Environmental Toxicology and Chemistry—Volume 00, Number 00—pp. 1–7, 2019
Received: 29 April 2019 | Accepted: 21 May 2019

Environmental Toxicology

Joint Toxicity of Acetamiprid and Co-Applied Pesticide Adjuvants on Honeybees under Semifield and Laboratory Conditions

Lang Chen,[a] Qingping Yan,[b] Jinzhen Zhang,[c,*] Shankui Yuan,[d] and Xingang Liu[a,*]

[a]State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China
[b]Environmental Safety Assessment Center, Hunan Academy of Agricultural Sciences, Changsha, China
[c]Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China
[d]Institute for Control of Agrochemicals, Ministry of Agriculture, Beijing, China

Abstract: The evaluation of adverse effects of pesticides, pesticide adjuvants, and their combination on honeybees is hampered by a lack of colony-level bioassays reflecting productivity and survival over longer term exposure. In the present study, the joint toxicity of acetamiprid and co-applied pesticide adjuvants (*N*-methyl pyrrolidone [NMP], Silwet L-77, and Triton X-100) to honeybees was determined both in the laboratory and under semifield conditions. The 3 pesticide adjuvants caused no significant acute toxicity to honeybees by themselves; however, in the laboratory tests, they significantly increased the acute contact toxicity of acetamiprid to honeybees. For the semifield tests, in the T2 group (treatment with 5% acetamiprid soluble concentrate [SL] containing 10% Silwet L-77), the mortality of honeybees was significantly higher ($p < 0.05$) than that of the blank control on the fourth day after application (DAA + 4), that of the T1 group (5% acetamiprid SL containing 10% NMP) on DAA + 4 and DAA + 7 (seventh day after application), and that of the T3 group (5% acetamiprid SL containing 10% Triton X-100) on DAA + 4. Furthermore, the flight intensity in the T2 group on DAA + 7, the colony intensity on DAA + 28 (28th day after application), and the mean areas covered by pupae on DAA + 15 (15th day after application) were significantly lower ($p < 0.05$) than those of the blank control. Therefore, pesticide adjuvants may be important factors in increasing the toxicity of neonicotinoids to honeybees. Measures should be taken to manage the environmental risk of pesticide adjuvants during the process of formulation development and registration. *Environ Toxicol Chem* 2019;00:1–7. © 2019 SETAC

Keywords: Ecotoxicology; Population-level effects; Insecticide; Pesticide adjuvants; Honeybee colony development; Flight intensity

INTRODUCTION

Adjuvants are commonly used during the processes of production and application of plant protection products for different purposes such as wetting, spreading, adhering, emulsifying, dispersing, preventing drift, defoaming, and increasing infiltration (Stevens 1993). The proportions of adjuvants in pesticide formulations range from several percentages to more than 90% (Bu et al. 2014). Therefore, with the use of plant protection products in agricultural activities, a large number of adjuvants are released into the environment every year. The impacts of adjuvants on ecosystems have drawn the attention of scientists. A number of recent studies have shown that pesticide adjuvants could also be toxic to ecosystems (Comber et al. 1993; Delvalls et al. 2002; Ciarlo et al. 2012; Zhu et al. 2014; Mullin et al. 2016), despite the widespread assumption that they are bioinert. Furthermore, the adjuvants may change the profiles of pesticide transport and transformation in soils, plants, and other materials and organisms, or may generate joint toxicity (such as a synergistic effect) with active ingredients. As a result, the bioavailability and ecotoxicity of the active ingredients to nontarget organisms are altered. Zhao et al. (2011) have reported that formulations of the same active ingredients with different adjuvants can produce significantly different acute toxicities to bees. It is obvious that risk assessments based only on the endpoints of active ingredients cannot accurately reflect the actual risks of the end products to nontarget organisms including humans (Mullin et al. 2016). For pesticides to be registered, ecotoxicology studies are usually required in the

* Address correspondence to 914341923@qq.com and xingangliu@caas.cn
Published online in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.4515

© 2019 SETAC

European Union and in many other countries for both active ingredients and formulations (commercial products, including the active ingredients, adjuvants, solvents, etc., to be tested as a whole). However, the different effects of different kinds of adjuvants on the ecotoxicity of active ingredients and the mechanism of their combined action can be complicated and have not yet been fully understood (Srinivasan 2008).

Bees are important insects for humans and the environment not only because they provide us with honey, pollen, royal jelly, propolis, and beeswax, but, more importantly, because they are the pollinators of many plants in nature and managed crops. Bee colony numbers in many parts of the world have been decreasing in recent years, a decline that potentially threatens the viability of agricultural crops and broader ecosystems. In addition to parasites, disease, and shrinking food resources, the use of pesticides such as neonicotinoids is believed to be one of the causes for bee colony decline (Fairbrother et al. 2014). The results of studies on acute and sublethal effects of neonicotinoids on honey bees, bumble bees, and other pollinators have led to regulatory restriction of this class of chemicals in some countries (Cresswell 2011; Goulson 2013; Stanley et al. 2015). "Clear evidence of harm" sufficient to trigger regulatory action has been declared by the European Food Safety Authority for 3 neonicotinoids (clothianidin, imidacloprid, and thiamethoxam; Cresswell 2011; Goulson 2013; Cressey 2017; European Food Safety Authority 2018).

