacloprid resulted in a worker mortality of 78, 41, 39, 17 and 0%, respectively.

There were also obvious sublethal effects on nest reproduction. The nests exposed to 100, 10, 1 and 0.5 ppm thiamethoxam showed a total loss of reproduction due to the high worker mortality (as described above), classifying thiamethoxam in class 4 of IOBC. A lower concentration of 0.1 ppm also resulted in strong detrimental effects as in these nests the numbers of drones were significantly ($p < 0.05$) lower and yielded only 14% of the controls (27.5 ± 2.0) (Fig. 1). When reducing the concentration to 10 ppb, the sublethal side-effects on reproduction were absent with equal ($p > 0.05$) numbers of drones as in the control nests. After probit analysis, the EC_{50} for thiamethoxam was 35 ppb (95% CI: 10–90 ppb; $R^2 = 0.94$); this corresponds to 1/2860 of the MFRC. As for thiamethoxam, oral exposure of workers to 120 and 60 ppm thiacloprid resulted in a total loss of nest reproduction because of the strong lethal effects in these nests (see above). With a 10 times lower concentration, 12 ppm, the nest reproduction was still significantly ($p < 0.05$) reduced by 36% as compared to the control nests (27.5 ± 2.0) (Fig. 1). But with lower concentrations of 1.2, 0.12 ppm and 12 ppb, there was no negative ($p > 0.05$) effect on reproduction. Based on these results the EC_{50} value was calculated to be 12 ppm (95% CI: 2.0–67 ppm; $R^2 = 0.97$), which corresponds to 1/10 of the MFRC. Compared to thiamethoxam, thiacloprid also posed harmful effects but these only appeared at higher concentrations; the NOEC for thiamethoxam was 10 ppb (=1/10000 MFRC), while this was 1.2 ppm (=1/100 MFRC) for thiacloprid.

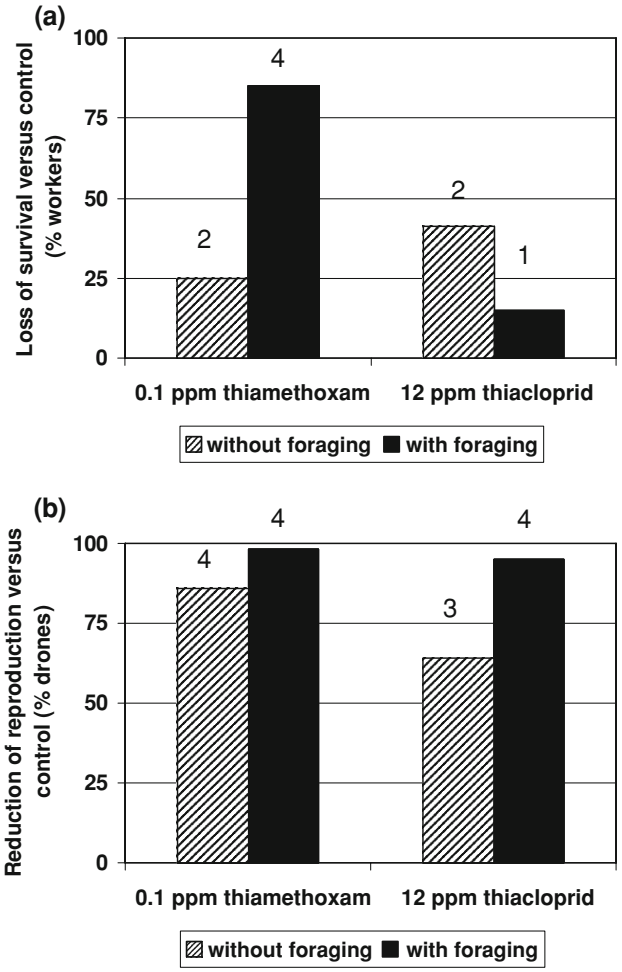

Fig. 1 Sublethal effects on survival of adult workers and nest reproduction by 0.1 ppm thiamethoxam and 12 ppm thiacloprid tested after oral exposure in treated sugar water via a chronic toxicity assay without and including foraging. **a** Loss of survival of adult bumblebee workers after 11 weeks. Data are given as percentage corrected mortality (Schneider-Orelli's formula). **b** Percentage reduction of numbers of drones produced per nest as compared to control nests after 11 weeks. In accord with IOBC classification, *1* not toxic, *2* weakly toxic, *3* moderately toxic, and *4* highly toxic

