Guidance on Exposure and Effects Testing for Assessing Risks to Bees

Office of Pesticide Programs
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1. Purpose

The intent of this document is to provide guidance to risk assessors within the U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) for determining which exposure and effects (toxicity) studies should be considered when characterizing the potential risk of pesticides to bees. This guidance supersedes the interim guidance[1] to risk assessors issued by the Environmental Fate and Effects Division (EFED) in 2011 and complements the broader risk assessment process outlined in the 2014 *Guidance for Assessing Pesticide Risks to Bees*[2], which was developed as a collaborative effort between the EPA, Health Canada Pest Management Regulatory Agency (PMRA) and the California Department of Pesticide Regulation (CDPR). Further information for risk managers within OPP to determine whether honey bee exposure and effects data are necessary for regulatory actions is provided in a companion guidance entitled, *Process for Requiring Exposure and Effects Testing for Assessing Risks to Bees during Registration and Registration Review* (also referred to as the implementation guidance). To provide the public with more insight into OPP's regulatory program, this document, along with the companion implementation guidance, are being made available on our website. By doing so, EPA expects the regulated community to become more familiar with how OPP plans to move forward with assessing risks to bees. It is important to note that the guidance documents EPA has developed focus primarily on studies that may be useful in evaluating the exposure and effects to honey bees of conventional pesticides. While they provide some guidance to risk assessors evaluating other types of pesticide products, further consideration is needed to determine whether the studies are appropriate for evaluating other types of pesticides or if different types of studies may be more useful. Therefore, this guidance document is focused on conventional pesticides; EPA discusses its current approach for other types of pesticides in section 4.

2. Background

Based on survey data collected through the U.S. Department of Agriculture National Agricultural Statistics Service (NASS[3]) on the number of managed honey producing colonies in the U.S., the numbers of such colonies have been in decline since the mid-1940s. The NASS survey reported in 1947 that there were approximately 5.8 million colonies used to produce honey[4]; however, as of 2016, there were roughly 2.59 million[5]. Additional information on declines in pollinator species such as the honey bee in North America was reported by the National Research Council[6] in 2007. In 2006, the magnitude of honey bee colony losses increased as beekeepers reported the sudden disappearance of adult bees from colonies in a phenomenon

[1] USEPA. 2011. Interim Guidance on Honey Bee Data Requirements.
[2] USEPA, PMRA, CDPR. 2014. Guidance for Assessing Pesticide Risks to Bees. Office of Pesticide Programs United States Environmental Protection Agency, Health Canada Pest Management Regulatory Agency (PMRA), California Department of Pesticide Regulation (CDPR). June 19, 2014. http://www2.epa.gov/sites/production/files/2014-06/documents/pollinator_risk_assessment_guidance_06_19_14.pdf (last accessed 06/28/2016).
[3] USDA. 2014. National Agricultural Statistics Service Bee and Honey Inquiry. https://www.nass.usda.gov/Surveys/Guide_to_NASS_Surveys/Bee_and_Honey/#skipnav (last accessed 06/27/2016).
[4] USDA. 1947. Bureau of Agricultural Economics, Washington DC. http://usda.mannlib.cornell.edu/usda/nass/HoneProd//1940s/1947/HoneProd-01-24-1947.pdf (last accessed 12/19/2015).
[5] USDA. 2016. National Agricultural Statistics Survey (NASS) Honey Bee Colonies. http://usda.mannlib.cornell.edu/usda/current/BeeColonies/BeeColonies-05-12-2016.pdf (last accessed 06/27/2016).
[6] National Research Council. 2007. Status of Pollinators in North America. Committee on the Status of Pollinators in North America Board of Life Sciences National Research Council of the National Academies. National Academies Press, Washington DC. http://www.nap.edu/openbook.php?record_id=11761 (last accessed 06/27/2016).

termed "Colony Collapse Disorder (CCD[7])" where colonies were left with insufficient numbers of bees to survive.

In 2007, Congress charged the USDA as the lead federal agency to determine the causes and potential mitigation measures for losses associated with CCD and more recently with general declines in honey bee health. Based on six years of research, the USDA has attributed losses to a number of factors including pesticides, pathogens (*e.g.*, fungal, bacterial, viral disease[s]), pests (Varroa mite; *Varroa destructor*), poor nutrition, and bee management practices)[8]. While no single factor has been identified as a specific cause of honey bee declines, available data suggest that CCD and declines in honey bee health are associated with combinations of these factors; however, the exact combination remains uncertain[9]. Pesticides have been identified as a factor, and EPA is responsible under Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for evaluating the potential for pesticides to adversely affect non-target organisms. Therefore, regulatory authorities in North America and elsewhere are implementing improved procedures for evaluating the potential risks of pesticides to bees.

In response to the uncertainties regarding the potential role that pesticides may play in pollinator declines, OPP established the Pollinator Protection Team in 2007 with representatives from each of the Divisions within OPP. This team was charged with:

- Advancing the Agency's scientific knowledge and assessment of pesticide risks to pollinators;
- Improving risk management tools for mitigating potential risks to pollinators; and,
- Increasing and broadening collaboration and communication with governmental and non-governmental organizations and the public in addressing pollinator issues.

Members of the Pollinator Protection Team have been and continue to be engaged in a broad range of efforts to advance the science, management and understanding of the extent to which pesticides play a role in any of the adverse effects being seen in pollinator populations. In 2014, EPA issued guidance to risk assessors in OPP for evaluating the potential risks of pesticides to bees[10]. The guidance identifies a tiered risk assessment process and the underlying data necessary to implement that process. The guidance is based on a White Paper[11] submitted to the FIFRA Scientific Advisory Panel (SAP) for review and comment in September 2012. The White Paper was in turn informed by efforts underway in Europe through the European and Mediterranean Organization for Plant Protection (EPPO[12]), the European Food Safety Authority (EFSA[13]) as well as the Organization for Economic Cooperation and Development (OECD). Non-governmental organizations such as the International Commission for Plant-Pollinator Relationships

[7] vanEngelsdorp, D., J. D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B. K, Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D. R. Tarpy, J. S. Pettis. 2009. Colony Collapse Disorder: A Descriptive Study. PLoSONE 4(8): e6481. Doi:10.1371/journal.pone.0006481 http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0006481 (last accessed 06/27/2016).

[8] USDA. 2013. Report on the National Stakeholders Conference on Honey Bee Health. National Honey Bee Health Stakeholder Conference Steering Committee. October 15 – 17, 2012. http://www.usda.gov/documents/ReportHoneyBeeHealth.pdf (last accessed 06.27/2016)

[9] Pettis, J. S. and K. S. Delaplane. 2010. Coordinated responses to honey bee decline in the USA. Apidologie 41: 256- 263. http://www.apidologie.org/articles/apido/pdf/2010/03/m09140.pdf (last accessed 06/27/2106).

[10] Ibid USEPA, PMRA, CDPR. 2014.

[11] USEPA. 2012. White Paper in Support of the Proposed Risk Assessment Process for Bees. Submitted to the FIFRA Scientific Advisory Panel for Review and Comment September 11 – 14, 2012. Office of Chemical Safety and Pollution Prevention Office of Pesticide Programs Environmental Fate and Effects Division, Environmental Protection Agency, Washington DC; Environmental Assessment Directorate, Pest Management Regulatory Agency, Health Canada, Ottawa, CN; California Department of Pesticide Regulation http://cues.cfans.umn.edu/old/pollinators/pdf-EPA/EAP-SAP-whitepaper.pdf (last accessed 06/27/2016).

