Honeybee Toxicity Testing Frequently Asked Questions – August 16, 2018

The FAQs below reflect EPA's responses to inquiries we commonly receive about protocols used to generate honeybee toxicity data submitted in support of pesticide registration. Registrants and contract laboratories that follow EPA's Pollinator Risk Assessment Guidance may find the FAQs useful when developing protocols. EPA encourages the regulated community to submit questions not answered here or in the Guidance to opppollinatortesting@epa.gov.

DISCLAIMER

These Frequently Asked Questions (FAQs) represent EPA's recommended procedures for honeybee toxicity study design and testing and are provided by the U.S. Environmental Protection Agency (EPA), for use by stakeholders conducting or planning to conduct said studies. These recommendations are not regulations and, therefore, do not add, eliminate or change any existing regulatory requirements. The recommendations are intended solely as guidance. These recommendations are not intended, nor can they be relied on, to create any rights enforceable by any party in litigation with the United States. EPA and stakeholders may decide to follow the recommendations provided in this document, or to act at variance with the recommendations, based on analysis of study-specific circumstances. These FAQs may be revised without public notice to reflect changes in EPA policy.

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General Study Questions

What studies have historically been required to evaluate the potential risk of pesticides to bees?

Studies required to support ecological risk assessments depend on the use pattern(s) of the pesticide and are delineated in Title 40 Part 158 Subpart G of the Code of Federal Regulations (abbreviated 40 CFR 158)

Can EPA require additional studies beyond those specified in the 40 CFR 158?

Yes, EPA has authority to require additional studies deemed necessary to evaluate potential risk.

What additional studies are now being recommended to evaluate the potential risk of pesticides to bees?

In 2011, EPA issued interim guidance on studies used to evaluate potential exposure and effects of pesticides on bees. In 2014, EPA in collaboration with Health Canada's Pest Management Regulatory Agency and the California Department of Pesticide Regulation developed the Guidance for Assessing Pesticide Risks to Bees (PDF)(59 pp, 2 MB) in which a tiered process is described along with the data used to inform that process. In 2016, EPA issued additional guidance for EPA risk assessors on Exposure and Effects Testing for Assessing Risks to Bees (PDF)(44 pp, 1 MB). Also in 2016, EPA issued guidance for risk managers on the Process for Requiring Exposure and Effects Testing for Assessing Risks to Bees during Registration and Registration Review (PDF)(26 pp, 394 K) identified in the 2014 Guidance.

Are there recommendations for adult and larval bee source colony maintenance?

The treatment and maintenance of hives should follow typical beekeeping practices. In cases where disease (*e.g.*, nosemosis, American foul brood) and/or pest (*e.g.*, Varroa mite, small hive beetle) control treatments are needed for the hives from which bees (adults and larvae) are collected, the date(s) of treatment application to the colony and the treatment product(s) should be reported. However, as specified in the Organization for Economic Cooperation and Development test guidelines and guidance documents, there should be no treatment(s) of the hives within the 4 weeks preceding the start of the tests (e.g., OECD Test no. 213)

Are measured test concentrations required?

As with toxicology studies of other taxa, EPA requires analytical verification of test concentrations in all Tier I, II and III toxicity tests. Ideally, all study measurement endpoints should be based in terms of measured concentrations rather than nominal concentrations.

The current guidelines and guidance documents say tests may be conducted using active ingredient or formulated product. Which should be used?

With the exception of the Residue Toxicity on Foliage study (850.3030), all of the laboratory-based studies of individual bees should be with technical grade active ingredient (TGAI); whereas, whole colony studies (with the exception of feeding studies) should be conducted with typical end-use product (TEP). However, there can be situations where the active ingredient has chemical/physical properties that render it difficult to test it as TGAI, and testing of a TEP may be warranted. If a test using a TEP is needed, the study authors should provide analytical results and justification for not testing with the TGAI. More discussion on this topic is provided in the following FAQs regarding chemical solubility and stability.

What if there are solubility, stability or homogeneity concerns with the preparation of the test diet?