Meanwhile, using pesticide usage data from the California Department of Pesticide Regulation during almond pollination (February and March), Mullin et al. (2016) reported that the recent decline of bee colonies in the United States may be related to the increased use of adjuvants, especially the organosilicon surfactant type. Apricot pollination in California is the largest pollination event in the world, involving the transport of more than 60% (1.5 million) of the bee colonies in the United States. Therefore, the report of Mullin et al. (2016) received a great deal of attention from researchers working on the causes of bee colony decline. Other investigators have also reported potential negative effects of pesticide adjuvants on bee colonies. Ciarlo et al. (2012) found that conditioned responses, olfactory learning, and foraging ability of honeybees decreased after ingestion of organosilicon surfactants (Comber et al. 1993). However, so far little research has been done on the effects of the interaction between pesticide adjuvants and active ingredients on honeybees, especially at the colony level.

We investigated the joint toxicity to honeybees of acetamiprid and 3 different co-applied pesticide adjuvants (*N*-methyl pyrrolidone [NMP], Silwet L-77, and Triton X-100). As a neonicotinoid insecticide, acetamiprid poses relatively lower toxicity to bees than other compounds in the same chemical class and is widely used (Stanley et al. 2015). In China, more than 600 pesticide products containing acetamiprid have been registered for pest control on vegetables (cucumber, cabbage, etc.) and fruits (citrus, etc.). The registered products have different formulations (soluble concentrate/powder, emulsifiable concentrate, suspension concentrate, water-dispersible granules, wettable powder, microemulsion, and others), and contain different adjuvant components. The effects of the 3 adjuvants (NMP, Silwet L-77, and Triton X-100) on bees and their potential joint toxicity with acetamiprid have not been investigated previously, and scientific data addressing these effects would be useful in evaluating the risk of the end products to bees. In the present study, we laboratory tested the acute contact toxicity to honeybees of the 3 adjuvants, 97.4% acetamiprid technical material and their 9 combinations, and 35% acetamiprid soluble concentration (SL) formulations containing 10% of the 3 adjuvants. (The other components remained identical in the 3 formulations tested.) In addition, we tested the toxic effects of 3 acetamiprid SL formulations on honeybee colonies using semifield tunnel tests. The main purpose of the tests was to investigate the joint toxicity of neonicotinoids and pesticide adjuvants, in an attempt to provide guidance for bee risk assessment of the products and for the development of formulations safe for bees.

MATERIALS AND METHODS

Test substance

The 97.4% acetamiprid technical material, NMP, Silwet L-77, Triton X-100, and 40% dimethoate emulsifiable concentrate were provided by the Institute for Control of Agrochemicals, Chinese Ministry of Agriculture and Rural Affairs (Beijing, China). The 3 5% acetamiprid SL formulations were specially prepared and provided by the Department of Pesticide Sciences, Institute of Plant Protection, Chinese Academy of Agricultural Sciences (Beijing, China). In these 35% acetamiprid SL formulations, NMP was present at 10% in the first formulation, Silwet L-77 at 10% in the second, and Triton X-100 at 10% in the third; the other components remained identical.

Instruments and reagents

We used the following instruments and reagents: a high-performance liquid chromatography (1200 series)–tandem level 4-pole mass spectrometer (model 6460; Agilent Technologies); an analytical balance (Sartorius); an ultrapure water system (Millipore); a centrifuge (Hitachi); a vortex generator (Kangjian Medical Apparatus); a single-nozzle knapsack sprayer (Agrolex AZ, LP 605, AD 400, 16-L tank volume); an Agilent Zorbax SB-C18 column (50 mm × 2.1 mm, 1.7 μm), with *N*-propyl-ethylenediamine (Agilent); acetamiprid (analytical standard, purity ≥98%); formic acid (high-performance liquid chromatography purity grade); acetic acid (analytical reagent grade); $MgSO_4$ (analytical reagent grade); sodium acetate trihydrate (analytical reagent grade); and ultrapure water. Analytical standard acetamiprid was dissolved in acetonitrile to obtain the stock solution (1.00 mg/mL). The stock solution was stored at –20 °C and further diluted with acetonitrile: water (20:80, v/v) to prepare working solutions, as required.

Test organism

Experiments were conducted with Italian honeybees (*Apis mellifera ligustica*). The adult bees used in the acute contact toxicity test were bred by the Institute of Plant Protection, Hunan

© 2019 SETAC

Academy of Agricultural Science (Hunan, China). In the semifield test, small healthy colonies with 5000–8000 honeybees per colony, 3 combs per colony were used. Bee colonies were as homogenous as possible and originated from local sources. The queens were from one breeding line, to guarantee uniform bee material in all treatments. The number of bees in each colony was estimated following the method described by Imdorf et al. (1987). Before transfer to the tunnels (on the day before first contact with the treated crop, or day after application [DAA]–1), there was no significant difference in the number of honeybees/hive, the coverage area of eggs, larvae, eggs, and pupae/hive, and the number of dead bees among all the honeybee hives. In addition, the flight activity of honeybees in each hive was normal (by visual observation, without flight intensity investigation or recording).

