TITLE

Honey bee Toxicity of Residues on Foliage (RT25) Study
Pollinator Research Task Force – Summary of Results and Recommendations

TEST GUIDELINE

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AUTHOR(S)

Joseph D. Wisk, BASF Corporation
Max Feken, Syngenta Crop Protection
Tim Joseph, Landis International
Verissimo Sa, Corteva Agriscience
Daniel Schmehl, Bayer CropScience
Rajwinder Singh, BASF Corporation
Hudson Tome, FMC Corporation

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PERFORMING LABORATORY/SPONSOR

Pollinator Research Task Force, LLC
c/o Landis International, Inc.
3185 Madison Highway
Valdosta, GA 31601

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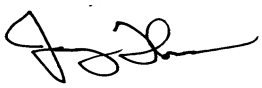

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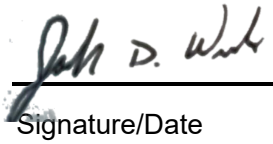
Jamey Thomas
Chair, Administrative Committee
Pollinator Research Task Force, LLC

Good Laboratory Practice Statement

This study was not conducted in accordance with the rules and regulations set forth under the EPA Code of Federal Regulation Title 40 Part 160 and differs in the following ways:

This study is not required to meet the standards of good laboratory practices because it does not meet the definition of a study contained in part 160.3 as there is no test material or experimentation.

STUDY DIRECTOR: As this study does not meet the definition of a study as defined in part 160.3 there is no study director of record.



June 13, 2022

Signature/Date

Joseph D. Wisk, Ph.D., DABT
BASF Corporation, Crop Protection
P.O. Box 13528
Research Triangle Park, NC 27709-3528

Background and Study Design

The honey bee toxicity of residues on foliage study (OCSPP 850.3030) is a laboratory/field test designed to determine the length of time over which field-weathered foliar residues remain acutely toxic to adult honey bees through contact exposure. The test substance (a typical end-use product; TEP) is applied to crop foliage (*e.g.*, alfalfa); the foliage is then harvested at predetermined intervals post-application, and test bees are caged along with the treated foliage for 24 h. If mortality of bees exposed to the foliage harvested 24 h after the application is greater than 25%, additional weathered, treated foliage samples continue to be taken every 24 h (*i.e.*, 48, 72, 96, 120 h, *etc*. post-application) and bees are then exposed to these additional samples for 24 h until mortality of bees exposed to the treated foliage is 25% or less. Results are expressed in terms of the length of time (in hours) required to reduce mortality in exposed bees to 25% or less following application at a specific rate of application (lb a.i./A).

Traditionally, the residual toxicity (RT_{25}) information has been considered useful to growers and beekeepers to ensure bee safety, as it can help them determine the appropriate amount of time between pesticide application and introducing bees into the field or orchard However, while compiling and reviewing the available RT_{25} data, U.S. EPA identified inconsistencies and variability in RT_{25} values between formulated products of the same pesticide active ingredient (personal communication, US EPA). EPA also noticed that these data did not appear to be correlated with chemical/physical characteristics of the pesticide active ingredient. Therefore, the Pollinator Research Task Force (PRTF), in collaboration with EPA, took on the task to review the U.S. EPA's test design (OCSPP 850.3030) and work with different stakeholders to improve the method, and attempt to ensure the reliability and predictive nature of RT_{25} data.

Test Design Improvements

When the PRTF reviewed the study design with various contract research organizations (CROs), the following potential sources of variation in the test design were identified:

- Use of variable test cage sizes which potentially lead to inconsistent exposure.
- Size (cut) and placement of treated foliage in cages.
- Inconsistencies in product application, crop condition, and ambient field conditions, including environmental parameters during weathering in the field. Examples of inconsistencies are listed below:
    - Crop grown in the field versus grown in flats in greenhouse.
    - Variable age of foliage used in the test. The type of alfalfa used, including smooth vs. hairy types, and erect vs. creeping.
    - Product application in the field versus application in lab using a spray booth.
    - Environmental conditions during application (e.g., wind speed, temperature, humidity)
    - No recommendation for environmental parameters during weathering in the field.