Science staff from the Environmental Fate and Effects Division (EFED) of the EPA Office of Pesticide Programs have participated in multiple discussions with contract research organizations (CROs) and the regulated community (registrants) on pollinator study design elements. Both CROs and registrants have expressed concern regarding the testing of chemicals with low aqueous solubility and/or high sorption properties. As discussed in the FAQs associated with each study type below, EPA recommends registrants submit protocols for review prior to initiating tests when there are likely to be issues related to measured versus nominal test concentrations/levels. At this point and until formal study guidance has been developed, these discussions are on a case-by-case basis and should be conducted with the chemical team.

For chemicals that are not readily solubilized, EFED will consider testing with a suspension as an acceptable means of delivery on a case by case basis. The registrant/CRO should demonstrate that the test chemical is stable and uniformly distributed within the diet suspensions (*e.g.*, sucrose solution, royal jelly). Preliminary tests involving the homogeneity and stability of the test material in the diet are useful in supporting discussions with EPA chemical teams. Efforts to demonstrate that the diet is homogeneous and stable should be concurrent with the study to demonstrate that homogeneity/stability are applicable to actual test conditions. As with testing any taxon, documenting exposure is a critical element; to the extent to which stock solutions may differ from actual dietary exposure levels, it is important to clearly document these differences and the efforts that were employed to ensure that measured exposure levels reflect nominal values. Including a discussion of the chemical/physical characteristics of the compound and why those characteristics dictated the use of a suspension rather than a true solution will be useful to the chemical team tasked with reviewing the protocol/study.

What are acceptable approaches to study design if a TGAI has issues with solubility in the diet matrix?

The functional solubility limit of the test substance should be determined in the diet without a co-solvent (*e.g.,* acetone) prior to initiating the biological phase of testing. Efforts should be made to maximize solubility (*e.g.,* saturation column, sonication, magnetic stirrer or increased sucrose concentration [67%]). If the functional solubility limit is high enough for the purposes of the risk

assessment without the use of a delivery agent, the definitive test should use the recommended TGAI as the test substance and minimize the use of solvents.

If the solubility of the TGAI in the larval diet is not high enough to reflect field exposure, then a delivery agent (*e.g.*, acetone) should be considered. Alternatively, the TEP containing the highest percentage of active ingredient can be used for testing instead; however, the registrant is strongly encouraged to discuss possible options in advance with the relevant EPA chemical team members. In this event, the study should be conducted with the TGAI at the functional solubility limit along with a progression of doses/dietary concentratons with the TEP.

What are appropriate solvent types and concentrations?

For technical products and substances of low water solubility, vehicles such as organic solvent, emulsifiers or dispersants of low toxicity to bees may be used (*e.g.*, acetone, dimethylformamide). If a solvent is needed, acetone (at no more than 5% of the test solution) is typically used; however, solvent selection may depend on test conditions and the effect on control bee survival and/or adult emergence. EPA recommends that if other solvents and/or percentage of the test solutions are proposed for use, these modifications should be discussed (and agreed upon) with EPA before the start of the study. If a solvent is used, then both a solvent control and a negative control should be included in the study. Ideally, solvents/emulsifiers/dispersants should not alter the absorption, distribution, metabolism and/or excretion (ADEM) of the test substance.

What if a chemical is unstable in the dietary matrix?

With respect to stability, EPA emphasizes the importance of demonstrating that diets containing the test material are homogenous and stable, especially given that the nature of diet makes it difficult to visually determine whether the test material is fully soluble/uniform. This can be done by measuring the batch diet on the day of preparation/administration and at the end of the study. If stability issues are identified, it is recommended that fresh batches of diet be prepared daily, and analytical verifications of concentrations in diet provided in fresh and old diets daily for the high and low test concentrations. It is recommended that aged samples from each of the test concentrations are also collected and if there is an issue with either the high or low concentration, then provide analysis of the other test concentrations.

EPA also recommends conducting a multi-residue analysis (*e.g.*, QuEChERs; including analysis for sulfonamide and chloramphenicol antibiotics) of the royal jelly batch to verify the absence of contaminants.