Test design

In the laboratory test, the acute contact toxicity on honeybees was determined for the 97.4% acetamiprid technical material, 3 pesticide adjuvants (NMP, Silwet L-77, and Triton X-100), 3 types of 5% acetamiprid SL formulations (one containing 10% NMP, one containing 10% Silwet L-77, and one containing 10% Triton X-100) and 9 mixtures of combinations of acetamiprid and 3 adjuvants in 3 proportions (1:0.1, 1:0.01, and 1:0.001). In the semifield trial, 5 groups of tests were created: treatment with 5% acetamiprid SL containing 10% NMP (T1 group), treatment with 5% acetamiprid SL containing 10% Silwet L-77 (T2 group), treatment with 5% acetamiprid SL containing 10% Triton X-100 (T3 group), a positive control treated with 40% dimethoate emulsifiable concentrate (R group), and a blank control (CK group). Three replications were performed simultaneously in each group. The spray application rate of acetamiprid in the T1, T2, and T3 groups was 30 g active ingredient/hectare (a.i./ha), which is consistent with the recommended dosage to control oilseed rape aphids, and the application rate of dimethoate in the R group was 240 g a.i./ha. For the CK group, only water was sprayed, with the volume of water sprayed equal to that of each treatment group, that is, 4.8 L/80 m^2 (equivalent to 600 L/ha).

Acute contact toxicity test

In each acute contact toxicity test, 5 to 6 doses of test substance in a geometric series (with a factor not exceeding 2.2), a blank control (CK), and a solvent (acetone) control were used according to Organisation for Economic Co-operation and Development guidelines (1998). Three replicate test groups, each containing 10 bees, were dosed with each test concentration. Acetamiprid technical material was dissolved in acetone to obtain a stock solution, and the 3 adjuvants, the 35% acetamiprid SL formulations, and the 40% dimethoate emulsifiable concentrate were separately dissolved in distilled water to obtain the corresponding stock solutions. Then the stock solutions (or mixtures of acetamiprid technical material stock solution and the adjuvant stock solutions in a corresponding proportion) were diluted into different test solutions by using their respective solvents (or solvent mixtures). After the adult bees had been anesthetized with carbon dioxide, they were individually treated by topical application. A droplet of 2 µL of solution containing the test substance at the appropriate test concentration was applied to the dorsal side of the thorax of each bee. After application, the bees were transferred to the test cages. The cages (40 × 100 × 100 mm) were constructed of stainless steel with 2 sides covered in 2-mm nylon mesh. The bees were supplied with sucrose solution (50% w/v) and kept in the dark at 25 ± 2 °C and 50 to 70% relative humidity. Mortality was recorded within 48 h after treatment. The median lethal doses (LD50s) and the corresponding 95% confidence intervals of the test substances (i.e., acetamiprid technical material, 3 pesticide adjuvants, and 3 acetamiprid formulations containing different adjuvants) were estimated using probit analysis (Data Processing System software program, Ver 9.5; Fujitsu).

Semifield test

Test location and test plant. The semifield trial was conducted near the city of Changsha, Hunan Province, China (28 °20′N, 113 °16′E). The test field (50 × 45 m, total area 2250 m^2) was subdivided into 15 tunnel tents (80 m^2 for each tunnel). The dimensions of each tunnel tent (covered plot) were 20 m long, 4 m wide, and 2.5 m high in the center. The tunnel tent frames were covered with insect-proof netting. The distance was at least 2 m between tunnels of the same treatment group and 3 m between tunnels of different treatment groups. A path in the middle of the tunnel was retained to facilitate evaluations. The crop used in the present study was oilseed rape (*Brassica napus L.*), which was sown in October 2013 and was in bloom between March and April 2014.

Application. Spray solutions (after 1000 times dilution of the test pesticide formulations) were applied with a single-nozzle knapsack sprayer for all treatments on 25 March 2014, during the time of full flowering of the crop. Before the applications, the sprayer was calibrated by measuring the total output to adjust the application speed. The actual amount of solution applied was determined by measuring the remaining solution after application. When the pesticide was applied, the wind speed in the tunnel was below 0.5 m/s, and the actual amount of each application that deviated from the target was less than 5%. The bee colonies were placed inside the tunnels after the application in the evening of 25 March 2014, to simulate the exposure risk of 3 different acetamiprid formulations to honeybees if appropriate risk reduction measures are taken to avoid spraying when bees are visiting the blooming crops.

Flight activity of bees in the tunnels. Assessments of flight activity started from the first day after the establishment of the colonies (DAA + 1) to the 7th d (DAA + 7). The flight intensity was recorded on a 1-m^2 area, at 3 different places regularly distributed in each tunnel. On each morning of observation, the numbers of bees that were foraging on the flowering oilseed rape or flying over the crop were counted for 1 min. In addition, the numbers of bees that came in and out of the hives were counted for 1 min.

