least 4 weeks before start of the study. Furthermore, the following criteria for the nuclei were guaranteed: (a) at least 2 brood combs with all brood stages; (b) at least 1 honey and pollen combs; (c) bees are free of symptoms of nosema and other bee diseases. Hives used in the study were selected without conscious bias but any that were obviously unhealthy or damaged were not used.

Crop

Cucumber (*Cucumis sativus* L.) seeds (Jinchun 4#) were provided by Nanjing Institute of vegetables and flowers. Cucumber was planted two months before the test, and was managed according to the good agriculture practice (GAP). In order to control the downy mildew, 44% Metalaxyl-M chlorothalonil SC was used for three times (9th, 18th and 25th of June) prior to the first application of the test chemicals.

Experiment procedure

Treatments

The treatments were carried out at full bloom, in the early morning, when cucumber flowers were open. At the time of applications, the environmental conditions were recorded. The application scenarios were as the following:

- Application rate: 75 g sulfoxaflor a.i./ha, with 2 applications at 6-day interval (T1);
- Application rate: 100 g sulfoxaflor a.i./ha, with 2 applications at 6-day interval (T2);
- Negative control (tap water): applied on the same days as sulfoxaflor;
- Positive control (40% dimethoate EC, 100 g product/mu): in order to validate the test system and the exposure process, dimethoate was selected to be the reference substance and was applied on the same day with the second application of sulfoxaflor.

Sulfoxaflor, the reference substance and the tap water in negative control were all applied through spraying.

Introduction, keep and removal of the colonies

The colonies were introduced into the tunnels one day after the first application in the evening (one colony per tunnel). Each tunnel had one entrance, and the colonies were placed at the side opposite to the entrance and in direction to the tunnel. On the day of the second application, the entrances of the test hives were closed in the early morning (prior to the bee flight) and the hives were covered with a plastic sheet, before treatments. After application of treatments, the entrances were opened and the bees were released from the hives. The colonies were left in the tunnels for 11 days. One open container with water was placed into each tunnel. The surface of the water was covered with grasses to prevent the bees from drowning. Before introduction to the tunnels, the hives were maintained in the apiary (Huangmei town, Jurong city, Zhenjiang city, Jaingsu province, about 60 km away from the field site). After the exposure phase of the bees (five days after the second application), the hives were transported back to the same apiary for continuing feeding and observation.

Assessments of the toxicity endpoints

Behaviour of honey bees:

In order to collect and record the dead bees in and outside the hive, a bee trap was set around the hive entrance. And the paths in the middle of the tunnel and around the tunnel were covered with blue nylon nets. The number of dead bees in the bee trap and on the blue nylon nets was recorded every day. On the day of application, the recording frequency increased, such as 2 h, 4 h and 6 h after application and in the nightfall when bees stop flying. Besides, aggressive behaviour and other toxic symptoms were recorded daily.

Flight intensity:

Bee flight intensities were observed in the morning, noon and evening from the day after bee introduction to the day before the second application, 5 days after the second application. And on the day of application, the recording frequency increased, such as 2 h, 4 h and 6 h after application. At each assessment time the number of bees that are both foraging on flowering cucumbers and flying over the crop were counted on a square of 1 m^2 for 1 minute. In each assessment time and plot, the square (3 sites) observed was chosen randomly.

Colonies conditions and sizes:

The conditions and sizes of the colonies were assessed for three times: before and after exposure, at the end of the field study.

The following parameters were visually estimated and recorded when assessing the colonies conditions: presence of the queen (healthy, presence of eggs, presence of queen cells); visual assessment of the pollen and nectar storage area; visual assessment of the area containing eggs, larvae and capped cells. For the estimation of colony conditions, each side of a comb was divided into 32 equally sized areas. The number of areas per comb side fully and/or half covered with eggs, larvae, capped cells, pollen, nectar and adult bees, were estimated and recorded. For each comb side, the number of areas was summed up to a maximum of the equally sized area number. This was carried out for all combs of each hive. Afterwards the mean values were calculated for each hive.

The size of each colony was measured by weighing. When bees were present on the walls inside the bee hives, they were estimated accordingly and were added to the number of areas of one or more of the comb sides.

Flower collections

Cucumber flowers were taken and analysed for sulfoxaflor. Sampling started from the beginning of the test till the end of exposure. Every day, 10 flowers (about 2 g) for each tunnel were collected. All samples were timely transported to the analysis site under low temperature and were subsequently stored deep frozen at about −18 °C.