Based on our review, the PRTF recommended the following:

- Application of test substance to field grown alfalfa which is between 20-40 cm high
- A one-pass application over the treated crop (alfalfa (*Medicago sativa*))
- Weathering of treated alfalfa in the field
- Analytical measurements of residues of the test substance on alfalfa leaves at various time points
- The use of test bioassay cages of similar size and dimension (transparent 32 oz plastic containers with upper diameter = approx. 11 cm, base diameter = approx. 9 cm; height = approx. 14 cm)
- Optimized size and placement of foliage cuttings (12-15 cm lengths and loosely placed 15 g portions upright/diagonally in each test cage)
- Test conducted using young adult worker honey bees that are of a similar age (three to five days post-emergence)

Results from PRTF-Sponsored Trials

The PRTF organized a ring test and additional method development trials with CROs in 2020 and 2021. In 2020, a ring test was conducted at three test facilities, two in the U.S. and one in Brazil using products containing the active ingredient dimethoate. The results of the trials were highly variable between the three facilities, with RT_{25} values ranging from between 6 to 24 hours to > 120 hours. In both trials at the U.S. facilities, analytical measurement of concentrations of dimethoate on treated alfalfa leaves as well as on randomly placed spray cards in the treated plots were carried out. Analytical measurements on the spray cards indicated that the facility which used a modified boom sprayer with a single pass application over the treated plot had more consistent residue coverage in the crop than the facility which used a backpack sprayer and conducted two passes over the crop. This indicated that the field application portion of the test was a significant source of variability. Therefore, a recommendation was made for conducting future trials with a single pass spray application.

Another potential source of variability between the trials was that they were conducted at different times with different weather conditions (hot and dry versus warm and humid). Therefore, the next phase of the project included an analysis of the effect weather conditions had on the results. A formal report of the 2020 ring test has been prepared and submitted to U.S. EPA (MRID #51646901).[1]

In 2021, the PRTF sponsored additional work to attempt to standardize the test design. Trials were sponsored at two U.S. laboratories, located in the State of North Carolina. Once again, a formulated product containing the active ingredient dimethoate was used. The close proximity of the two facilities to one another allowed for the sharing of treated alfalfa samples to conduct bioassays on each facility's treated alfalfa. This would help confirm whether the bioassays themselves were a source of variability. Two separate time coordinated applications were made at each facility, one in early June, when it was expected to be hot and dry and a second in mid-September, when it was expected to be more humid. A total of four bioassays were conducted at each facility.

The results from the June applications and bioassays at each facility provided consistent results, with the laboratories reporting RT_{25} values between 6 and 24 hours for all four bioassays. For the September applications, the bioassays on treated alfalfa from one of the two facilities produced results that were consistent with the results from the samples from the June applications (i.e., RT_{25} values between 6 and 24 hours). However, the bioassays on the alfalfa samples from the second facility produced results that were inconsistent with the other CRO's alfalfa sample bioassays and with the results from the samples from the June applications. Once again, the analytical results from spray cards and alfalfa samples indicated inconsistent spray coverage on the test plot for this application.

The overall results indicate that bioassay portion of the test was consistently harmonized and any variability is likely due to the field application portion of the study. A formal report of the 2021 trials has been prepared and submitted to U.S. EPA (MRID #...).[2]

Usefulness of Results within US EPA's Risk Assessment Process for Pollinators

The U.S. EPA's pesticide risk assessment process has significantly evolved over the past decade with the release of the Agency's proposed risk assessment process for bees[3] and guidance for assessing pesticide risk to bees.[4] The process requires acute and chronic toxicity data for both adult and larval honey bees as well as exposure estimates for a screening-level risk assessment. The guidance also outlines steps to refine the screening-level risk assessment through the conduct of higher-tier semi-field and field studies to measure exposure to and/or effects on honey bee colonies. The results of the various studies not only determine the potential risk of a pesticide product to honey bees or other insect pollinators under worst-case conditions, but also helps to determine whether a product can be applied during bloom (including night time applications) without presenting a high risk to honey bee colonies.

The usefulness of the Toxicity of Residues on Foliage study is somewhat limited in the overall risk assessment process since the test only evaluates effects on adult honey bees and only considers the contact route of exposure. Even if the results from this test showed low residual toxicity (e.g., RT_{25} < 6 hours) for a highly toxic pesticide, it's unlikely that the product could be registered for a bloom-time application to a highly bee attractive crop unless results from the formal risk assessment, including the results from higher-tier exposure and/or effects studies indicated a low risk to honey bee colonies. However, the RT_{25} study could be a useful screening tool to evaluate whether it could be possible to allow bloom time applications (including applications at night) on a crop that is highly attractive to bees. For example, if the results of the study indicate low residual toxicity, it could be followed up with higher-tier exposure or effects studies to confirm a low risk to honey bee colonies. On the other hand, if the results of the study indicate long residual toxicity (i.e., RT_{25} > 24 hours), the results could shift the focus to evaluating the risk of applications outside of the bloom period (e.g., pre-bloom) for crops that are highly attractive to bees.