 © 2019 SETAC

Mortality investigation. The assessments of the numbers of dead bees were carried out in the morning from DAA–1 to DAA + 7. To note and count the numbers of dead honeybees that were carried out from the hives in all bee colonies, dead bee traps containing gauze were fixed at the entrance of each hive. The gauze at the top allowed the leaving forager bees to pass only without load (i.e., dead bees). The dead bees that were carried out by the leaving bees were dropped inside the traps and could then be counted. The dead bees were separated into adult worker bees, males, larvae, pupae, and freshly emerged (young) bees during each assessment. In addition, the dead honeybees were counted on gauze, which was spread out in the middle of each tunnel.

Brood assessment. The condition of all colonies and the brood development were assessed on DAA–1, DAA + 7, DAA + 15, DAA + 21, and DAA + 28 (DAA–1 was the day before the first contact with the treated crop). For all combs (both sides) in each hive, the strength of the colony was assessed by visual estimation of comb areas covered by bees and comb areas containing eggs, larvae, and pupae (in %/comb) following the methods described by Imdorf et al. (1987). The mean values were calculated for each hive at each assessment time.

Pollen collection behavior of bees. To study the pollen collection behavior of honeybees, on each investigation day (DAA + 1, DAA + 2, DAA + 4, and DAA + 7) a pollen collector was placed outside each hive, and pollen was collected into brown bottles after 2 h and stored immediately at –20 °C for subsequent analysis.

Determination of acetamiprid in pollen: Sample preparation. Approximately 0.2 to 0.4 g of pollen was placed in a polypropylene centrifuge tube. A total of 2 mL of water, 4 mL of acetonitrile, 1.5 g $MgSO_4$, and 0.4 g CH_3COONa was added and mixed. The mixture was immediately hand-shaken for 1 min and centrifuged (4000 rpm for 5 min). Then aliquot portions of the supernatant were transferred to a 10-mL centrifuge tube containing 50 mg primary secondary amine, 50 mg C18, and 150 mg $MgSO_4$. The tube was vortexed for 1 min and centrifuged for 5 min at 4000 rpm. A total of 200 µL of the supernatant was transferred into a sample vial and mixed with 800 µL of water.

High-performance liquid chromatography conditions. The column temperature was fixed at 30 °C. The injection volume was 5 µL. Mobile phase A was 0.1% formic acid aqueous solution, and mobile phase B was acetonitrile. The flow rate was 200 nL/min. The gradient elution program was as follows: 0 to 1.5 min, 90% A; 1.5 to 4.0 min, 90% A to 40% A; 4.0 to 5.0 min, 40% A to 10% A; 5.0 to 5.01 min, 10% A to 90% A; 5.01 min to 6.0 min, 90% A.

Liquid chromatography–tandem mass spectrometry with multiple reaction monitoring. Sample ionization was performed by electrospray ionization in the positive ion detection mode. The capillary voltage was set at 3.5 kV, and the desolvation gas flow rate was maintained at 5 L/min and 325 °C. The flow rate of the sheath gas was 12 L/min (400 °C). The collision gas pressure was 50 psi. The quantitative ion was 223 > 126, and the qualitative ion was 223 > 56.0 for liquid chromatography–tandem mass spectrometry with multiple reaction monitoring. During the test, the recovery concentrations used were 20 to 100 µg/kg. The mean recovery from 6 replicate fortifications on each test batch was in the range of 73.5 to 79.4%, and the relative standard deviation was in the range of 1.3 to 2.7%. The limit of quantification was 20 µg/kg (based on the 10 × noise ratio).

Statistical methods. In the semifield trial, data were analyzed using SPSS Ver 16.0. Before statistical analysis, normal distribution and homogeneity of variances were tested. Differences between each group at each time point were compared by one-way analysis of variance (ANOVA). In the case of a significant ANOVA, post hoc analysis was performed using Tukey's test (or Dunnett's T3 test when data or data after ln $(x + 1)$ transformation did not meet the normal distribution and homogeneity of variance assumptions). The differences in pupae coverage areas between each treatment group and CK were determined by one-way ANOVA, followed by Dunnett's test. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Acute contact toxicity tests

The 3 pesticide adjuvants had very low acute toxicities to honeybees (Table 1). The LD50s of the 3 tested formulations ranged from 4.79 to 10.73 µg acetamiprid/bee, similar to that of

TABLE 1: Test results on bees of toxicity after acute contact with acetamiprid, 3 kinds of pesticide adjuvants, and their preparations

Treatment group	LD50 (µg a.i./bee)	95% Confidence limits (µg a.i./bee)
97.4% Acetamiprid technical material	26	18–34
NMP	>2000	—
Silwet L-77	357	306–410
Triton X-100	1436	1214–1874
5% Acetamiprid SL containing 10% NMP	6.44	2.03–10.15
5% Acetamiprid SL containing 10% Silwet L-77	4.79	0.51–9.03
5% Acetamiprid SL containing 10% Triton X-100	10.73	1.64–18.90
Acetamiprid:NMP = 1:0.1	15.91	8.23–120
Acetamiprid:NMP = 1:0.01	0.96	0.03–1.91
Acetamiprid:NMP = 1:0.001	1.21	0.15–2.69
Acetamiprid:Silwet L-77 = 1:0.1	4.43	3.24–5.70
Acetamiprid:Silwet L-77 = 1:0.01	1.52	0.16–3.00
Acetamiprid:Silwet L-77 = 1:0.001	3.26	1.12–6.05
Acetamiprid:Triton X-100 = 1:0.1	7.28	4.81–11.10
Acetamiprid:Triton X-100 = 1:0.01	0.86	0.02–2.19
Acetamiprid:Triton X-100 = 1:0.001	3.62	1.25–5.96

LD50 = median lethal dose; a.i. = active ingredient; NMP = *N*-methyl pyrrolidone; SL = soluble concentrate.