LC-MS/MS analysis of flowers

Weigh 2 g sample of cucumber flower and put the sample into 80 mL centrifuge tube, add 10.0 mL ace-

tonitrile, 1 g anhydrous magnesium sulphate and 1 g sodium chloride successively and mix them, use homogenizer to homogenize and extract them for 2 minutes with the speed of 15000 rpm, use supercentrifuge to centrifuge for 5 minutes with the speed of 8000 rpm, get 2 mL supernatant and put the supernatant into 10mL centrifuge tube, and add 0.05 g PSA, 0.01 g active carbon and 0.15 g anhydrous magnesium sulphate to purify, swirl them for 3 minutes and centrifuge them for 5 minutes with the speed of 8000 rpm, get supernatant to filter through 0.22 μm filter membrane and wait to be measured by LC-MS/MS.

Statistical analysis

All data were expressed as means ± SD. SPSS 22.0 was used to perform statistical analysis. The relevant values were analysed through One-way Analysis of Variance (ANOVA) followed by LSD test. Statistical significance was considered at $P < 0.05$ and highly significant difference was considered at $P < 0.01$.

Results

Behaviour of the bees

During the exposure period, many bees were clustering at the top of the tunnel, and it is bees' native response when they were confined within the tunnels. Except that, not any other abnormal activities were observed in the sulfoxaflor treatment groups and the blank control groups. But bees in the positive control groups were fairly irritable after the application of reference substance (dimethoate).

Mortalities

After being introduced into the tunnels, a small number of bees died. One day after the introduction (1DAE), large number of bees died in each group for the confinement of the tunnels. The number of dead bees of T1 group was the highest, which was extremely significantly different with that of the blank control ($P < 0.01$). And the number of dead bees of untreated positive control group was lower than that of T1 group, but was also extremely significantly different with that of the blank control ($P < 0.01$). The number of dead bees of T2 group was lower than that of the untreated positive control group but apparently higher than that of the blank control ($P > 0.05$). It could be supposed that the bee colonies were confined in the tunnels. Two days after the introduction (2DAE), the numbers of dead bees of blank control group and T2 group increased, while the number of dead bees of T1 and the untreated positive control decreased. The numbers of dead bees of T1 group and T2 group were still higher than that of the blank control, but the differences were not significant ($P > 0.05$). Three days after the introduction (3DAE), the numbers of dead bees of all groups all decreased, the numbers of dead bees of T1 group and T2 group were obviously higher than that of the blank control, and the differences were extremely significant ($P < 0.01$). Four days after the introduction (4DAE), the numbers of dead bees of all groups continued decreasing, the numbers of dead bees of T1 group and T2 group were still higher than that of the blank control, and the difference between T2 group and blank control was significant ($P < 0.05$). Five days after the introduction (5DAE), the numbers of dead bees of all groups continued decreasing, the numbers of dead bees of T1 group and T2 group were still higher than that of the blank control, but the differences were not significant ($P > 0.05$). Six days after the introduction (6DAE), the second application was conducted; meanwhile, the positive control substance (dimethoate) was applied. After the application, the numbers of dead bees of all groups increased significantly, among which the number of dead bees of positive control group was the highest, the number of dead bees of T1 group was lower than that of the positive control group but much higher than that of the T2 group. The numbers of dead bees of positive control group, T1 group and T2 group were extremely significantly higher than that of blank control group ($P < 0.01$). When comparing with the positive control group, the difference between the T1 group and the positive control group was not significant ($P > 0.05$), but the difference between the T2 group and the positive control group was significant ($P < 0.05$) with the number of dead bees of T2 group significantly lower than that of the positive control group. One day after the second application (7DAE), the numbers of dead bees of all groups all decreased, but the number of dead bees of positive control group was still the highest, the number of dead bees of T2 group was lower than that of the positive control group but much higher than that of the T1 group. The differences between the positive control group, the T2 group and the blank control group were extremely significant ($P < 0.01$), and the differences between the T1 group and the blank control group were significant ($P < 0.05$). The numbers of dead bees of T1 group and T2 group were significantly lower than that of the positive control group ($P < 0.05$). Two days after the second application (8DAE), the numbers of dead bees of all groups continued decreasing, the number of dead bees of positive control group was still the highest, and the number of dead bees of T1 group was higher than that of the T2 group. The difference between the positive control group and the blank control group was extremely significant ($P < 0.01$). The difference between the T1 group and the blank control group was significant ($P < 0.05$). The difference between the T2 group and the blank control group was not significant ($P > 0.05$). The number of dead bees of T2 group was significantly lower than that of the positive control group ($P < 0.05$). Three days after the second application (9DAE), the numbers of dead bees of all groups continued decreasing, the number of dead bees of positive control group was still the highest, and the number of dead bees of T1 group was still higher than that of the T2 group. The difference between the positive control group and the blank control group was extremely significant ($P < 0.01$). The difference between the T1 group and the blank control group was significant ($P < 0.05$). The difference between the T2 group and the blank control group was not significant ($P > 0.05$). The numbers of dead bees of T1 group and T2 group were significantly