Chronic toxicity assay including foraging behavior

It was clear that imidacloprid was highly toxic for the bumblebee workers in the behavior test. In the nests treated with 200, 20, 2 and 0.2 ppm, 100% worker mortality was observed and this was after a few hours, 7, 14 and 49 days, respectively, classifying it in IOBC class 4 being highly toxic. Exposure to 20 ppb resulted in 50% mortality after 49 days, but with a lower concentration of 10 ppb there was no more worker mortality in treated than in control nests. After probit analysis, the LC_{50} value for imidacloprid

was 20 ppb (95% CI: 19–21 ppb; $R^2 = 0.99$), corresponding to 1/9850 of the MFRC (Table 2). The NOEC was 10 ppb.

Strong sublethal effects were observed on the nest reproduction. In the nests treated with imidacloprid at 200, 20, 2 and 0.2 ppm and 20 ppb, 0 ± 0, 0 ± 0, 0 ± 0, 4.8 ± 4.0 and 7.0 ± 6.4 drones were observed, respectively, which was in all cases much lower as compared with 28.4 ± 2.9 drones in the controls (ANOVA: $F = 50.7$, $df = 47$, $p < 0.001$). As given above, the total loss with 200, 20 and 2 ppm was due to the high worker mortality. For imidacloprid at 0.2 ppm and 20 ppb, significantly ($p < 0.05$) lower numbers of drones were produced as a consequence of the high worker mortality in these nests (ANOVA: $F = 35.3$, $df = 23$, $p < 0.001$). With the lowest concentration tested, 10 ppb, significantly ($p < 0.05$) lower numbers of drones (10.8 ± 7.2) were observed compared to the controls (28.4 ± 2.9) (ANOVA: $F = 25.8$, $df = 15$, $p < 0.001$). In addition, it was typical that these workers were less inclined to forage and feed, and the building up of the nest and their travel times were much longer when compared with workers in the control nests. After probit analysis, the EC_{50} value for imidacloprid using this bioassay with foraging was 3.7 ppb (95% CI: 2.5–5.5 ppb; $R^2 = 0.99$), implying that the NOEC should be below 2.5 ppb.

As above, two other neonicotinoid insecticides were also tested for comparison. With thiamethoxam at 0.1 ppm and thiacloprid at 12 ppm in the behavior bioassay, 85 and 15% of worker toxicity were observed, respectively (Fig. 1). In addition, there were strong significant sublethal effects ($p < 0.05$) as the drone production was very low: only 2 and 5% of the numbers of drones in the control nests were observed (29.8 ± 9.0 drones), respectively (Fig. 1).

Finally, in the behavior bioassay the daily consumption of sugar water per bumblebee worker was determined as 277 ± 16 µl.

Chronic test in the greenhouse, including foraging behavior

Imidacloprid at 20 and 10 ppb affected all (100%) the workers in the treated hives after 2 weeks of exposure via treated sugar water, representing class T (harmful) in accordance with the IOBC classification. In detail, we scored an effective kill of workers (62 ± 1 and 92 ± 5% mortality with 20 and 10 ppb, respectively), and all the other workers from these hives were totally apathic with any signs of movement and foraging. In addition it should be mentioned that with 20 ppb, nearly all dead worker bees were found around the sugar water and the pollen, whereas with 10 ppb all dead workers were found inside the hives, indicating that with 10 ppb of imidacloprid the worker bees were able to fly back to their hive. In contrast, at a lower concentration of 2 ppb imidacloprid, there were no lethal side-effects as the percentage of worker mortality yielded 5 ± 2%, representing class H (harmless), which was equal to that in the controls (6 ± 1%) after 2 weeks.