© 2019 SETAC

acetamiprid 20% soluble powder (9.26 µg acetamiprid/bee) reported by the European Food Safety Authority (2016), but 2.4 to 5.4 times lower than that of the technical material (26 µg/bee; Table 1). The 5% acetamiprid SL containing 10% Silwet L-77 induced the lowest LD50 among the 3 acetamiprid formulations. Almost all LD50s of the 9 mixtures of acetamiprid and adjuvants were lower than that of acetamiprid technical material and those of the formulations (except for 2 combinations: acetamiprid:NMP = 1:0.1 and acetamiprid:Triton X-100 = 1:0.1). These laboratory test results showed that the 3 pesticide adjuvants increased the acute contact toxicity of acetamiprid to bees in varying degrees.

Semifield tests

Effects on flight intensity and mortality. During the entire test period, the daily mortality of honeybees in the R group was higher than that in the CK group, and the difference was statistically significant on DAA + 3 ($p < 0.05$; Figure 1, designation b). On DAA + 4, the number of dead bees in the T2 group was nearly 3 times higher than that in the CK and R groups, and there were significant differences in number of dead bees in the T2 group and those in the CK, T1, and T3 groups ($p < 0.05$). On DAA + 7, the number of dead bees in the T2 group was approximately equal to that of the R group and significantly higher than that in the T1 group ($p < 0.05$; Figure 1). From DAA + 1 to DAA + 5, the flight intensity of bees in the R group was significantly lower than that in the CK group ($p < 0.05$), and no flight was observed on DAA + 6 (Figure 2). At the initial stage after spray application (i.e., on DAA + 1), the flight intensity of bees in the T3 group was lower than that in all other groups except for the R group (not statistically significant, $p > 0.05$). On DAA + 7, the flight intensity of bees in the T2 group was nearly 80% lower than that in the CK group ($p < 0.05$).

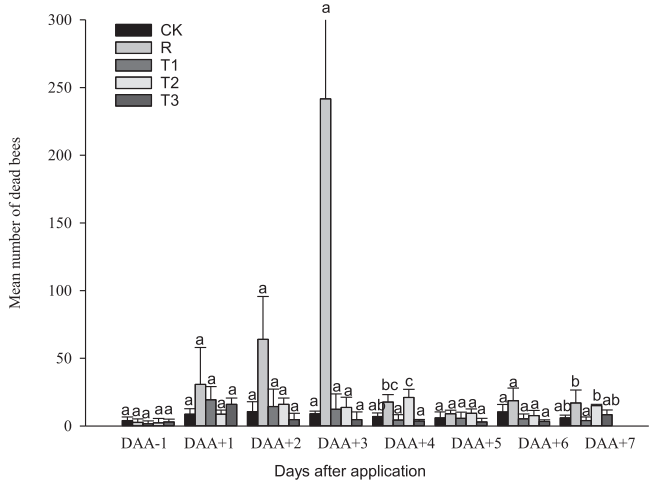

FIGURE 1: Number of dead bees/hive on different assessment dates. Data were obtained from triplicate assays and are given as mean ± standard deviation. Bars with the same letters are not significantly different ($p < 0.05$). CK = blank control; R = positive control (40% dimethoate emulsifiable concentrate); T1 = treatment group with 5% acetamiprid soluble concentrate (SL) containing 10% *N*-methyl pyrrolidone; T2 = treatment group with 5% acetamiprid SL containing 10% Silwet L-77; T3 = treatment group with 5% acetamiprid SL containing 10% Triton X-100.

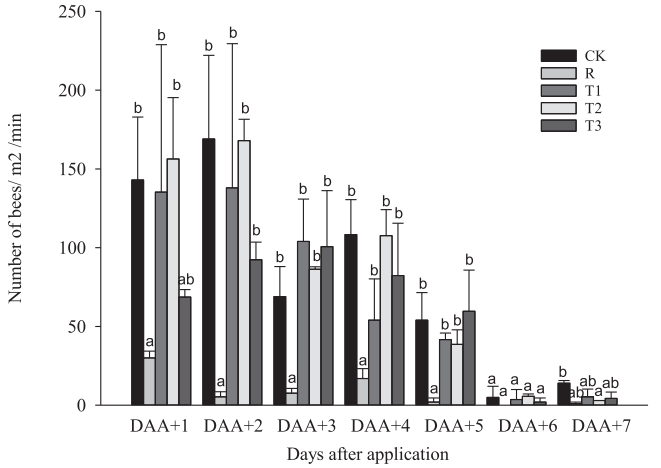

FIGURE 2: Flight intensity of honeybees on different assessment dates. Data were obtained from triplicate assays and are given as mean ± standard deviation. Bars with the same letters are not significantly different ($p < 0.05$). CK = blank control; R = positive control (40% dimethoate emulsifiable concentrate); T1 = treatment group with 5% acetamiprid soluble concentrate (SL) containing 10% *N*-methyl pyrrolidone; T2 = treatment group with 5% acetamiprid SL containing 10% Silwet L-77; and T3 = treatment group with 5% acetamiprid SL containing 10% Triton X-100.