Conclusions and Recommendations

The efforts of the PRTF have effectively enhanced the science of bee testing in harmonizing the test design for the Honey Bee Toxicity of Residues on Foliage (RT_{25}) study. However, as with any study with a field application component, there will always be some uncontrolled variability

that could affect the overall results. Interpretation of the results was greatly aided with the inclusion of the analysis of residues on the treated alfalfa and the inclusion of the analysis of spray cards in the trials.

Although results from this study can inform the environmental hazard label language for bees, it is important to note that this study does not produce an endpoint that drives EPA's risk assessment process for bees. It should be viewed as an advanced screening tool that helps inform higher tier testing programs to evaluate risk of products to honey bee colonies when applied to bee-attractive blooming crops.

Based on the results from PRTF sponsored research over two testing seasons, a proposed test protocol for future studies is included in this document as Appendix A.

References

1. Clark, S.L. 2021. Results of the Honey Bee (*Apis mellifera*) Toxicity of Residues on Foliage (RT_{25}) Ring Study, MRID #51646901.
2. Clark, S.L. 2022. Results of the Honey Bee (*Apis mellifera*) Toxicity of Residues on Foliage (RT_{25}) Ring Study, Phase II, MRID #51895501.
3. White Paper in Support of the Proposed Risk Assessment Process for Bees 2012. U.S. Environmental Protection Agency Office of Pesticide Programs Environmental Fate and Effects Division, Health Canada Pest Management Regulatory Agency Environmental Assessment Division, and California Department of Pesticide Regulation. Submitted to the FIFRA Scientific Advisory Panel, September 11 – 14, 2012.
4. Guidance for Assessing Pesticide Risk to Bees 2014. U.S. Environmental Protection Agency Office of Pesticide Programs, Health Canada Pest Management Regulatory Agency, and California Department of Pesticide Regulation.

Appendix A

Proposed Protocol for Honey Bee Toxicity of Residues on Foliage (RT25) Study

Proposed Protocol:
Honey Bee Toxicity of Residues on Foliage (RT_{25}) Study

Based on EPA's Ecological Effects Test Guideline OCSPP 850.3030, dated January 2012, with modifications

1. **Purpose:** This guideline is intended for use in developing data on the residual toxicity to honey bees of chemical substances and mixtures ("test chemicals" or "test substances") subject to environmental effects testing requirements. This guideline describes a toxicity test in which honey bees are exposed to weathered residues of a test substance on treated foliage.

2. **Definitions:**

   a) Acute Residual Toxicity is the adverse effects occurring over a period of time (hours or days) from a single dose of the test substance to foliage.

   b) Dose is the amount of test substance applied. Dose is expressed as a mass, pounds of test substance per acre (lbs/A) and for a pesticide, pound(s) of active ingredient applied per acre (lbs a.i./A). The dose used in this test should be the maximum, single application dose allowable according to the end-use product labeling.

   c) Mortality: an animal is recorded as dead when it is completely immobile (*e.g.*, no movement within 5 seconds).

   d) RT_{25} is the residual time needed to reduce the activity of the test substance and bring bee mortality down to 25% in cage test exposures to field-weathered spray deposits (see paragraph (e)(2) of this guideline). The time period represented by this toxicity value (RT) is considered to be the length of time (in hours) that the test substance is expected to remain toxic by contact to bees in the field, when bees are exposed to weathered residues of the test substance on vegetation at an expressed rate of application (lb a.i./A). Exposure to weathered residues in the laboratory are a surrogate for field conditions.

3. **Summary of test:** The honey bee (*Apis mellifera*) foliar residue study is a laboratory test designed to determine the length of time over which field-weathered foliar residues remain toxic by contact to honey bees. The test substance (*e.g.*, a typical end-use product) is applied to crop foliage, the foliage is harvested at predetermined intervals post-application, and test bees are caged on the treated foliage. Results are expressed in terms of the length of time (observed time interval) following application, during which residues continue to cause 25% mortality (RT_{25}) in test populations at an expressed rate of application (lb a.i./A).

4. **General test guidance:** Based on EPA's Ecological Effects Test Guideline OCSPP 850.3030, dated January 2012, with some modifications.