[12] EPPO. 2010. Efficacy Evaluation of Plant Protection Products: Side-effects on Honey bees. PP 1/170 (4). OEPP/EPPO Bulletin 40: 313–319

[13] EFSA. 2013. Guidance on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus spp*. and solitary bees). European Food Safety Authority. EFSA Journal 11(7): 3295. https://www.efsa.europa.eu/en/efsajournal/pub/3295 (last accessed 06/27/2016).

(ICP-PR) and the Society of Environmental Toxicology and Chemistry (SETAC) also have sponsored efforts to advance the science of assessing risks to bees from pesticides.

In June 2014, President Obama issued a memorandum[14] establishing the Pollinator Health Task Force and requiring the development of a federal strategy to protect the health of bees and other pollinators. The Pollinator Health Task Force identified in the strategy is co-chaired by EPA and USDA and is tasked with collecting, collating and disseminating information on stressors (*e.g.*, pesticides) that may affect bees and other pollinators. In May 2015, the White House published the National Strategy to Promote the Health of Honey Bees and other Pollinators[15] document.

Assessing risks to bees is a complex matter. There are many factors that contribute to potential adverse effects on bees. Consistent with EPA's process for evaluating risk to other taxa, it relies on multiple studies identified in Title 40 (Protection of the Environment) of the Code of Federal Regulations, Part 158 (Data Requirements for Pesticides; abbreviated as 40 CFR Part 158[16]). OPP's assessments of risk must adjust as science evolves. As an Agency, EPA is committed to using the best available and sound science in its decision making process. As such, data requirements will change over time. The number and nature of studies needed to support regulatory decisions has continued to evolve as the science evolves and the process for implementing those studies through rule making (*e.g.*, codifying in the 40CFR158) can take considerable time. EPA also recognizes that the capacity of contract research organizations (CROs) to conduct new studies on behalf of the regulated community depends on the nature of the studies and the laboratory's familiarity with the test species and/or study conditions.

The honey bee exposure and effect studies discussed in this guidance reflect the evolving science and EPA's effort to address uncertainties regarding the extent to which bees may be exposed and the nature of potential effects on bees at differing stages of development (*e.g.*, larval *vs.* adult bees) and social organization (*e.g.*, individual bee *vs*. bee colony). In the absence of data, there will be uncertainties regarding the potential for exposure and effects to bees. For pesticides where bees are not considered likely to be exposed or in situations where acute and/ or chronic toxicity is not expected based on other lines of evidence (*e.g.*, mode of action, toxicity data for other related taxa), additional data may not be warranted to support regulatory decisions. Decisions to proceed with a particular regulatory action will consider the nature of the uncertainties (*e.g.*, which data may not be available), the benefits associated with the use, whether there are alternatives and the potential risks associated with those alternatives, and the extent to which mitigation measures can reduce exposure/effects from the pesticide undergoing the registration action. Further information for determining whether the honey bee exposure and effects studies described in this document are required for various regulatory actions is provided in the companion guidance document, *Guidance for Implementation of Exposure and Effects Testing for Assessing Risks to Bees*.

[14] White House. 2014. Presidential Memorandum Creating a Federal Strategy to Promote the Health of Honey Bees and Other Pollinators. Memorandum for Heads of Executive Departments and Agencies. June 20, 2014. http://www.whitehouse.gov/the-press-office/2014/06/20/presidential-memorandum-creating-federal-strategy-promote-health-honey-b (last accessed 06/27/2016).

[15] White House. 2015. National Strategy to Promote the Health of Honey Bees and other Pollinators. Pollinator Health Task Force, May 19, 2015. https://www.whitehouse.gov/sites/default/files/microsites/ostp/Pollinator%20Health%20Strategy%202015.pdf (last accessed 06/27/2016).

[16] CFR. 2016. Code of Federal Regulations Title 40 (Protection of the Environment) Chapeter I (Environmental Protection Agency) Subchapter E (Pesticide Programs) Part 158 (Data Requirements for Pesticides) http://www.ecfr.gov/cgi-bin/text-idx?tpl=/ecfrbrowse/Title40/40cfr158_main_02.tpl (last accessed 06/27/2016).

3. USEPA Toxicity Testing Requirements for Bees

3.1. Statutory/Regulatory Provisions

In general, pesticides can only be sold and distributed in the United States if they have been registered by EPA. Prior to EPA granting a registration, each applicant must establish that its product meets the standards set forth in FIFRA section 3(c)(5) and/or 3(c)(7). These standards include finding that when a pesticide is used in accordance with widespread and commonly recognized practice, it will not generally cause unreasonable adverse effects on the environment. FIFRA also provides for regular review of existing pesticide registrations. FIFRA section 3(g) and its implementing regulations at 40 CFR Part 155 set forth the process for the reevaluation of currently-registered pesticides (*i.e.*, Registration Review).

FIFRA's implementing regulations at 40 CFR Part 158 set forth the data requirements for pesticide registration. Additionally, these regulations discuss the flexibility EPA has in evaluating when data may be required for pesticide registrations. Under 40 CFR Part 158.30, EPA may determine to modify the data requirements on an individual or case-by-case basis to fully characterize the effects of a pesticide product. Additionally, these regulations make clear the data routinely required under Part 158 may not always be sufficient to assess whether there are unreasonable adverse effects on the environment. Under 40 CFR Part 158.30(b) and 40 CFR Part 158.75, EPA may require additional information to better characterize the potential risks.

As noted earlier, EPA has developed guidance documents[17] [18] for risk assessors that identify additional data that may be useful in evaluating effects of pesticides on honey bees. These guidance documents identify three tiers of data, and currently provide the most up-to-date information on the data that might be needed by EPA. Given the advancement of the science, EPA believes that there are benefits associated with revising the existing insect pollinator data requirements in part 158. The enhanced clarity and transparency of the information presented in part 158 should enhance the ability of industry to efficiently manage their registration submissions. Applicants for registration may save time and money by understanding when higher-tiered studies are needed. Having all required studies available to EPA at the time of application should halt potential delays in the registration process. This should enable the registration of products that could decrease risks to pollinators and therefore allow such products to enter the market earlier.

EPA intends to codify all of the data required to support each tier of the risk assessment process for bees in 40 CFR Part 158. EPA initiated the rulemaking process in 2015 with the understanding that the process can take time to complete. EPA's process for developing a rulemaking is intended to assure that the action (1) is supported by strong analysis, (2) is developed via an open process, and (3) meets the requirements of the Administrative Procedures Act. To support the science analysis for this rulemaking, EPA's research on insect pollinator issues has included a presentation to a peer review panel (*i.e.*, the FIFRA SAP). For a rulemaking, EPA also prepares an economic analysis (EA) to describe the costs and benefits of the action.

[17] *Ibid* USEPA 2011
[18] *Ibid* USEPA 2014.