Effects on brood development. At the first brood assessment before the hives were established on DAA–1, the mean strengths of the colonies were 7042, 6375, 7583, 6542, and 6167 bees/hive in the CK, R, T1, T2, and T3 treatment groups, respectively. At the last brood assessment on DAA + 28, the mean strengths of the colonies were 10 125, 8917, 9458, 7063, and 8208 bees/hive in the CK, R, T1, T2, and T3 treatment groups, respectively. Apart from the number of bees in the T2 treatment group being significantly ($p < 0.05$) lower than that in the CK group on DAA + 28, there were no significant differences in colony size among any of the groups from DAA–1 to DAA + 28 (Figure 3). For the mean area/hive covered by eggs and larvae, treatment-related responses were not observed in any of the treatment groups during the entire assessment period (Figure 4). For pupae, the mean covered areas/hive decreased in the T2 group from DAA + 7 to DAA + 28 compared with the CK group, and showed significant differences from those of the CK group on DAA + 15 (Dunnett's test, $p < 0.05$).

Effects on pollen yield. The average amount of pollen collected by bees was 0 g in the R group during the entire test period. There was no significant difference in pollen yields among the T1 and T3 groups and the CK group during the test period. The average amount of pollen collected by bees in the T2 treatment group was always lower than that in the CK group from DAA + 1 to DAA + 7, although no statistical difference was measured (Table 2).

Residues of acetamiprid in pollen. There was no significant difference in the initial measured concentrations of acetamiprid among the 3 treatment groups (2432 ± 502 µg/kg in the T1, T2, and T3 groups). The 3 different acetamiprid formulations had similar active contents and similar residues in pollen after application; however, under semifield conditions, they had different

 © 2019 SETAC

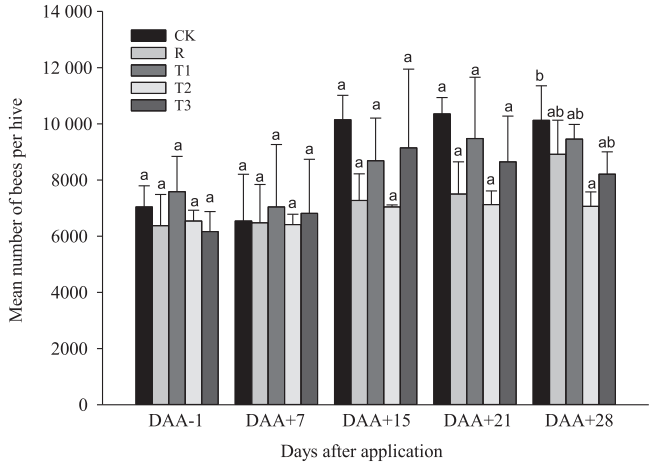

FIGURE 3: Number of honeybees/hive on different assessment dates. Data were obtained from triplicate assays and are given as mean ± standard deviation. Bars with the same letters are not significantly different ($p < 0.05$). CK = blank control; R = positive control (40% dimethoate emulsifiable concentrate); T1 = treatment group with 5% acetamiprid soluble concentrate (SL) containing 10% *N*-methyl pyrrolidone; T2 = treatment group with 5% acetamiprid SL containing 10% Silwet L-77; T3 = treatment group with 5% acetamiprid SL containing 10% Triton X-100.

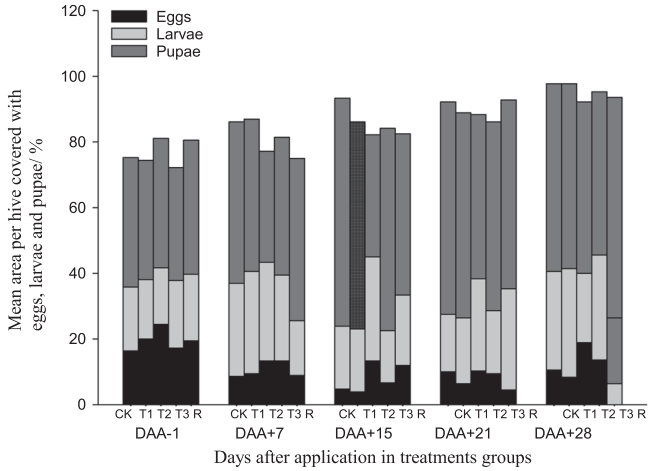

FIGURE 4: Area/hive covered with eggs, larvae, and pupae during the assessment period. Data were obtained from triplicate assays and are given as mean ± standard deviation. ♦ = significantly lower than that in CK ($p < 0.05$). CK = blank control; R = positive control (40% dimethoate emulsifiable concentrate); T1 = treatment group with 5% acetamiprid soluble concentrate (SL) containing 10% *N*-methyl pyrrolidone; T2 = treatment group with 5% acetamiprid SL containing 10% Silwet L-77; T3 = treatment group with 5% acetamiprid SL containing 10% Triton X-100.

toxicities to honeybees. Therefore, adjuvants might influence the toxicity of acetamiprid to bees.