As a consequence of the lethal effects, there were obvious sublethal effects by 20 and 10 ppb imidacloprid with a total loss of reproduction. Only with 2 ppb there were no harmful sublethal effects on the colony performance over the 2 weeks of the experiment. The hives of the 2 ppb treatments increased in net fresh weight with 24.0 ± 3.0 g over the 2 weeks, which was equal ($p = 0.33$) as in the control series (38.5 ± 13.0 g), the numbers of newborn workers and the total amount of brood were equal as in the control hives (respective $p = 0.93$ and 0.16), and the numbers of filled sugar water pots per hive (56 ± 7) and that of dead larvae (71 ± 7) were equal from the controls (77 ± 9, $p = 0.09$, and 63 ± 8, $p = 0.51$, respectively).

In addition we scored the foraging behavior and potential sublethal effects by imidacloprid as the numbers of workers entering and leaving the hive during 30 min intervals. At the start of the experiment in the greenhouse, the foraging behavior was significantly equal in all the hives used for 20, 10 and 2 ppb and controls (ANOVA; $F = 2.888$, $df = 23$, $p = 0.06$). At the end, i.e. after 2 weeks, all workers were affected and/or dead with 10 and 20 ppb which is a situation that did not allow to score the foraging behavior. With 2 ppb imidacloprid, the foraging behavior (24.2 ± 3.7 workers in and out per 30 min) was equal ($p = 0.70$) as in the controls (22.0 ± 1.0).

Finally in the greenhouse behavior test, the mean weekly sugar water consumption for the three hives exposed to 2 ppb imidacloprid was 474 ± 39 g and this was significantly ($p = 0.49$) equal to the control hives (508 ± 7 g). Here the daily consumption of sugar water per bumblebee was calculated to be 244 ± 28 µl in the controls.

Discussion

In this paper a risk assessment bioassay "behavior test" for bumblebees is presented that includes the foraging behavior to evaluate side-effects of pesticides against these beneficial pollinators. This bioassay allowed queenless microcolonies of 5 bumblebee workers to be used under standardized conditions and to perform adequate comparisons between contaminated food treatments. In these experiments, the overall toxicity of the tested neonicotinoid insecticides was different between the chronic toxicity assay without foraging and that including foraging. For imidacloprid the first assay demonstrated a median lethal