Honey bee (*Apis mellifera*) Acute Contact Toxicity Test (Adult) (OCSPP 850.3020) – Tier I

What is the current guideline to use for this test?

Honey Bee Acute Contact Toxicity" Ecological Effects Test Guidelines OCSPP 850.3020

Alternatively, OECD Test Guideline 214 can be relied on.

OECD Guidelines for the Testing of Chemicals. Test Number 214, Acute Contact Toxicity Test [EXIT]

Is it necessary to submit a protocol to EPA prior to conducting the study?

No, since a formal OECD Test Guideline has been established and is consistent with the OCSPP 850.3020, a protocol is not needed for submission and review prior to conducting the study.

What is the appropriate study duration?

As stated in the guideline, the test consists of the administration (single dose) of the test substance followed by an observation period of 48 hours. If mortality increases by more than 10% between 24 and 48 hours, the test duration should be extended up to a maximum of 96 hours, provided that control mortality does not exceed 20%. As with acute toxicity testing of with other taxa, any sublethal effects should be reported.

Honey bee (*Apis mellifera*) Acute Oral Toxicity Test (Adult) – Tier I

What is the current guideline to use for this test?

OECD Guidelines for the Testing of Chemicals. Test No. 213: Honeybees, Acute Oral Toxicity Test [EXIT]

Is it necessary to submit a protocol to EPA prior to conducting the study?

No, a protocol is not needed for submission and review prior to conducting the study. EPA recommends registrants contact the Agency prior to initiating tests when there are likely to be issues related to topics addressed in the General Study Questions above (*e.g.*, difficult to test substances).

What is the appropriate study duration?

As stated in the OECD guideline, mortality is recorded daily during at least 48 hours and compared with control values. If the mortality is increasing between 24 and 48 hours while control mortality remains at an accepted level, *i.e.*, ≤10%, it is appropriate to extend the duration of the test to a maximum of 96 hours. Additionally, if there is evidence of delayed mortality in other tests, then the test design should include observations up to 72 or 96 hours (to ensure any delayed mortality is captured). As with acute toxicity testing of with other taxa, any sublethal effects should be reported.

Honey bee (*Apis mellifera*) Acute Oral Toxicity Test (Larval), single exposure – Tier I

What is the current Guideline to use for this test?

Formal EPA guidelines for the acute oral larval toxicity test have yet to be published; however, the protocol development and study conduct should rely upon the published OECD Test Guideline No 237 guideline, see:

OECD Guidelines for Testing Chemicals. Test No. 237: Honey Bee (Apis Mellifera) Larval Toxicity Test, Single Exposure [EXIT]

Is it necessary to submit a protocol to EPA prior to conducting the study?

No, a protocol is not needed for submission and review prior to conducting the study in accordance to the OECD guideline. EPA recommends registrants contact the Agency prior to initiating tests when there are likely to be issues related to topics addressed in the General Study Questions above (e.g., difficult to test substances).

How is a replicate defined for this test?

The OECD guideline states that the larval bees should be collected from three different source colonies, and individuals from each colony should be allocated to one and only one replicate. Thereby, in the OECD review, each replicate is comprised of multiple individuals collected from a different source colony. EPA also recommends three source colonies, however, the individual bee is considered the replicate based on the dose being administered to each well which only contains a single individual. For the definitive test, it is recommended that 36 wells (12 larvae/source colony x 3 source colonies) of each 48-well cell plate used in the test be placed (grafted) within grafting cells; the remaining 12 wells per plate should be filled with water. Different treatment levels should not be mixed on the same well plate.

Should sublethal effects be recorded?

Mortality is the only measurement endpoint listed in the current guideline; however, consistent acute toxicity tests with other taxa, sublethal effects (*e.g.*, low food consumption, small size and delayed development) should be reported as well. Also, according to OECD TG 237, mortalities are recorded on Day 5, 6 and 7 (*i.e.*, 24, 48 and 72 hours after exposure to test substance). EFED recommends that the proposed acute toxicity test report mortality and sublethal effects on Day 5, 6 and 7 (*i.e.*, 24, 48 and 72 hours after exposure).