5. **Definitive test**: The goal of the definitive test is to determine the 24-h RT_{25}, length of time post-application that residues of the test substance on foliage are toxic to honey bees. For this determination, one treatment level, the maximum application rate on the label, and at least three different time intervals between application and harvest are typically used. The test substance should be evaluated at the labeled maximum, single application rate. A summary of test conditions is provided in Table 1, and validity elements for an acceptable definitive test are listed in section 11 of this protocol.

6. Test specifications:

6.1. **Test organism**:

a) **Species:** Honey bee, *Apis mellifera*, is the test species.

b) **Source:** Bees may be obtained from on-site colonies or from a commercial apiary. All control and treatment bees used in a test should be from the same source and breeding lineage. Bees are emerged from brood frames taken from the source colonies in an incubator (34-35 °C, 45-90% humidity) and reared for three to five days with "bee bread" (pollen that is already stored on the brood frame) supplemented with pollen patty and 50% w/v sucrose in water solution. In order to obtain a sufficient number of bees with known age (3-5 days post-emergence), brood frames can be collected from multiple colonies within the same apiary. Collection in early spring or late autumn should be avoided, as the bees have a changed physiology during this time.

c) **Age:** The test should be conducted using young adult worker bees that are of a similar age (three to five days post-emergence) and feeding status.

d) **Health status:** Bees used in the test should be in apparent good health. Only bees from apparently disease-free colonies should be used, and they should be kept in conditions conforming to proper culture practices. Bees from hives treated with chemical substances, such as antibiotics, anti-varroa, *etc*., should not be used for toxicity tests for four weeks from the time of the end of the last treatment.

e) **Care and handling:** During holding and testing, bees should be shielded from excessive activity, handling stress or other disturbances and kept in the dark. Bees should be handled only as much as is necessary to conform to test procedures.

f) **Diet and feeding:** A 50% weight/volume (w/v) or weight/weight (w/w) solution of sugar/water (500 grams/liter) is provided *ad libitum* throughout the holding and test periods. Purified or distilled water should be used for preparation of the sugar solution. Top feeding is preferred, so for the ring test, the feeding syringe/tube should be inserted through an opening in the top of the test cage. Attention should be paid to avoid any contact between the feeders and the treated foliage.

6.2. **Test crop:** The test crop is alfalfa (*Medicago sativa*). Alfalfa should be grown in an unshielded open outdoor field location. Foliar applications of the test substance should be performed when the alfalfa crop is between 20-40 centimeters in height. To ensure harvest is not impeded by excessive weed growth, pre-emergence and early post-emergence herbicide applications may be made to the cropped area. Applications of any maintenance pesticides (herbicides, fungicides, insecticides) must not be made within 4 weeks of the start of the study. Fertilizer and irrigation treatments may be made as needed consistent with good agronomic practices up to 24 hours before start of the study but must not be made during the study. All agronomic practices, variety of alfalfa, the seeding rate, date of planting, fertilizer, irrigation and pesticide treatment history for the three years prior to the start of the study, should be reported. If seeds treated with seed-applied pesticides are used to establish the crop, the field should not be used for RT_{25} studies for 1 year from planting.

6.3. **Test duration**: The test starts with the placement of weathered treated foliage into cages with bees, followed by a 24-h observation period during which mortality and clinical signs of toxicity are recorded at 4±1 and 24±1 h post-exposure.

6.4. **Post-treatment weathering intervals**: The treated foliage should be harvested at minimum three mandatory intervals of 3±1, 6±1 and 24±1 h post-application, and placed in cages to expose young adult honey bees to the weathered residues of test substance. If mortality of bees exposed to the foliage harvested 24 h after the application is greater than 25% (control-corrected), weathered, treated foliage samples should continue to be collected and tested at 24-h intervals until the mortality is ≤25% (control-corrected), up to five days post-application.

6.5. **Observation period**: Bees are observed for 24 h after the bees and treated foliage are placed onto the cages.

6.6. **Test facilities:** Test substance application and weathering should occur outdoors under natural field conditions. The bee exposure portion of the test should be conducted indoors to control lighting and other environmental variables, while bees are being maintained in small test cages. The cages containing honeybees should be placed in an environmental chamber to control temperature and relative humidity.

6.7. **Test cages**: Transparent 32 oz plastic containers (upper diameter = approx. 11 cm, base diameter = approx. 9 cm; height = approx. 14 cm) (see Fig .1). The top of the test cage is covered with a screened lid to allow ventilation and has an opening for inserting a feeding syringe/tube.