The draft notice of proposed rulemaking and the EA are reviewed via an EPA-internal review process and via an external interagency review process before publication in the Federal Register. EPA will publish the proposed rule and proposed EA so that members of the public can consider the proposal and send their comments to us. EPA accepts comments via the official docket at http://www.regulations.gov/. EPA, considers, reviews and evaluates all comments submitted on the proposed rule and EA, and determines whether or not any changes are needed. Then, drafts of the final documents are prepared and these draft final documents also undergo the internal-EPA and external interagency review processes before publication in the Federal Register. The final rule is not effective until the new regulatory text becomes part of CFR, *i.e*., the new data requirements are codified in the 40 CFR Part 158. This happens 60 days after publication of the final rule. EPA expects to publish the proposed new data requirements in 2016, which would be followed by the public comment period. The timing of the codification of the new data requirements depends somewhat on the number and complexity of the comments submitted, as well as other external factors. EPA projects the new rule to be effective by mid-to-late 2017.

While the Agency's data requirements are established to provide the information needed by EPA to make decisions about whether new pesticide products and new uses of existing products should be registered, EPA may determine that additional data (*e.g*., bee studies) are required to support an existing registration of a pesticide. In such cases, the EPA notifies registrants of the pesticide through issuance of a Data Call-In Notice or DCI under FIFRA section 3(c)(2)(B). The DCI requires each affected registrant to provide evidence within 90 days that the affected registrant is taking appropriate steps to respond to the DCI. Additionally, the Notice sets deadlines for data submission and may specify interim deadlines. Before issuing a DCI, OPP must submit to the Office of Management and Budget (OMB) its justification for requiring the additional information. Once OMB has approved the DCI, OPP may issue the order.

The next sections discuss the current data requirements and also explain what and why additional data may be needed on a case-by-case basis.

3.2. Conventional Pesticides: Current Data Requirements (40 CFR Part 158, Subpart G)

The current EPA data requirements for insect pollinator testing, for conventional pesticides, are specified in the 40 CFR Part 158 Subpart G (Ecological Effects) §158.630 (Terrestrial and Aquatic Non-target Organism Data Requirements Table).[19] Data specified in the 40 CFR Part 158 are used to inform regulatory decisions under FIFRA about the risks and benefits of pesticide products. Current toxicity testing data requirements specified in 40 CFR Part 158 for insect pollinators are shown in **Table 1.**

[19] CFR40. 2016. Part 158, subpart G, §158.630 http://www.ecfr.gov/cgi-bin/text-idx?SID=3da251be263b16deffd269aa64e0098c&mc=true&node=sp40.24.158.g&rgn=div6 (last accessed 06/27/16).

Table 1. Toxicity Testing Requirements for Insect Pollinators as Specified in 40 CFR Part 158, Subpart G.

Guideline Number	Data Requirement	Use Pattern						Test substance	Test Note No.
		Terrestrial	Aquatic	Forestry	Residential Outdoor	Green-house [5]	Indoor		
Insect Pollinator Testing									
850.3020	Honey bee adult acute contact toxicity	R	CR	R	R	NR	NR	TGAI	1
850.3030	Honey bee toxicity of residues on foliage	CR	CR	CR	CR	NR	NR	TEP	2
850.3040	Field testing for pollinators	CR	CR	CR	CR	NR	NR	TEP	3

Definitions: R = Required; CR = Conditionally Required; NR = Not Required; TGAI = Technical Grade of the Active Ingredient; TEP = Typical End-Use Product

Test Notes:

1. Data using the TGAI are required to support all outdoor end-use product uses. Data are generally not required to support end-use products in the form of a gas, a highly volatile liquid, a highly reactive solid, or a highly corrosive material.
2. Data are required only when the formulation contains one or more active ingredients having an acute LD_{50} of <11 micrograms per bee as determined in the honey bee acute contact study and the use pattern(s) indicate(s) that honey bees may be exposed to the pesticide. (Note that in the regulatory text this is actually Test Note 24.)
3. Required if any of the following conditions are met: (Note that in the regulatory text this is actually Test Note 25.)
   i. Data from other sources (Experimental Use Permit program, university research, registrant submittals, *etc*.) indicate potential adverse effects on colonies, especially effects other than acute mortality (reproductive, behavioral, *etc*.);
   ii. Data from residual toxicity studies indicate extended residual toxicity.
   iii. Data derived from studies with terrestrial arthropods other than bees indicate potential chronic, reproductive or behavioral effects

As indicated in **Table 1**, current data requirements specified under Part 158 include the honey bee acute contact toxicity test (OCSPP Guideline 850.3020)[20], the honey bee toxicity of residues on foliage test (OCSPP Guideline 850.3030)[21] and field testing for pollinators (OCSPP Guideline 850.3040)[22] when certain pesticide use patterns or triggers are met. These data are used to provide risk assessors with an understanding of the effects of pesticides to which non-target insects are exposed through contact with residues on various surfaces or through direct contact via spray and/or dust. The honey bee acute contact toxicity test is required for pesticide technical grade active ingredients (TGAI) with terrestrial, forestry and residential outdoor uses and is conditionally required for pesticides with aquatic uses as a Tier 1 screen conducted under laboratory conditions. EPA will consider limit tests (100 μg a.i./bee) in the acute toxicity tests; however, the registrant should provide a rationale for conducting a limit test. The rationale should demonstrate that the limit test is protective for the highest estimated environmental exposure level for individual bees. If the results of the honey bee acute contact toxicity test indicate that a pesticide has a median acute lethal dose to 50% of the animals tested, *i.e*., the LD_{50} value, of less than (<) 11 micrograms (µg) per bee, and the use pattern indicates that honey bees may be exposed, then the toxicity of residues on foliage test is conditionally required as a laboratory-based test using the technical end-use product (TEP). Toxicity of residues on foliage studies are designed to determine the time required for fewer than 25% of bees exposed via contact with aged residues on foliage to die (RT_{25}). Notably, information indicates that

[20] USEPA. 2012a. "Honey Bee Acute Contact Toxicity" Ecological Effects Test Guidelines OCSPP 850.3020. EPA 712-C-019 Web: http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines (last accessed 06/27/2016).
[21] USEPA. 2012*b*. "Honey Bee Toxicity of Residues on Foliage." Ecological Effects Test Guidelines OCSPP 850.3030. EPA 712-C-018. Web. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines (last accessed 06/27/2016).
[22] USEPA. 2012*c*. "Field Testing for Pollinators." Ecological Effects Test Guidelines OCSPP 850.3040. EPA 712-C-017. Web. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines (last accessed 06/27/2016).

the RT_{25} value may be strongly dependent on the formulation and environmental conditions. The Interim Guidance on Honey Bee Data Requirements[23] identified several other types of studies that can be used to assess the persistence of pesticide residues on foliage, including plant metabolism studies (OCSPP Guideline 860.1300[24], rotational crop studies (OCSPP Guideline 860.1900[25]) and magnitude of residue studies (OCSPP Guideline 860.1500[26]). This information can be coupled with acute contact toxicity data to obtain estimates of the period over which residues may be toxic to bees and thus, may provide an alternative to RT_{25} estimates. Notably, however, obtaining and analyzing these crop residue studies for determination of foliar dissipation half-life values can be challenging and resource intensive.