Does EPA accept an 8-day repeat-dose larval study instead of a single-dose larval study (*i.e.*, OECD 237) to represent the acute larval single exposure based endpoint?

Data obtained from the single-dose acute larval study are used in estimating acute risk to individual larval bees based on a single exposure event. Since the interpretation of effects based on repeated

exposures cannot readily be translated into a single dose, EPA requires the submission of a single-dose study. While risk estimates are based on an individual exposure basis, other lines of evidence (such as a repeated exposure study) may also be considered to characterize risks and to help determine whether higher-tier studies are needed at the whole colony level. If the registrant wishes to use a repeat-dose study as a surrogate for a single-dose study, then such request should be made to EPA. EPA would then consider using the endpoint and assume that it represents a single dose, thereby reflecting some conservatism in its use for risk assessment.

Honey bee (*Apis mellifera*)10-Day Chronic Adult Toxicity Test – Tier I

What is the current guideline to use for this test?

Formal EPA guidelines for the chonic oral adult toxicity test has yet to be published; however, the protocol development and study conduct should rely upon the published OECD Test Guideline No 245 guideline, see:

OECD Guidelines for Testing Chemicals. Test No. 245: Honey Bee (Apis Mellifera L.), Chronic Oral Toxicity Test (10-Day Feeding) [EXIT]

Is it necessary to submit a protocol to EPA prior to conducting the study?

No, a protocol is not needed for submission and review prior to conducting the study in accordance to the OECD guideline. EPA recommends registrants contact the Agency prior to initiating tests when there are likely to be issues related to topics addressed in the General Study Questions above (*e.g.*, difficult to test substances).

Does EPA require hypopharyngeal gland development studies?

EPA is not requesting honeybee histology data for the laboratory-based studies. While efforts have been made to link this measurement endpoint (hypopharyngeal acini diameter) to assessment endpoints of impaired whole organism growth, survival and development, these linkages can be tenuous. Meaningful impacts to the gland may be more apparent in the other measurement endpoints evaluated in the 10-day study. Furthermore, the current ring-tested study design did not include any determination of the ideal diet (*i.e.*, protein requirement) for examining hypopharyngeal gland development and [currently] relies on feeding 50% sucrose alone when protein may be a critical dietary component when evaluating hypopharyngeal gland development

Honey bee (*Apis mellifera*) 22-day Chronic Larval Toxicity Test – Tier I

What is the current guideline to use for this test?

Formal EPA guidelines for the chonic oral larval toxicity test has yet to be published; however, the protocol development and study conduct should rely upon the published OECD Guidance Document 239 guidance, see:

OECD Test No. 239. Guidance document on honey bee larval toxicity test following repeated exposure [Exit] (PDF)(41 pp, 1.6Mb)

Is it necessary to submit a protocol to EPA prior to conducting the study?

No, a protocol is not needed for submission and review prior to conducting the study in accordance to the OECD guidance. EPA recommends registrants contact the Agency prior to initiating tests when there are likely to be issues related to topics addressed in the General Study Questions above (e.g., difficult to test substances).

Is it necessary to sample, quantify and analyze the remaining diet that may be remaining in wells prior to supplying the next dose of diet?

EPA is not recommending that registrants/CROs analyze diet sitting in the exposure wells. Rather, they should analyze the batch diet at each treatment level, similar to what is done for other taxa. They should demonstrate that the test material in the actual diet is stable and homogeneous over the period in which that diet is being used.

Although EPA initially considered evaluating the quantity of diet remaining in larval cells, EPA is not recommending the quantification of diet remaining in each cell. To the extent possible, the study should report at each of the observation intervals whether larvae are or are not consuming diet.

How is a replicate defined for this test?