6.8. **Collection of bees**: The day prior to exposure, young bees should be collected from frames kept in the incubator and acclimated for approximately 24 hours. The bees can be acclimated in bulk or acclimated in the actual test cages. If the acclimation occurs in the test units/cages, dead and impaired bees should be removed and, if needed, replaced by healthy bees from the same pool of newly emerged bees prior to the introduction of the test foliage. If acclimation occurs in the test cages, it is recommended that excess bees be acclimated in excess test cages in case there is a need to replace dead or impaired bees prior to test initiation. Introduction of bees into the test cages shall be done in an indiscriminate manner. During transfer to the exposure cages, immobilization of bees with cold temperatures, carbon dioxide gas (CO_2) or nitrogen gas (N_2), may be necessary but should be kept to the minimum.

6.9. **Controls:** Paired negative (untreated) controls are included in the test. Control crop foliage is treated with water only and identically to treatment plots, except for applications of the test substance. Control and test bees are kept under the same environmental conditions.

6.10. **Number of test organisms and replicates**: Six replicates should be assigned to each treatment and control group at each post-application interval, with a minimum of 25 bees for each replicate. Test organisms should be impartially assigned to different treatment groups.

6.11. **Test substance**: A description of the test substance should include: identification, source, name of active ingredient(s), lot or batch number, purity, and expiration date.

6.12. **Application of test substance**: The test substance should be applied at the maximum single application rate for the use(s) to be evaluated. A single application should be made in the morning after the dew has dried and when alfalfa crop is between 20-40 centimeters in height. Application should be made in the field with a tractor mounted or hand-held boom sprayer, using standard nozzles in accordance with regionally accepted practices. The sprayer should be calibrated on the day of, or a day prior to the spraying of the plants. Spray tank solutions should be continuously stirred or circulated prior to and during use. Nozzle height above the crop during application

should be maintained consistent with manufacturer recommendations. Wind speed should be less than 3 m/sec during application. Spray equipment should produce a wide enough swath so that the alfalfa plots can be treated in single-pass spray. Detailed aspects of the application should be reported including nozzle type, spacing, height above crop canopy, flow rate, pressure, application speed and pass times, nominal and actual volumes applied, results of equipment calibration, volumes and concentrations of spray solutions prepared. Environmental conditions during application should be recorded including air temperature, relative humidity, soil moisture, presence/absence of dew or moisture on the crop, cloud cover, wind speed, application time of day (beginning and end of spraying), time of sunrise and sunset and any other relevant observations that may affect the interpretation of the results.

6.13. **Field plots and harvest of foliage**: Plots should be at least 1 m^2 (10.8 square feet) in alfalfa grown according to standard agricultural practices. Applications of any maintenance pesticides (herbicides, fungicides, insecticides) must not be made within 4 weeks of the start of the study. At a minimum, nine test substance treatment plots are used to obtain three plots for harvesting at each time interval (3±1, 6±1 and 24±1 h post-application). After test substance residues have aged (weathered) for the appropriate time period, alfalfa foliage sufficient to place in six treatment cages (approximately 180 g fresh weight or 6,000 cm^3 total), should be harvested in 12-15 cm long sections from three treated test plots using hand equipment, placed individually in labeled bags and returned immediately to the laboratory for processing and placement in test cages. Treated foliage should be collected, using a random sampling scheme, from the top 15 cm of the canopy. Minimum distance of 10 m should be kept between treatment and control plots to avoid potential contamination of control plots due to drift. At each of the minimum time intervals, three alfalfa samples are harvested from the control plot using a random sampling scheme, to obtain sufficient foliage to place in six control cages. If additional harvest intervals are required beyond the minimum two, control samples must be collected and tested also at each harvest interval.

6.14. **Preparation of treated foliage:** Samples of foliage are returned to the laboratory in bags and transported in coolers that should be held between 8 and 12 °C once the coolers are filled and closed. Temperature data loggers should be included in the coolers. The samples for each treatment are mixed thoroughly and then divided into approximately 15 g or 500 cm^3 portions. Leave the foliage in 12-15 cm lengths and loosely place 15 g portions upright/diagonally in each test cage to maximize the exposure.

6.15. **Introduction of the bees to the treated foliage in the cages:** Bees should then be released on the top of the foliage or the treated foliage added directly into the test cages if the bees are being acclimated in the test cages. Special attention should be paid to avoid any direct contact between the sugar solution feeders and the treated foliage.