concentration (LC_{50}) of 59 ppb, whereas this was 20 ppb in the behavior test. In a similar manner, the median sublethal effect concentration (EC_{50}) for imidacloprid was 37 ppb without foraging and 3.7 ppb including foraging. Therefore, the behavior bioassay including foraging was 3 to 10 times more sensitive than the test without foraging. In addition, we evaluated the results obtained in the laboratory with the behavior test including foraging under more field related conditions in a greenhouse with use of queenright colonies of *B. terrestris* and wherein workers needed to forage/fly over a distance of 3 m to collect food. Here 10 ppb imidacloprid resulted in a severe lethal effect on the workers whereas this concentration was found to be the NOEC in the behavior test, but this can be explained by the stringent conditions of the greenhouse experiment wherein the food was put at a distance of 3 m from the hives. However the most important message here is that for what concerns the sublethal effects the NOEC for different parameters on the queenright colony performance obtained in the greenhouse test (2 ppb) correlated very well with the NOEC as determined in the new behavior bioassay with queenless micro-colonies in the laboratory (<2.5 ppb). Overall, these results showed that the new behavior bioassay can be used for a true risk assessment of sublethal impairments of foraging behavior and this under laboratory conditions. Moreover, the risks as scored in the behavior test agree with experiments by other investigators in bumblebees and also in honeybees. Tasei et al. (2000) reported in *B. terrestris* colonies a loss of 10% in worker survival and a significant decline of about 40% in the numbers of adults produced upon exposure to imidacloprid at 6–10 ppb in the syrup and pollen. Similar sublethal effects were also seen in other bumblebee species like *Bombus occidentalis* and *Bombus impatiens*: exposure to 30 ppb imidacloprid had a significant negative effect on the foraging rates (Morandin and Winston 2003). These results on risks by imidacloprid also agree with data in honeybees. Schmidt (1996) reported that imidacloprid is highly toxic in feeding tests with an oral LD_{50} of 3.7 ng per honeybee. On the sublethal effects, Schmuck (1999) observed a decline of foraging honeybees already after an exposure of 20 ppb imidacloprid for 30–60 min and reported that imidacloprid affected the preciseness of communication related to the distance of the food source from the nest. Recently, Decourtye et al. (2004b) determined the lowest effect concentration of imidacloprid on the foraging behavior to be 24 ppb in honeybees that were subjected to PER under laboratory conditions and this was also the case in free flying honeybees under semi-field conditions. In addition, no recovery of the foraging activity was seen at this concentration (Decourtye et al. 2004b). As suggested by Thompson (2003) these disturbances on the foraging behavior are a consequence of imidacloprid on the motor neuron signal transmission. The data presented suggest that the chronic feeding bioassay without foraging underestimated the risks of side-effects. The behavior test is more stringent and may be considered as a worst case bioassay in the laboratory to assess potential risks on lethal and sublethal effects including foraging behavior, especially for compounds that are expected/suspected to impair with the foraging behavior. This newly developed behavior test also has a great practical advantage in that a flight cage is not necessary as the presence of a 20-cm long plastic tube is sufficient to reveal impairments of foraging behavior, allowing a first screening assay to be performed relatively easily in the laboratory. These results can then be used in a tiered-kind approach. For instance, in order to evaluate the effect of a chronic exposure to low doses of pesticides under practical conditions, a long-term toxicity test should be performed under more natural conditions as in the field bumblebees need to collect food over a longer distance than 20 cm. Indeed, recently Wolf and Moritz (2008) determined the mean foraging distance of *B. terrestris* workers to be 267 m with a maximum of 800 m. This is more than 1,000 times the travel distance in the behavior test with the 20-cm tube. In addition, apart from nest reproduction, sublethal effects may also affect other behavior traits and it would be useful to evaluate how flower visitation and homing ability of queenright bumblebee colonies might be affected by foraging on flower-containing fields.

Secondly, the concentrations that we observed as detrimental in the behavior test, are environmentally relevant. In this behavior test a chronic exposure of 10 and 20 ppb imidacloprid in the sugar water caused 0 and 50% lethal effects and 66 and 75% sublethal effects, respectively. The lethal LC_{50} is 20 ppb and the sublethal EC_{50} 3.7 ppb; the respective NOECs are 10 ppm and <2.5 ppm. Based on Tasei et al. (2000) who reported a daily intake of 4.8 ng imidacloprid per worker when exposed to treated syrup (10 ppb) and treated syrup (6 ppb), we can estimate a respective daily uptake of imidacloprid of 2.8 ng and 5.2 ng per worker with a chronic exposure of 10 and 20 ppb imidacloprid in the sugar water as we measured a daily sugar water consumption of 277 µl per bumblebee in the laboratory behavior bioassay. Interestingly, the latter value also agrees with the greenhouse experiment wherein a daily consumption of 244 µl per worker was measured. As compared to the imidacloprid amounts recovered in pollen loads collected by honeybees, these amounts are environmentally realistic. A field survey conducted over 3 years in French apiaries to monitor the weakness of honey bee colonies, in which pollen loads were collected from traps at four different times per year, showed that residues of imidacloprid and 6-chloronicotinic acid (imidacloprid metabolite) were found in 69% of the samples,

and imidacloprid contents were quantified with values ranging from 1.1 to 5.7 µg per kg pollen (=ppb) (Chauzat et al. 2006). Similarly, in a large greenhouse with sunflower plants wherein the seeds (*Helianthus annuus*) had been dressed with commercial 700 g/kg Gaucho® in a manner comparable to commercial practice, the residues of entire non-metabolized imidacloprid in the nectar and pollen extract reached 1.9 and 3.3 µg/kg, respectively (Schmuck et al. 2001). However little is known about the future of residues of pesticides when contaminated pollen and nectar are brought to the hive and stored, and whether they are conserved or metabolized.