The OECD guideline states that the larval bees should be collected from three different source colonies, and individuals from each colony should be allocated to one and only one replicate. Thereby, in the OECD review, each replicate is comprised of multiple individuals collected from a different source colony. EPA also recommends three source colonies, however, the individual bee is considered the replicate based on the dose being administered to each well which only contains a single individual. For the definitive test, it is recommended that 36 wells (12 larvae/source colony x 3 source colonies) of each 48-well cell plate used in the test be placed (grafted) within grafting cells; the remaining 12 wells per plate should be filled with water. Different treatment levels should not be mixed on the same well plate.

At what stage are bees considered “emerged” for taking bee weights?

Bees that survive until Day 22 are considered “emerged” and are removed from the test well and weighed. Some study designs include a lid over the pupal plate that prevents adult bees from

emerging. This practice is important because each plate represents multiple replicates from 3 different source hives. Once bees emerge from the cell and move around the chamber they can no longer be tracked back to the source hive.

Honey bee (*Apis mellifera*) toxicity of residues on foliage (RT_{25})- (OCSPP 850.3030) – Tier I

What is the current guideline to use for this test?

Honey Bee Toxicity of Residues on Foliage. Ecological Effects Test Guidelines OCSPP 850.3030.

What is RT_{25}?

This is the length of time post-application that aged residues of a test substance on foliage are lethal to >25% of adult honey bees tested, due to contact exposure. It is a numeric value (time to 25% mortality) referred to as the RT_{25}.

When is this study triggered?

If the honey bee adult acute contact toxicity study has an LD_{50} <11 µg/bee and a use pattern that indicates that honey bees would be exposed, then the study may be required using a typical end-use product (TEP).

Is it necessary to submit a protocol to EPA prior to conducting the study?

No, a protocol is not needed for submission and review prior to conducting the study. EPA recommends registrants contact the Agency prior to initiating tests when there are likely to be issues related to topics addressed in the General Study Questions above (*e.g.*, difficult to test substances).

What is the test material that should be used?

The 850.3030 guideline specifies that a representative end-use product is used as the test substance.

What is the preferred test crop that should be used?

The 850.3030 guideline indicates that alfalfa is the preferred test crop.

How long should the study be continued for?

If mortality of bees exposed to the foliage harvested 24 hours after the application is greater than 25%, bees should continue to be exposed to aged (weathered) residues on foliage samples collected every 24 hours (*i.e.,* 48, 72, 96, 120 hours, etc., after the application) until the mortality is <25%.

What are the limitations of the RT_{25} data?

The RT_{25} values are a function of a number of factors including application rate, physical-chemical properties, weather/environmental conditions, crop, and pesticide formulation. Thus, there may be considerable variability in RT_{25} values within a single formulation, between formulations, between crops, and across application rates.

What is the RT_{25} used for?

The RT_{25} value is used by EPA to inform label language and provide lengths of time that pesticide products may remain toxic to bees and other pollinators following application.

Tier II – Semi-Field/Tunnel Colony Level Toxicity Studies

What is the current guideline to use for these tests?

Formal EPA guidelines for semi-field or tunnel colony level toxicity tests have yet to be published; however, information that can help guide the development of either a semi-field tunnel test protocol can be found at OECD 75 and EPPO 170(4), see:

OECD Test No. 75. Guidance document on the honey bee (*Apis mellifera* L.) brood test under semi-field conditions [Exit] (PDF)(27 pp, 278 kb)

EPPO 2010. Side-effects on honeybees. European and Mediterranean Plant Protection Organization. PP 1/170 (4).

For short-term field-feeding studies see: Oomen *et al.* 1992:

Oomen, P. A. A. DeRuijter and J. Van der Steen. 1992. Method for honey bee brood feeding tests with insect growth-regulating insecticides. Bul OEPP/EPPO Bulletin 22: 613 – 616.

Is it necessary to submit a protocol to EPA prior to conducting the study?

Yes, a protocol should be submitted to EPA for review prior to conducting these complex studies. A registrant/CRO may submit a general protocol for review, after which all subsequent protocols that do not deviate from the reviewed general protocol would not need to be submitted and reviewed.