Influence of the 3 adjuvants on the toxicity of acetamiprid to honeybees. As a cyano-substituted neonicotinoid insecticide, acetamiprid exhibited a much lower bee toxicity than the nitro-substituted compounds such as imidacloprid, thiamethoxam, and dinotefuran. Iwasa et al. (2004) suggested that the cytochrome P450 enzymes might be an important mechanism for acetamiprid detoxification and thus a reason for its low toxicity to honeybees. However, cyano-substituted neonicotinoids have appeared to be more likely to interact with other compounds and show synergistic effects. Research undertaken by Iwasa et al. (2004) showed that piperonyl butoxide, triflumizole, and propiconazole increased the toxicity of acetamiprid by 6.0-, 244-, and 105-fold in honeybees, respectively. Silicone surfactant is presently the most effective pesticide adjuvant and has exhibited synergistic toxicity to insects (e.g., boll weevils, *Anthonomus grandis* Boheman) when co-applied with insecticides (e.g., phorate and Zectran; Mullin et al. 2016). It has been proposed that the silicone surfactant Silwet L-77 increases the lethality rate of imidacloprid to Asian citrus psyllid (Srinivasan 2008) and that of horticultural oils to mites (Cating et al. 2015). According to Srinivasan (2008), a combination of Silwet L-77 with imidacloprid afforded adequate control of Asian citrus psyllid nymphs in both laboratory tests and field trials even at a reduced rate (one-tenth the lowest label rate). Therefore, based on the role of organosilicon adjuvants in improving the adhesion, permeability, and bioavailability of neonicotinoid insecticides to bees, a potential synergistic effect between pesticide adjuvants such as Silwet L-77 and acetamiprid is to be expected; however, the synergistic mechanism requires further study. In the present study, although the 3 types of pesticide adjuvants exhibited no obvious toxicity when honeybees were exposed to each one individually in the laboratory, they significantly increased the acute contact toxicity of acetamiprid to honeybees. Furthermore, in the semifield trial, compared with the CK group, Silwet L-77 co-applied with acetamiprid (the T2 group) showed a synergistic toxic effect on honeybees by increasing mortality (on DAA + 4) and decreasing flight intensity (on DAA + 7), colony

TABLE 2: Pollen collected by bees during the assessment period in the control and treatment groups

	Amount of collected pollen (g, mean ± standard deviation)			
Treatment group	DAA + 1	DAA + 2	DAA + 4	DAA + 7
CK	0.464 ± 0.271	0.192 ± 0.295	0.840 ± 0.356	0.901 ± 0.254
T1	0.222 ± 0.075	0.134 ± 0.179	1.262 ± 0.767	0.465 ± 0.560
T2	0.262 ± 0.109	0.072 ± 0.0640	0.281 ± 0.486	0.127 ± 0.219
T3	0.644 ± 0.408	0.285 ± 0.0555	0.728 ± 0.633	0.650 ± 0.307
R	0.000	0.000	0.000	0.000

DAA + X = X day after application; CK = blank control; R = positive control (40% dimethoate emulsifiable concentrate); T1 = treatment group with 5% acetamiprid soluble concentrate (SL) containing 10% *N*-methyl pyrrolidone; T2 = treatment group with 5% acetamiprid SL containing 10% Silwet L-77; and T3 = treatment group with 5% acetamiprid SL containing 10% Triton X-100.

© 2019 SETAC

size (on DAA + 28), and pupae coverage (on DAA + 15). The mortality of honeybees in the T2 group was higher than that in the T1 group on DAA + 4 and DAA + 7, and higher than that in the T3 group on DAA + 4. Therefore, pesticide adjuvants such as Silwet L-77 might be important factors in increasing the toxicity of neonicotinoids to honeybees.

When there is no better alternative in agricultural production and neonicotinic insecticides must still be used, measures should be taken to optimize the formulation of neonicotinic insecticide preparations by developing and using bee-friendly adjuvants to minimize their survival threats to these helpful pollinators.

CONCLUSIONS

Silwet L-77 has wide application as a pesticide adjuvant and is considered to possess low toxicity; however, limited research has been undertaken at the field level to understand its possible adverse effects on nontarget species. In the present study, adjuvants including Silwet L-77 at the normal concentration level in pesticide formulations showed joint adverse effects on honeybees when co-applied with the neonicotinoid acetamiprid. Silwet L-77 not only increased the acute contact toxicity of acetamiprid to honeybees in the laboratory, but also significantly increased the mortality rate of honeybees and reduced the colony size and brooding (e.g., pupae coverage) when co-applied with acetamiprid under semifield conditions. According to the semifield test results in the present study, if appropriate risk reduction measures are taken (e.g., notification of beekeepers before application, and avoidance of spraying during bee flight), the risks to honeybees of 5% acetamiprid SL containing the other 2 adjuvants (NMP and Triton X-100) could be acceptable under the recommended spray application used to control oilseed rape aphids.