Thirdly, based on our results the three neonicotinoids tested can be classified in two groups according to their activity towards bumblebees. From our data it was apparent that neonicotinoid insecticides containing a nitro group such as imidacloprid and thiamethoxam caused the greatest side-effects, while the cyano group with thiacloprid possessed lower activity. In our chronic toxicity test without foraging the respective LC_{50}s for imidacloprid and thiamethoxam were 59 (52–68) ppb and 120 (40–380) ppb, ranking them both in class 4 of highly toxic according IOBC, while this LC_{50} was 18 (3.8–85) ppm for thiacloprid. These median lethal concentrations correspond to 1/3390; 1/833 and 1/7 of the respective MFRC of each neonicotinoid insecticide. Here based on LC_{50} values, thiacloprid was 305 times less toxic than imidacloprid. This ranking was also identified for sublethal side-effects against nest reproduction (EC_{50}): imidacloprid (37 ppb = 1/5410 MFRC) = thiamethoxam (35 ppb = 1/2860 MFRC) << thiacloprid (12 ppm = 1/10 MFRC), and was also confirmed in the behavior assay although in that assay the effective doses were 3 to 10 times lower. Based on the ratios of the MFRC it is clear that thiacloprid can be considered as safer in an environment with bumblebees. These findings are also in accordance with Iwasa et al. (2004) who demonstrated that the LD_{50} for thiacloprid in honeybees was 816 times higher than for imidacloprid. As discussed by Jones et al. (2006) the lower side-effects of the cyano group may be explained by the existence of different nicotinic acetylcholine receptor (nAChR) subtypes. A high diversity of nAChRs in honeybees was already shown via genome sequencing. In addition, although Suchail et al. (2004b) reported that the absorption in the honeybee is similar for both neonicotinoid groups, there may be significant differences in metabolism and/or the toxicity of the formed metabolites for the two neonicotinoid groups. According to Burnet et al. (2005) the rapid metabolism of a cyano neonicotinoid (^{14}C-acetamiprid), i.e. more than 50% was broken down in less than 30 min, was responsible for the low toxicity. In contrast, ^{14}C-imidacloprid demonstrated a much longer half-life in honeybees, namely 4.5–5 h (Suchail et al. 2004a, 2004b). In addition, Nauen et al. (2001) and Suchail et al. (2001) reported the occurrence of a peak of two metabolites of imidacloprid at 4 h after oral uptake: olefin and 5-hydroxyimidacloprid, and also these metabolites are toxic for honeybees. For thiamethoxam, previous work demonstrated an oral LD_{50} in honeybees of 30 ng/bee (Iwasa et al. 2004) which agrees with the LC_{50} of 120 ppb (=33 ng per worker) in bumblebees in this study. Also in the behavior test, 0.1 ppm thiamethoxam caused 85% worker mortality and a significant loss of nest reproduction. Nauen et al. (2003) and Tan et al. (2007) reported that, although thiamethoxam is not a direct agonist or antagonist of the nAChR, it is its metabolite clothianidin that is highly active as antagonist and therefore highly toxic which explains the results in this study.

Finally as a general conclusion, the experiments in this paper using a simple behavior assay in the laboratory showed that concentrations of pesticides, that may be considered safe for bumblebees in a classical toxicity assay, can have a negative influence on their foraging behavior, leading to a loss of worker survival and nest reproduction. This is particularly true for compounds that are expected/suspected to impair with the foraging behavior like the neonicotinoid insecticides. Therefore, it is recommended that behavior tests should be included in risk assessment tests for highly toxic pesticides because impairment of the foraging behavior can result in a decreased pollination, lower reproduction and finally in colony mortality due to a lack of food. Here, imidacloprid and thiamethoxam were indicated as highly hazardous, while thiacloprid is suggested to be safe. However, before making final conclusions, a good knowledge of environmentally relevant concentrations of these pesticides is necessary and the pesticides as well as combinations of pesticides should also be evaluated in more realistic field situations for the assessment of potentially deleterious effects on foraging behavior using whole bee colonies.

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