What considerations should be made when deciding which reference standard(s) to use in these studies?

In moving from laboratory-based studies of individual bees to colony-level studies under semi- or full-field conditions, an effort should be made to refine the risk hypothesis and to focus on remaining uncertainties. If there are uncertainties regarding effects on adult bees, then the reference toxicant used in semi-field studies is typically dimethoate. If the effect of concern is on brood development, then fenoxycarb or diflubenzuron is typically used as a reference toxicant. Reference toxicants are not typically used for full-field studies as non-target organisms within the study area may be adversely affected.

Is a second brood evaluation required?

Generally, for the tunnel studies, EPA recommends two full brood cycles (42-days) post-exposure in order to incorporate the potential for delayed effects on brood development. EPA understands that extending the observation period to include a second brood cycle involves additional cost and resources. Should this be a concern, EPA recommends that this issue be raised to EPA before the definitive study is initiated.

Does EPA ask for overwintering in tunnel studies?

Generally, an overwintering component is not required of Tier 2 tunnel studies. Such studies are typically conducted to evaluate the effect of short-term exposures on the colony health and brood development. Since such exposures usually occur many months prior to overwintering, the value added of including an overwintering component is less certain. The feasibility of including an overwintering component is also difficult since small nucleus colonies ("nucs") are typically used which may not achieve adequate strength to successfully overwinter (*i.e.*, to achieve a sufficiently large cluster to maintain colony temperature). Overwintering has been a component of the other "Tier 2" study, *i.e.*, feeding studies, which involve longer exposure durations (*e.g.*, 6 weeks) and may include larger-sized colonies. If EPA determines that an overwintering component is necessary, it will work with the registrant to ensure that the study is initiated early enough in the year to enable the colony to increase in size sufficiently to sustain itself during winter. Nevertheless, if overwintering is being considered as a component of a Tier 2 tunnel study, it is recommended that this issue be discussed with EPA prior to study initiation.

Does EPA want brood termination rates measured in tunnel studies?

If the objective of the tunnel study includes evaluation of effects on brood development, then the EPA generally recommends that brood development indices such as brood termination rate be included, per OECD Guidance Document 75. EPA recognizes that brood termination rate in control hives can be impacted when colonies are confined in tunnels and that efforts are underway to attempt to improve the success of brood indices in colony-level tunnel studies through the International Commission on Plant-Pollinator Relationships (ICPPR).

What is a Tier II Semi-Field Colony Feeding Study? Does EPA have general study design considerations for protocol development?

In 2016, EPA issued additional guidance for EPA risk assessors on Exposure and Effects Testing for Assessing Risks to Bees (PDF)(44 pp, 1 MB) . This guidance provides, as an appendix, details on the design, and considerations for Tier II semi-field feeding study protocol development.

It is strongly recommended that the proposed study protocol be submitted to, reviewed and approved by, EPA prior to study initiation.

Tier II – Residues in Pollen and Nectar Studies/Field Residue Analysis

In 2016, EPA issued additional guidance for EPA risk assessors on Exposure and Effects Testing for Assessing Risks to Bees (PDF)(44 pp, 1 MB). This guidance provides, as an appendix, details on the design and considerations for Tier II residues in pollen and nectar study protocol development.

It is strongly recommended that the proposed study protocol be submitted to, reviewed and approved by, EPA prior to study initiation.

Tier III – Full Field Colony Level Toxicity Studies

EPA guidance is available for full-field pollinator testing (OCSPP 850.3040) and in 2016, EPA issued additional guidance for EPA risk assessors on Exposure and Effects Testing for Assessing Risks to Bees (PDF)(44 pp, 1 MB). This guidance provides, as an appendix, details on the design and considerations for Tier III full-field colony level toxicity study protocol development.

As Tier III full-field tests are intended to address specific uncertainties which have been identified in lower-tier tests, the design of these studies depends to large extent on the specific hypotheses being tested. It is strongly recommended that the proposed study protocol be submitted to reviewed and approved by, EPA prior to study initiation.