As specified in 40 CFR § 158.630[27], field testing of pollinators is required if any of the following conditions are met:

- Data from other sources (Experimental Use Permit program, university research, registrant submittals, *etc*.) indicate potential adverse effects on colonies, especially effects other than acute mortality (reproductive, behavioral, *etc*.);
- Data from residual toxicity studies indicate extended residual toxicity; or,
- Data derived from studies with terrestrial arthropods other than bees indicate potential chronic, reproductive or behavioral effects.

Field testing of pollinators may include semi-field/feeding (Tier 2) or full-field studies (Tier 3) although historically, studies conducted under OCSPP Guideline 850.3040 have focused on full-field testing. Full-field studies are intended to represent real world conditions and are considered the highest level of refinement (Tier 3) for bee toxicity testing according to the White Paper[28] and the 2014 Bee Risk Assessment Guidance[29] document. As such, full-field studies should ideally be designed to address specific uncertainties that have been identified in lower-tier tests. Pollinator full-field study designs received by OPP to date have varied considerably; therefore, rather than a rigid study methodology, study design elements that should be considered for these studies are provided to risk assessors (**Appendix 1**). Variability has on occasion resulted from study designs attempting to collect too much information where colonies are stressed by the collection of repeated measures. At a full-field level, study colonies are also vulnerable to the same factors (*e.g.*, disease, pests, poor nutrition) that have been associated with declines and honey bee health and these can confound efforts to conduct such studies. Therefore, study protocols for Tier 3 tests should be developed by pesticide applicants/registrants to address the specific hypothesis being tested. Such protocols should ideally be reviewed by EPA staff prior to study initiation.

[23] USEPA 2011. Interim Guidance on Honey bee Data Requirements. Memorandum from Donald Brady, Director, Environmental Fate and Effects Division, dated October 19, 2011.
[24] USEPA. 1996. Residue Chemistry Test Guidelines. OPPTS 860.1300 Nature of the Residue—Plants, Livestock. Office of Chemical Safety and Pollution Prevention formerly the Office of Prevention, Pesticides and Toxic Substances (7101) EPA 712-C-96-172. August 1996. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-860-residue-chemistry-test-guidelines (last accessed 06/27/2016).
[25] USEPA. 1996. Residue Chemistry Test Guidelines OPPTS 860.1900 Field Accumulation in Rotational Crops. Office of Chemical Safety and Pollution Prevention formerly the Office of Prevention, Pesticides and Toxic Substances (7101) EPA 712-C-96-189 August 1996. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-860-residue-chemistry-test-guidelines (last accessed 06/27/2016).
[26] USEPA. 1996. Residue Chemistry Test Guidelines OPPTS 860.1500. Crop Field Trials. Office of Chemical Safety and Pollution Prevention formerly the Office of Prevention, Pesticides and Toxic Substances (7101) EPA 712-C-96-183. August 1996. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-860-residue-chemistry-test-guidelines (last accessed 06/27/2016).
[27] *Ibid* CFR40 2016.
[28] *Ibid* USEPA. 2012
[29] *Ibid* USEPA *et al*. 2014.

3.3. Conventional Pesticides: Additional Bee Testing Guidance

After considering input from the SETAC Pellston, EPPO and EFSA (discussed above), in 2011, EFED issued interim guidance[30] for ecological risk assessors to determine whether and what additional honey bee studies beyond the existing data requirements may be needed to better characterize the potential hazard (*i.e.*, adverse effects) of chemicals to honey bees. That guidance also more clearly establishes a tiered structure for assessing effects of pesticides on bees. Relevant studies for Tier 1 include the acute and chronic oral toxicity studies with adult bees and acute and chronic toxicity studies with larval bees, in addition to the currently required honeybee acute contact toxicity study. For Tier 2, relevant data may include semi-field effects studies conducted with full colonies (*e.g.,* field feeding and tunnel studies) as well as studies quantifying pesticide concentrations in pollen and nectar from treated plants. Finally, Tier 3 may involve full-field testing with honeybee colonies, similar to the existing field testing for pollinator data requirement. As EPA gains experience evaluating these data, it may be possible to consider data on similarly structured chemicals with a common mode of action to that of the chemical under review depending on the quality of those data.

The following subsections discuss the types of studies that are required and the additional studies that may be needed on a case-by-case basis to fully characterize risk in various tiers of the risk assessment process for bees. The decision to recommend additional data should be based on the extent to which bees are considered likely to be exposed and if so, whether there are any existing data on the chemical or similarly structured chemical with respect to exposure and effects. Additional considerations may include whether mitigation has been imposed/proposed that would reduce the likelihood of exposure/effects.

Bees can be exposed to pesticides through multiple pathways including contact with sprays and dusts and through ingestion of residues in food/water (*e.g.*, pollen/nectar and water used to maintain colony temperature). Worker bees foraging on flowers for pollen and nectar can be exposed to residues in pollen and nectar either through direct contamination of these matrices by foliar sprays and/or dusts through translocation of residues via systemic transport of the active ingredient. Residues can in turn be brought back to bee colonies where in-hive bees including young adults and developing brood (*i.e.*, eggs, larvae and pupae) may be exposed. EPA guidance[31] on assessing the risk of pesticides to bees identifies a suite of laboratory-based studies intended to serve as the foundation for screening chemicals for potential acute and chronic effects to individual adult and larval bees.

With respect to the acute oral and chronic toxicity tests with adult bees, terrestrial invertebrates are likely to be impacted if exposed to pesticides in various use settings. Pesticide residues may be transferred to or come in contact with pollen and/or nectar of treated plants and ingested directly by individual bees or for social species brought back to the colony. Therefore, potential acute effects to adult honey bees and other insect pollinators from oral exposure to some pesticides could exist. The acute contact toxicity test does not fully address possible effects of oral exposure on adult terrestrial insect survival. Because of the potential for pollen and nectar to be contaminated with pesticide residues, and subsequently brought back to the hive and ingested, it is important to determine the acute oral toxicity to adult honey bees and other insect pollinators for chemicals where exposure is considered possible.

[30] *Ibid* USEPA. 2011.
[31] *Ibid* USEPA *et al*. 2014.

With respect to the acute and chronic toxicity tests with larval bees, these studies evaluate both oral and contact toxicity since larvae both ingest residues and are in contact with the treated diet during the study. Similar to the potential route of exposure described for adult bees, pesticide residues may be transferred to or come in contact with pollen and/or nectar of treated plants and these contaminated food items may in turn be ingested by developing larvae during certain stages of their development. The acute contact toxicity test with adult bees may not adequately address uncertainties related to differential sensitivity of different life stages of bees. The chronic toxicity test provides a means of evaluating other endpoints (*e.g.*, growth, development and survival) of individual bees as they transition from larvae, through pupation and emergence as adults.