6.16. **Sampling for residue analysis:** Collect an approximately 15-g sample of the treated and untreated control foliage immediately after the spray has dried (approximately 1 hour ± 30 minutes) and at each harvest to confirm test substance concentration. If the study extends past 24 hours, then continue to take samples of foliage at each 24-h interval thereafter, to correspond with the exposure, up to 5 days post-application. Fresh sample weights should be recorded before freezing the samples. In addition, analytical evaluations should also be conducted on spray solution (*i.e.*, tank mix). The spray solution sample should be collected after completion of the application. Samples are to be transported from the field and subsequently deep frozen until shipment to the

designated analytical laboratory. Samples should be transferred to the designated analytical laboratory deep frozen.

6.17. **Spray cards:** Although not required by the test guideline, the study Sponsor may include the placement and analysis of spray cards in the study. This would be done to evaluate the consistency of test substance coverage on the treated crop. If spray cards are included, the recommendation is to have at least three spray cards (preferably glass fiber discs) placed randomly in the test plots for the application. The spray cards should be held in a horizontal position at the top height of the crop canopy so that it gets the full rate of the spray without interception by the crop. At the time of collection, the spray cards should be folded and placed into plastic bags similar to those used for foliage collection.

6.18. **Environmental conditions**:

a) **Environmental conditions during application and weathering in the field:** Sunlight, precipitation and temperature are three extremely important factors in the dissipation of pesticide residues. Test substance application should be made preferably on clear days with maximum temperatures ranging between 20-40 °C and <30% chance of precipitation. Application should happen in the morning after dew or moisture from any overnight rains has dried off. Test plots should be protected from direct precipitation for at least 3 h (up to 6 h) following the application. If rainfall should occur, the test plots should be sheltered from direct rainfall using a tarp or other suitable canopy. If a canopy is used, it should be removed 3 h (up to 6 h) after application to allow full effect of natural weathering to take place (*i.e.*, direct sunlight). Also, application should be avoided in windy conditions (*i.e.*, average wind speed >3 m/s) to avoid contamination of untreated control plots. Treated test crop should be allowed to weather outdoors under natural field conditions.

b) **Environmental conditions during exposure phase**: Environmental parameters in the laboratory during the bioassays should be maintained as follows:

I. Temperature and humidity. Temperature should be maintained at 33 $\pm$ 2°C, with relative humidity between 50% and 80%.

II. Lighting and photoperiod. It is recommended that test bees be maintained in the dark except during transfer to test cages and observations.

III. Test cages, including treated and control cages, are placed within the incubator in a randomized pattern which is also recorded.

7. Observations:

7.1. **Analysis for test substance concentrations:** Test substance residues on treated foliage are expressed in parts per million (ppm; mg ai/kg foliage) fresh weight. Concentrations in spray solution (*i.e.*, tank mix) should be expressed as mg a.i./L. If spray cards are used and analyzed, results should be reported as mg a.i./cm^2 and in units of lb a.i./acre.

7.2. **Field site conditions**: Environmental conditions should be monitored at the field site at the time of test substance application and during weathering period. Environmental information to be collected should include daily minimum and maximum air temperature, precipitation, and relative humidity. Wind speed and estimated cloud cover should be recorded at least at the time of application. A data-logging weather

station shall be placed on site, within 1 km of the application area, to collect environmental data.

7.3. **Conditions during exposure in the lab**: Temperature and relative humidity should be recorded during the bee exposure in laboratory test cages.

8. **Measures of Effects**:

8.1. **Mortality:** For a given weathered residue treatment or control, bees should be observed for mortality at least once at 4±1 h after exposure and at exposure termination at 24 h. Dead bees should not be removed from the test cages until the test is terminated.

8.2. **Appearance and behavior**: For a given weathered residue treatment or control, bees should be observed for all clinical signs of intoxication and any other abnormal behavior once during the first 4±1 h after exposure and at test termination (24 h). Observations should be recorded by treatment level and by time of occurrence. Signs of intoxication are those behaviors apparently due to the test substance and may include a wide variety of behaviors, such as ataxia, lethargy, excessive cleaning, tremors, convulsions and hypersensitivity (agitation). Prior to the evaluation at test termination, observations should be made without disturbing or removing bees from the test chambers; for these observations, estimates of mortality and effects are sufficient.