The present study enhanced our comprehension of the potential affects of adjuvants on neonicotinoid toxicity to honeybee colonies, and may contribute to our understanding of the complex relationships between the composition of pesticide formulations and harm to bees. Our results underline the importance of considering pesticide adjuvants in honeybee risk assessments, because the joint toxicity effects of acetamiprid and the co-applied adjuvants (e.g., Silwet L-77) were observed not only at the individual organism level, but also at the colony level. To provide guidance for the development and usage of environmentally friendly pesticide formulations, further research is required on the environmental safety of adjuvants and their interactions with active ingredients in terms of nontarget species. In addition, to obtain more accurate information and a scientific theoretical basis for guiding the safe use of pesticides and pesticide adjuvants, further studies are required to determine the sublethal toxicity, long-term (semi-) field effects, mechanism of action, and residue levels in nectar, pollen, foliar, dead bees, honeybees, and honey hives.

Acknowledgment—The present study was financially supported by the National Key R&D Program of China (grant 2018YFD0200100) and the Natural Science Foundation of China (Grants 31201533 and 31801768).

Data Accessibility—Research Data pertaining to this article are located at figshare.com (https://doi.org/10.6084/m9.figshare.7730279).

REFERENCES

Bu YQ, Wang ZC, Zhi Y, Wang JY, Shan ZJ. 2014. Investigation and risk analysis of pesticide inert ingredients in pesticide products (in Chinese). *Agrochemicals* 53:932–936.

Cating RA, Palmateer AJ, Hoy MA, Gazis R. 2015. Management of orchid pests with Silwet® L-77 and horticultural oils. Report PP287. Plant Pathology Department, University of Florida, Gainesville, FL, USA.

Ciarlo TJ, Mullin CA, Frazier JL, Schmehl DR. 2012. Learning impairment in honey bees caused by agricultural spray adjuvants. *PLoSOne* 7:e40848.

Comber MHI, Williams TD, Stewart KM. 1993. The effects of nonylphenol on *Daphnia magna*. *Water Res* 27:273–276.

Cressey D. 2017. The bitter battle over the world's most popular insecticides. *Nature* 551:156–158.

Cresswell JE. 2011. A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees. *Ecotoxicology* 20:149–157.

Delvalls TA, Forja JM, Gomezparra A. 2002. Seasonality of contamination, toxicity, and quality values in sediments from littoral ecosystems in the Gulf of Cádiz (SW Spain). *Chemosphere* 46:1033–1043.

European Food Safety Authority. 2016. Conclusion on the peer review of the pesticide risk assessment of the active substance acetamiprid. EFSA supporting publication 2016:EN-4610. Parma, Italy.

European Food Safety Authority. 2018. Evaluation of the data on clothianidin, imidacloprid and thiamethoxam for the updated risk assessment to bees for seed treatments and granules in the EU. EFSA supporting publication 2018:EN-1378. Parma, Italy.

Fairbrother A, Purdy J, Anderson TD, Fell RD. 2014. Risks of neonicotinoid insecticides to honeybees. *Environ Toxicol Chem* 33:719–731.

Goulson D. 2013. Neonicotinoids and bees: What's all the buzz? *Significance* 10:6–11.

Imdorf A, Buehlmann G, Gerig L, Kilchenmann V, Wille H. 1987. A test of the method of estimation of brood areas and number of worker bees in free-flying colonies [Liebefeld method] (in German). *Apidologie* 18:137–146.

Iwasa T, Motoyama N, Ambrose JT, Roe RM. 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot* 23:371–378.

Mullin CA, Fine JD, Reynolds RD, Frazier M. 2016. Toxicological risks of agrochemical spray adjuvants: Organosilicone surfactants may not be safe. *Front Public Health* 4:92–92.

Organisation for Economic Co-operation and Development. 1998. Test No. 214: Honeybees, acute contact toxicity test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.

Srinivasan R. 2008. Laboratory and field evaluations of Silwet L-77 and kinetic alone and in combination with imidacloprid and abamectin for the management of the Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Psyllidae). *Fla Entomol* 91:87–100.

Stanley J, Sah K, Jain SK, Bhatt JC, Sushil SN. 2015. Evaluation of pesticide toxicity at their field recommended doses to honeybees, *Apis cerana* and *A. mellifera* through laboratory, semi-field and field studies. *Chemosphere* 119:668–674.

Stevens P. 1993. Organosilicone surfactants as adjuvants for agrochemicals. *Pestic Sci* 38:103–122.

Zhao S, Lin BQ, Li XZ, Hui GL, Li F. 2011. Study and application of the technology of progressive and large diameter buried drainage pipe by crossing-hole. *Procedia Eng* 26:852–862.

Zhu W, Schmehl DR, Mullin CA, Frazier JL. 2014. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. *PLoS One* 9:e77547.

 © 2019 SETAC