Although EPA guidelines have not been developed for these studies, the Organization for Economic Cooperation and Development (OECD) has developed a formal test guideline for an acute oral toxicity study with adult bees (OECD 213[32]) as well as a test guideline for an acute oral toxicity study with honey bee larvae (OECD 237)[33]. OECD also has a test guideline for assessing acute contact toxicity with young adult bees (OECD 214[34]), that may provide sufficient data to fulfill the 40 CFR Part 158 adult contact toxicity test requirement (OCSPP Guideline 850.3020). Although the OECD has not yet finalized test guidelines for chronic studies, efforts are currently underway to develop standardized guidelines for assessing the effects from chronic exposures to adult and larval honey bees in the laboratory. For Tier 2, EPA does not have a formal guideline; however, OECD 75[35] and Oomen *et al*. 1992[36] represent useful guidance that can help shape the conduct of a tunnel or feeding study. As stated in 40 CFR Part 158.70(d)(2), registrants/applicants can satisfy EPA data requirements by conducting studies in accordance with OECD requirements and recommendations. Importantly, non-guideline studies should not automatically be classified as supplemental by data reviewers. Scientifically sound, non-guideline studies may be considered acceptable if tests were conducted according to OECD guidelines or test protocols were previously reviewed by EPA.

Tier 1 of the risk assessment process consists of acute and chronic laboratory toxicity studies of individual bees (adults and larvae) and is used as a basic screen. Depending on whether screening-level risk estimates exceed EPA levels of concern (LOCs) and the extent to which additional information is needed to inform risk management decisions, more refined (or higher-tier) studies, may be required. Higher-tier studies examine the whole colony rather than individual bees and may be conducted under relatively controlled environmental conditions where colonies are confined to tunnels/enclosures (Tier 2), are left unconfined and fed pesticide-spiked diets (Tier 2) or are allowed to freely forage in unconfined areas where pesticide applications may be made (Tier 3). **Appendix 2** contains example justification tables for exposure and effects studies with bees which are intended for use in Registration Review Problem Formulation documents.

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[32] OECD. 1998*a*. OECD Guidelines for the Testing of Chemicals. Honeybees, Acute Oral Toxicity Test. 213. http://www.oecd-ilibrary.org/environment/test-no-213-honeybees-acute-oral-toxicity-test_9789264070165-en (last accessed 06/27/2016).

[33] OECD. 2013. OECD Guidelines for Testing Chemicals. Honey bee (*Apis mellifera*) larval toxicity test, single exposure. http://www.oecd-ilibrary.org/environment/test-no-237-honey-bee-apis-mellifera-larval-toxicity-test-single-exposure_9789264203723-en (last accessed 06/27/2016).

[34] OECD.1998*b*. OECD Guidelines for the Testing of Chemicals. Test Number 214, Acute Contact Toxicity Test. http://www.oecd-ilibrary.org/environment/test-no-214-honey_bees-acute-contact-toxicity-test_9789264070189-en;jsessionid=43gvto47wnue9.delta (last accessed 06/27/2016).

[35] OECD. 2007. Guidance document on the honey bee (*Apis mellifera* L.) brood test under semi-field conditions. Series on Testing and Assessment No. 75. ENV/JM/MONO(2007)22.

[36] 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.

Screening-level Toxicity Studies (Tier 1)

The additional toxicity data for honey bees at Tier 1 may apply to situations where exposure of bees to the pesticide is considered likely. As noted earlier, the decision to recommend specific studies should be based on the extent to which bees are considered likely to be exposed and if so, the extent to which other scientifically relevant information may not be available to address uncertainties regarding exposure and effects to bees. For pesticides where acute or chronic toxicity is not expected, based on other lines of evidence (*e.g.*, mode of action, toxicity data for other related taxa), the assessor may determine that a limit test[37] according to OCSPP or OECD guidelines is appropriate prior to performing a definitive test. If the limit dose causes increased mortality to bees, then a definitive test would be triggered. Use of a limit test for the chronic larval toxicity test may be less labor intensive studies compared to the other Tier 1 bee toxicity tests. If there are data to indicate that a TEP is potentially more toxic than the TGAI and there is reason to believe that bees may come directly in contact with the intact TEP, then testing of such formulated products should be considered.

Acute Oral Adult Toxicity

The acute oral toxicity study with young (newly emerged) adult bees provides median lethal dose (LD_{50}) value for honey bees (*A. mellifera*) based on acute oral exposure following OECD test guideline 213[38]. These data are used in conjunction with acute contact LD_{50} data obtained through OCSPP 850.3020[39] to estimate the acute toxicity of the technical grade active ingredient (TGAI) to individual young adult honey bees. The studies also provide slopes for the dose-response curves that can in turn be used for estimating the likelihood of individual effects. In addition, sublethal effects observed in the study (*e.g.,* abnormal behavior or movement) are used to further characterize effects following a single exposure to the technical grade material. Data obtained from this study are used in estimating acute risk to individual adult bees based on ingestion of residues. Risk estimates based on these data are considered along with other lines of evidence (*e.g.*, the likelihood of colony exposure and the potential magnitude of effect based on toxicity data collected on individual bees) to determine whether higher-tier studies are needed at the whole colony level.

[37] Limit testing should ensure that the highest level tested accounts for high-end exposure levels that be encountered at the maximum application rate of the compound. Tier 1 exposure modeling estimates based on the 2014 Bee Risk Assessment Guidance should be used in determining the limit test.
[38] *Ibid* OECD 1998*a*
[39] *Ibid* USEPA 2012*a*.

Acute Contact Adult Toxicity

This acute contact toxicity study with young (newly emerged) adult bees is currently an EPA guideline (OCSPP 850.3020[40]) as well as an OECD test guideline (OECD 214[41]). Data from the acute contact toxicity test are used in conjunction with acute oral LD_{50} data obtained through OECD 213[42] to estimate the acute toxicity of the TGAI to individual young adult honey bees. The studies also provide slopes for the dose-response curves that can be used for estimating the likelihood of individual effects. In addition, sublethal effects observed in the study (*e.g.,* abnormal behavior or movement) are used to further characterize effects following a single exposure to the technical grade material. Data obtained from this study are used in estimating acute risk to individual adult bees based on contact exposure. Risk estimates based on these data are considered along with other lines of evidence to determine whether higher-tier studies are needed at the whole colony level. For highly volatile chemicals used as fumigants, this test can be adapted to address exposure through the vapor phase.

Acute Larval Toxicity

The 7-day single dose study with larval bees provides a 96-hr LD_{50} for larval bees following OECD test guideline 237[43]. Data obtained from this study are used in estimating acute risk to individual larval bees based on ingestion of residues. Risk estimates based on these data are considered along with other lines of evidence to determine whether higher-tier studies are needed at the whole colony level.

10-day Adult Chronic Toxicity Study

The 10-day toxicity study with young adult bees (guideline under development by OECD) provides a no-observed adverse effect level (NOAEL) and lowest-observed adverse effect level (LOAEL) for assessing chronic effects. Although the study focuses primarily on survival and growth (weight of bees), sublethal effects on behavior and food consumption can be obtained as well. Data obtained from this study are used in estimating chronic risk to individual adult bees. Risk estimates based on these data are considered along with other lines of evidence to determine whether higher-tier studies are needed at the whole colony level.