9. **Treatment of results**:

9.1. Descriptive summary statistics:

a) Environmental conditions: Data should be summarized in tabular form, showing the range and mean temperature, precipitation, relative humidity, and wind speed.

b) Mortality. Data should be summarized in tabular form, showing for each weathered age of foliage treatment and control the number of bees initially exposed, mortality at each observation time, and the percent mortality. Correct the mortality observed in the treatments for average mortality using Abbott's formula.

c) Appearance and behavior. Data should be summarized in tabular form, showing for each weathered age of foliage, appearance and behavior at each observation time. Statistical analysis of sublethal effects are not conducted.

9.2. Residual Time (RT_{25}): A test for comparing two paired populations (*e.g.*, paired t-test) should be performed to detect significant ($p<0.05$) difference of treatments from controls. Abbott's correction should be used in the event of control mortality. Additional discussion about measurement endpoints and statistical procedures is found in OCSPP 850.3000.

10. **Tabular summary of test conditions**: Table 1 lists the important conditions that should prevail during the definitive test. Meeting these conditions will increase the likelihood that the completed test will be acceptable or valid.

Table 1. Summary of Test Conditions for Honey Bee Toxicity of Residues on Foliage Test

Test type	Toxicity of residues on foliage
Test duration	24 h observation period for each aged residue interval (3±1, 6±1 and 24±1 h aged residue intervals are tested; additional 24 h residue intervals may be appropriate).
Temperature during laboratory exposure	33 ± 2°C
Relative humidity during laboratory exposure	50 – 80%
Lighting	Darkness, except during transfer of bees to treatment cages and observations
Test chamber	32 oz plastic cages with an upper diameter approximately 11 cm, base diameter of approximately 9 cm and height of approximately 14 cm
Foliage cutting length and placement	Foliage lengths of 12-15 cm; upright/diagonally placed in test cages
Test substance application	15-g or 500-cc portions of treated foliage placed in a test cage
Age of test bees	Young adult worker bees of similar age (1-5 days post-emergence) and feeding status
Number of bees per chamber	25 (minimum)
Number of bees per treatment and control	150 (minimum)
Number of treatments	Minimum of 2 treatment groups (3±1, 6±1 and 24±1 h post-application of maximum single application rate) which includes the negative control(s). Additional intervals may be appropriate if mortality is >25% for the 24 h post- application treatment
Feeding	50% sugar/water (w/v) solution *ad libitum*
Measure of Effect or Measurement Endpoint	RT_{25} based upon mortality at 24 h after bees are exposed to foliage. If mortality of bees exposed to the foliage harvested 24 h after the application is greater than 25%, additional weathered, treated foliage samples taken every 24 h.

11. **Test validity criteria:** The definitive test is considered invalid if one or more of the following conditions occurred -

    a) Test bees were not of similar age and feeding status.

    b) More than 20% mortality averaged across control treatments.

    c) All bees in a test were not from the same source (apiary) and breeding lineage.

    d) Concurrent negative (untreated) controls were not included in the test.

    e) Environmental conditions (temperature, precipitation, relative humidity, wind speed and cloud cover) at the field site were not monitored/reported.

    f) Test organisms were not impartially assigned to test cages.

    g) Substances, other than the test pesticide were applied to the growing alfalfa within 4 weeks of test initiation.

12. **Reporting**:

    12.1. **Protocol deviations:** Include a description of any deviations from the test protocol or any occurrences which may have influenced the results of the test.

    12.2. **Test substance:**

    a) End-use product (name, state or form, source), its purity (for pesticides, the identity (common name, IUPAC and CAS names, CAS number) and concentration of active ingredient(s)) and known physical and chemical properties that are pertinent to the test.

    b) Storage conditions of the test substance.

    c) Methods of preparation of test substance for application onto foliage, the maximum label rate, and the actual application rate (lb a.i./A) with the finished spray volume per acre.

    d) Describe the stability of the test substance under storage conditions.

    12.3. **Test organisms:**

    a) Scientific name, race, and source.

    b) Culture method and conditions.

    c) Health status of colonies used for collection of test bees (*e.g.*, any adult diseases, use and application date(s) of any prophylactic or preventative treatments).

    d) Collection method and date of collection.

    e) Holding period.

    f) Age at initiation of exposure to an aged residue treatment.

    12.4. **Test system and conditions**:

    a) Description of housing conditions: type, size, and material of test cages.

    b) Description of any feeding during the test (if applicable), including: method, type of food, source, amount given and frequency.

    c) Common and scientific name of treated crop.

    d) Plot size, and method and time of administration of test pesticide on plots.

e) Number of aging intervals tested.

f) Time after application to plot of foliage collection (age intervals tested) and placement of foliage in test chambers.

g) Plots per aging interval and negative control.

h) Number of bees per test cage.

i) Number of cages (replicates) per aging interval plot and negative control plot.

j) Methods used for test cage and treatment randomization as well as methods for impartial assignment of bees to test cages.

k) Exposure duration to a given aged residue and duration of the study.

l) Methods and frequency of environmental monitoring performed on treated plots during administration of test substance and weathering period for temperature and precipitation, and any other known weather conditions that would impact initial concentration or stability of residue levels on treated plots.

m) Methods and frequency of environmental monitoring performed during the definitive study or positive control study for test room temperature, humidity and lighting.

n) For the definitive test, all analytical procedures and preservation methods should be described. The accuracy of the method, method detection limit, and limit of quantification should be given.

12.5. **Results**:

a) Laboratory environmental monitoring data results (test room temperature, humidity and lighting) in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, maximum).

b) Field site environmental monitoring data results (temperature, precipitation, wind speed, relative humidity, cloud cover) in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, maximum).

c) For the bioassays, the number of dead bees which were observed at least once during the first 4 hours of exposure and at 24 h (provide the raw data).

d) For the bioassays, a description of signs of intoxication and other abnormal behavior, including time of onset, duration, severity, and number affected at each aged residue treatment and control(s) (provide the raw data).

e) Provide 24-h RT_{25} values.

f) Description of method used, including software package, for determining the 24-h RT_{25} value.

g) Results of analysis of variance (ANOVA) to detect significant differences of treatment groups from the controls.

13. **References**: The references in this paragraph should be consulted for additional background material on this test guideline.

a) Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18:265-267.

b) Johansen, C. *et al*., 1977. Bee Research Investigations. Dept. of Entomology, Washington State University, unpublished, 22 pp.

c) Lagier, R.F. *et al*., 1974. Adjuvants Decrease Insecticide Hazard to Honey Bees. College of Agriculture Research Center, Washington State University Bulletin 801, 7 pp.

d) Mayer, D. and C. Johansen, 1990. Pollinator Protection: A Bee & Pesticide Handbook. Wicwas Press. Cheshire, CT.

e) Mayer, D. (approved by), 1996. Standard Operating Procedure (SOPs) – Residue Bioassay. The Bee Group-Irrigated Agriculture Research and Extension Center. Prosser, WA.

f) U.S. Environmental Protection Agency, 1982. Pesticide Assessment Guidelines Subdivision L Hazard Evaluation: Nontarget Insects. Office of Pesticides and Toxic Substances, Washington, D.C., EPA-540/9-82-019.

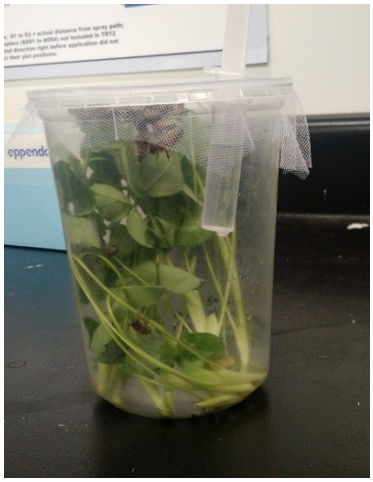
g) U.S. Environmental Protection Agency, 1985. Hazard Evaluation Division Standard Evaluation Procedure, Honey Bee—Toxicity of Residues on Foliage. Office of Pesticides Programs, Washington, D.C., EPA-540/9-85-003.

h) USEPA 2012. Ecological Effects Test Guidelines OCSPP 850.3030: Honey Bee Toxicity of Residues on Foliage. Office of Chemical Safety and Pollution Prevention (7101). EPA 712-C-018. January 2012.

i) USEPA. 2012. Ecological Effects Test Guidelines OCSPP 850.3020: Honey Bee Acute Contact Toxicity Test. Office of Chemical Safety and Pollution Prevention (7101). EPA-712-C-019. January 2012.

j) EPA. 2017. U.S. Environmental Protection Agency's policy to mitigate the acute risk to bees from pesticide products. Office of Pesticide Programs. January 12, 2017. EPA-HQ-OPP-2014-0818-0477.

Fig 1. Test cage



Test cages are transparent 32 oz plastic containers (upper diameter = approx. 11 cm, base diameter = approx. 9 cm; height = approx. 14 cm). The top of the test cage is covered with a screened lid to allow ventilation and has an opening for inserting feeding syringe/tube.