21-day Larval Toxicity Study

Developing bee brood (*i.e*., larvae and pupae) can be exposed to the active ingredient through residues brought back to the colony by worker bees foraging in areas where the pesticide has been applied. While larvae are typically fed royal or brood jelly during their early stages of development, worker bee (females) and drone (males) larvae are also fed pollen/honey (bee bread) directly by in-hive nurse bees. The 21-day larval toxicity study (guidance under development by OECD) provides chronic toxicity data on developing bee brood, expressed in terms of a 21-day no-observed adverse effect level (NOAEL) and lowest-observed adverse effect level (LOAEL), for assessing chronic effects. Effects on survival and development (adult bee emergence and body weight) from repeat exposures to the active ingredient are used in estimating

[40] *Ibid* USEPA 2012*a*.
[41] *Ibid* OECD 1998*b*.
[42] *Ibid* OECD 1998*a*
[43] *Ibid* OECD 2013.

chronic risk to individual brood. In some cases, it may be possible to document food consumption by larvae during the feeding component of the study. Risk estimates based on data from this study are considered along with other lines of evidence to determine whether higher-tier studies are needed at the whole colony level. Special considerations for the design of the 21-day larval feeding study are provided in **Appendix 3.**

When all of the Tier 1 data are not available to evaluate potential exposure and effects to bees, it may be difficult to develop suitable mitigation measures for some compounds (*e.g.*, systemic insecticides) especially when the use is on an indeterminate blooming plant (*e.g.*, cotton, cucurbits) which is attractive to pollinators. If the EPA cannot evaluate the potential exposure and effects to bees, EPA may not be able to make the necessary determination under FIFRA to register the pesticide or the new use.

Tier 2 Toxicity Testing

As is the case with current data requirements for pollinators, the need for Tier 2 studies to more fully characterize risk is based on the outcome of the screening-level assessment where acute and/or chronic risk LOCs have been exceeded for bees. Bees are likely to be impacted if exposed to pesticides in various use settings. For social bees, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to the hive and may adversely affect developing brood (egg, larvae, and pupae) and adult bees. Screening-level (Tier 1) studies of individual bees are not meant to fully address possible effects and/or exposure to pesticide residues at the colony-level, and for many pesticides, assessing effects at the colony-level may not be necessary (*e.g.*, when RQs do not exceed LOCs or when the potential for exposure can be mitigated). Because of the potential for pollen and nectar to be contaminated with pesticide residues, and subsequently brought back to the hive, it may be important on a case-by case basis to determine whether bee colonies may be negatively affected under relatively controlled exposure conditions of a semi-field study. In addition to providing effects data, these studies can provide data on pesticide residues in pollen/nectar of treated plants.

Semi-field Testing with Honey Bee Colonies

If screening-level RQ values exceed the acute risk LOC (RQ ≥0.4) and/or chronic risk LOC (RQ≥1.0) and depending on the need for additional information to characterize risk, higher-tier studies may be required to examine potential effects at the colony level. At Tier 2, semi-field studies are conducted under relatively controlled conditions (*i.e.*, through use of enclosures/tunnels or outdoor feeding studies) to better ensure that bees are confined to the treatment area and that exposure has taken place. Depending on the risk management question, semi-field studies can be conducted with TEP at the maximum application rate on a pollinator-attractive crop or multiple exposure levels can be tested to enable the development of concentration-response data.

Honey Bee Brood Study (OECD 75)

Although a general study guidance for conducting a semi-field study is still under development, the OECD 75 guidance document on honey bee brood testing[44], and the European and Mediterranean Plant Protection Organization (EPPO) 170 describe basic semi-field study elements that should be considered.

In a tunnel study, there is typically a pesticide exposure period in the tunnel and an extended observation period when test bees are allowed to freely forage from the landscape. While typically honey bee colonies can only be maintained within enclosures for a limited exposure time (~10 days), these colonies may be monitored following their removal from the enclosure to evaluate chronic effects resulting from the exposure period or delayed exposure from ingestion of stored pollen/nectar. However, the tunnel studies are conducted with smaller colonies (referred to as nucleus "nuc" colonies) and can only accommodate relatively short exposure periods in the tunnel (*e.g.*, up to 10 days or so) due to confinement-related stress on the bees. If overwintering is an additional measurement endpoint (not identified in OECD 75[45]), the colonies must be provided appropriate time and forage to buildup sufficiently to test their ability to overwinter successfully. Typical endpoints measured in tunnel studies include adult mortality, flight activity, brood development, hive strength (numbers of adult bees and brood; food reserves), and abnormal behavior. These endpoints are typically expressed in terms of the pesticide application rate used in the study, although measurement and expression of results in terms of measured pesticide residues may also be conducted.

Feeding Studies

The feeding study methodology described by Oomen *et al*. 1992[46] and the extended-feeding field study design proposed in the SAP White Paper[47] may also be considered useful for assessing the potential effects of pesticides on bees at the colony level. Rather than restricting bees to tunnel enclosures with a treated crop, colonies are unrestricted and fed food sources spiked with known concentration of pesticides. The amount of pesticide in the diet and the quantity of diet consumed by the bees can be monitored to provide an estimate of an overall amount of pesticide "dose" consumed in hives. These studies are intended to provide a NOAEC and LOAEC based on a range of measurement endpoints including colony strength, *i.e*., numbers of adult bees and brood (eggs, larvae, and pupae) covering each frame of the colony, as well as sublethal endpoints (*e.g*., foraging behavior). Unlike the typical tunnel study designs, feeding studies are designed to provide a dose-response relationship between pesticide residues in diet and effects on the colony. The NOAEC and LOAEC values from feeding studies can then be compared to pesticide residues measured in pollen and/or nectar of crops to qualitatively characterize risk and identify risk mitigation options. Furthermore, feeding studies can provide information on the effects to honey bees over longer durations of exposure compared to tunnel studies. However, it is worth noting that the feeding study design also has some uncertainty with respect to how well it mimics actual forage activity and pesticide exposure experienced by colonies with actual pesticide-treated crops. Considerations for the design of the colony feeding study are provided in **Appendix 4.**

[44] *Ibid* OECD. 2007.
[45] *Ibid* OECD. 2007.
[46] *Ibid* Oomen, *et al*., 1992.
[47] *Ibid* USEPA 2012

Tier 3 Toxicity Testing

As is the case with current data requirements for pollinators, whether Tier 3 studies may be necessary to fully characterize risk is based on the outcome of the screening-level assessment (Tier 1) where acute and/or chronic risk LOCs have been exceeded for terrestrial invertebrates and where Tier 2 studies either under semi-field tunnel conditions and/or feeding studies have indicated potential adverse effects at the colony level. Available toxicity studies from lower-tier studies may not address uncertainties related to possible effects and/or exposure to pesticide residues at the colony-level under actual pesticide use conditions and where specific uncertainties regarding the likelihood of exposure and/or effects remain. Full-field studies also provide an opportunity to measure residues in pollen and nectar as well as various matrices (beebread, honey, wax) within the colony to obtain a more realistic understanding of exposure. Because EPA guideline 850.3040 is relatively broad, additional information on Tier 3 study design elements to consider when recommending/reviewing such studies are provided in **Appendix 1**.

Full-field Testing with Honey Bee Colonies

If Tier 2 semi-field studies indicate a likelihood of adverse effects at the colony level, then Tier 3 studies with TEP may be needed to address specific uncertainties that are identified in the lower-tier studies. With each progressive tier, the study design should be increasingly refined to address specific questions while the study is increasingly realistic, *i.e.*, representative of actual use conditions and likely exposure scenarios. Therefore, the full-field study protocol cannot be standardized given that it is intended to address specific uncertainties identified in lower-tier studies. In general, full-field studies offer the advantage of capturing exposure and effects of a pesticides on honey bee colonies under real-world conditions, since bees are free to forage for pollen and nectar without constraints or supplemental feeding. However, careful design of the field study is necessary to ensure that the range of exposures expected in agricultural ecosystems are adequately represented. Historically, many full-field studies have used treated fields of relatively small size which tend to underestimate honey bee exposure in larger agriculturally- dominated ecosystems where a large percentage of the crop may be treated with the pesticide of interest. **Appendix 1** of this document provides study design elements to consider in Tier 3 studies.

Table 2 lists the additional bee testing data described above that may be required on a case-by-case basis and their respective triggers. When determining whether data listed in **Table 2** are necessary, the table can provide useful information on study tiers and triggers. As noted previously, the decision to recommend additional data should be based on whether exposure of bees is considered likely and/or whether other scientifically relevant information may be available to address uncertainties. As discussed in the risk assessment guidance[48], any proposed mitigation measures should be evaluated prior to determining whether additional data are recommended. Additional considerations may include an analysis of the benefits associated with the proposed chemical/use as well as the alternatives and their associated risks.

[48] *Ibid* USEPA 2014.

Table 2. Additional Requirements for Bee Exposure and Effects Testing.[a] [b]

Study	Study Type	Test substance	Table Note No.
Non-Guideline Study (Tier 1) [c]	Honey bee adult acute oral toxicity	TGAI	1
Non-Guideline Study (Tier 1) [d]	Honey bee larvae acute oral toxicity	TGAI	1
Non-Guideline Study (Tier 1) [c] [g]	Honey bee adult chronic oral toxicity	TGAI	1
Non-Guideline Study (Tier 1) [e] [g]	Honey bee larvae chronic oral toxicity	TGAI	1
Non-Guideline Study (Tier 2) [f] [g]	Semi-field testing for pollinators (tunnel or colony feeding studies)	TEP (tunnel) or TGAI (feeding)	2

Definitions: TGAI = Technical Grade of the Active Ingredient; TEP = Typical End-Use Product

[a] Recommendations for bee toxicity data may be modified for certain types of outdoor residential uses for which exposure is considered extremely limited (*e.g.*, crack and crevice treatment, spot treatment, *etc*). In such cases, acute toxicity data may still be warranted but chronic toxicity data may be of limited value in the risk assessment.

[b] For greenhouse uses that involve bee pollination, Tier 1 and Tier 2 bee exposure and effects data may be required.

[c] Honey bee acute oral toxicity test protocol available through OECD TG 213.[49] For aquatic uses, acute oral toxicity data are needed to evaluate exposure of bees through drinking water and in evaporative cooling of the hive and for exposure through systemic transport into food items (pollen/nectar).

[d] Honey bee acute larval toxicity test protocol available through OECD TG 237.[50]

[e] Draft test protocols are currently being finalized through the OECD..

[f] Semi-field tunnel study protocol available through OECD Guidance 75.[51]

[g] Study protocol should be submitted for review prior to conduct of the study.

Test Notes:

1. Data using the TGAI are required to support all outdoor end-use product uses. Data are generally not required to support end-use products in the form of a gas, a highly volatile liquid, a highly reactive solid, or a highly corrosive material. For greenhouse use patterns, data are required for crops that require pollination (*e.g.*, tomatoes); for aquatic use patterns, data are required if bees are likely to be exposed as a result of the proposed use.
2. Tier 2 studies may be required pending the results and evaluation of Tier 1 studies. Tier 2 studies may be required if the ratio of the EEC and larval or adult bee acute LD_{50} >0.4 or the ratio of the EEC and chronic NOAEC >1. Tier 2 may be required if data from other sources (Experimental Use Permit program, university research, open literature, registrant submittals, adverse effect incident reports, *etc*.) indicate the potential to adversely affect bee colonies, especially effects other than acute mortality (*e.g.*, reproductive, behavioral, *etc.*). Tier 2 studies may also be required if data derived from studies with terrestrial arthropods other than honeybees indicate potential chronic, reproductive, or behavioral effects.

When determining whether additional data are necessary for risk characterization, it is important that the assessor consider the nature of any uncertainties from existing data for the chemical or similarly structured chemicals. As noted earlier, if there are data to indicate that a TEP is potentially more toxic than the TGAI and there is reason to believe that bees may come directly in contact with the intact TEP, then testing of such formulated products should be considered. Also, as is the current practice, the additional data, if required, would be tiered. At Tier 1 (screening-level), the focus is on laboratory-based studies of acute and chronic exposure with individual bees (adults and larvae). Conditioned on the outcome of these laboratory studies and the likelihood of exposure, semi-field and full-field studies may be required where the focus is on whole honey bee colonies. As the assessment process is refined (*i.e.*, moving to higher tier studies), tests are intended to reflect increasingly realistic exposure conditions and to address specific risks/uncertainties

[49] *Ibid* OECD 1998*a*.
[50] *Ibid* OECD 2013.
[51] *Ibid* OECD 2007.

(*e.g.*, decreased brood production) identified in the lower-tier studies. As noted in **Tables 1** and **2**, higher-tier testing at the semi-field and full-field level are typically conducted with TEP; however, feeding studies (Tier 2) are usually conducted with the TGAI but may also be conducted with the TEP when bees may be orally exposed to TEP.

There may be situations in which registrants/applicants submit/request the use of surrogate species such as aquatic invertebrates to serve as a means of estimating risk to bees when bee-specific data are not available. At the present time, information is lacking on the ability of toxicity data from other taxa to predict acute or chronic toxicity of pesticides to adult and larval honey bees. Specifically, it is not known how well pesticide toxicity data for aquatic invertebrates (*e.g., Daphnia*, mysid shrimp) or other terrestrial arthropods (*e.g.,* parasitic wasps) are correlated with toxicity to honey bees. In addition, predictive toxicity tools based on chemical structure (*e.g.,* quantitative structure activity relationships [QSARs]) or toxicological mechanisms are either not available or in the early stages of development. Further exploration of these and other predictive toxicity tools is expected as additional data become available on the comparative toxicity of pesticides with different modes of action to bees and related taxa. As with any recommendations made by risk assessors for additional toxicity testing, the ultimate decision to require testing is based on discussion with the risk manager and the need for additional data to inform the regulatory When appropriate, risk assessors may determine it is appropriate to bridge toxicity data for bees based on other pesticides with the same mode(s) of action and similarity in chemical structure.

3.4. USEPA Residue Chemistry Requirements for Pollen and Nectar (Subpart O)

In addition to the bee-specific effects data, other data may be helpful in determining whether a pesticide may have the potential to cause adverse effects. The next section discusses how residue chemistry data may be helpful in this regard.

Bees may be impacted if exposed to pesticide residues in various use settings. As noted earlier, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to hive where all life stages of bees may be exposed. For some pesticides, the quantification of pollinator-relevant residues in treated flowering plants should be measured, since pollinators will be exposed to residues from either current or prior season applications (due to the potential for residues to accumulate in plants and trees). Residues in edible/transportable-to-hive parts of treated trees and plants, including (where appropriate), but not limited to, guttation water, sap/resins, whole plant tissue (*e.g.,* leaves, stems), as well as blooming, pollen-shedding, and nectar producing parts (*i.e.*, flowers and, if present, extra-floral nectaries) of plants may inform the potential for exposure and subsequent risk.

Measured residues in pollen and nectar can serve as a means though which screening-level RQs may be refined. Initially, risk estimates are based on exposure values generated using conservative models or default values. However, risk estimates can be refined using measured residue values in pollen and/or nectar collected by bees. Such data may be available by modifying existing residue chemistry data requirements, such as the magnitude of residue crop trial (OCSPP 860.1500[52]) and the field rotational crop trials (OCSPP 860.1900[53]); alternatively, residue data for pollen and nectar for specific crops and methods of application may be necessary on a case-by-case basis. **Table 3** depicts selected pertinent test requirements from the 40

[52] USEPA. 1996. Residue Chemistry Test Guidelines OPPTS 860.1500. Crop Field Trials. EPA 712-C-96.183. August 1996. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-860-residue-chemistry-test-guidelines (last accessed 06/27/2016).
[53] USEPA. 1996. Residue Chemistry Test Guidelines OPPTS 860.1900 Field Accumulation in Rotational Crops. EPA 712-C-96-189. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-860-residue-chemistry-test-guidelines (last accessed 06/27/2016).

CFR Part158 Subpart O (Residue Chemistry §158.1410[54]). For studies examining residues in pollen and/or nectar, protocols should be submitted for EPA review prior to initiating the studies.

Table 3. Requirements for Residue Data Similar to Existing Requirements in 40 CFR Part 158 Subpart O.

Guideline Number	Study Type	Use Pattern					Test substance	Test Note No.
		Terrestrial Food or Feed	Aquatic Food	Greenhouse Food	Indoor Food	Residential Outdoor		
Magnitude of residue								
860.1500	Crop field trials	R	R	R	CR	CR	TEP	a
860.1900	Field rotational crops	CR	CR	NR	NR	NR	TEP	a

Definitions: R = Required; CR = Conditionally Required; NR = Not Required; TGAI = Technical Grade of the Active Ingredient; TEP = Typical End-Use Product

[a] see 40CFR158 §158.1410; http://www.ecfr.gov/cgi-bin/text-idx?SID=167bce653abc0770ce9748e9d28ff832&mc=true&node=sp40.24.158.o&rgn=div6 (last accessed 06/27/2016).

Table 4 below shows data that may be necessary on a case-by-case basis for characterizing risk from residues in pollen and nectar. Since risk is a function of both exposure and toxicity, measured residues in various plant matrices can provide exposure data to refine risk estimates. These data can also be useful in determining uptake and decline curves for residues of concern in pollen/nectar and for determining the extent to which a compound is distributed systemically. Alternatively, data on residues in pollen and nectar may be available through the ecological effect studies where the pesticide is applied under semi- and full-field conditions (**Tables 1** and **2**). As with other environmental fate studies where the registrant must demonstrate that the methods of chemical analysis are appropriate (*i.e.*, reliable and sensitive), to support residue analysis in pollen and nectar, the registrant must provide evidence that suitable environmental chemistry methods (ECM) with independent laboratory validation (ILV) have been used to quantify residues. It is important to keep in mind that sampling colonies for pollen, nectar, wax and beebread can be destructive to the colony as food reserves and comb are collected as samples. Depending on the sample sizes and the frequency of sampling, such efforts may be disruptive to studying the adverse effects on the bees/colony as a whole. Depending on the study design, separate colonies for collecting residue exposure data may be needed where pollen traps are used to collect incoming pollen from the legs (corbicula) of forager bees or nectar from the honey stomach of forager bees. Pollen and/or nectar (beebread and/or honey) collected from the comb may require that the desired sample is gouged from the comb until sufficient sample size is obtained. Additional information on exposure study design elements to consider when recommending/reviewing such studies is provided in **Appendix 5**.

[54] CFR40. 2016. Title 40 (Protection of Environment), Part 158 (Data Requirements for Pesticides), Subpart O (Residue Chemistry) §158.1410 (Residue chemistry data requirements table. http://www.ecfr.gov/cgi-bin/retrieveECFR?gp=&SID=fc0257afaa2feb94488f8f1c4af6d3e6&n=pt40.24.158&r=PART&ty=HTML#se40.24.158_12150 (last accessed 06/27/2016).

Table 4. Requirements for Residue Data Measured in Pollen and Nectar.

Study	Study Type	Test substance	Test Note No.
Non-Guideline Study (Tier 2)	Field trial of residues in pollen and nectar	TEP	1, 2

Definitions:; TEP = Typical End-Use Product

[1]Field studies that quantify pesticide residues in pollen/nectar may be required to refine screening level exposure estimates, depending on the results and evaluation of Tier 1 studies. Pollen and nectar residue studies may be required if the ratio of the EEC and larval or adult bee acute LD_{50} >0.4 or the ratio of the EEC and chronic NOAEC >1. Incident data and/or compelling open literature studies can also serve as rationale for requiring pollen and nectar residue studies. These data can be collected at any point during the tiered process; prior consultation with the Agency is recommended to determine when to collect the data, and test protocols must be submitted for Agency review prior to initiation of the study. For greenhouse use patterns, data are required for crops that require pollination (*e.g.*, tomatoes); for aquatic use patterns, data are required if bees are likely to be exposed as a result of the proposed use (*e.g.,* riparian vegetation).

[2]Protocol should be submitted for EPA review prior to initiating study.

4. Non-Conventional Pesticides

Some of the studies noted in this paper may be useful for reviewing effects of other (non-conventional) pesticides, but how and whether they will be appropriate will require further consideration. Other types of pesticides may vary from conventional pesticides in their use patterns, modes of action, likelihood of exposure, and other unique characteristics. The following sections explain EPA's current approach to evaluating effects of non-conventional pesticides on honey bees and other pollinators.

4.1 Antimicrobial Pesticides

For antimicrobial pesticides, exposure to pollinators may result from compounds used as wood preservatives or any product which can be used *"for beehive applications when the beehive (empty or occupied) may be treated"*. In 40 CFR Part 158 Subpart W[55], the Tier 1 studies [honey bee acute contact data (OCSPP 850.3020) and toxicity of residues to honey bees (OCSPP 850.3030)] are required for all wood preservatives and conditionally required for products used for beehive applications. The acute contact study is routinely required of all wood preservatives and for hive treatments. The toxicity of residues study is intended to provide risk information for hives constructed of treated wood and is rarely required since EPA's Biological and Economic Analysis Division (BEAD) provided information that hives are not constructed of treated wood because the potential exposure of wood preservatives to bees is too high.

The additional studies listed in **Table 2** are not applicable to antimicrobial compound use patterns because exposure from antimicrobial uses is expected to be minimal. Honey bees may rest on treated surfaces, but would not be attracted to feed or to gather treated materials for transport to the hive.

4.2 Biochemical Pesticides

For biochemical pesticides, exposure to pollinators may result primarily from foliar ground and aerial applications, as well as from products used within beehives to control pathogens or parasites. In 40 CFR

[55] CFR40. 2016. Title 40 (Protection of Environment), Part 158 (Data Requirements for Pesticides), Subpart W (Antimicrobial Pesticide Data Requirements), §158.2240 (Non-target organisms.
http://www.ecfr.gov/cgi-bin/text-idx?SID=fc0257afaa2feb94488f8f1c4af6d3e6&node=sp40.24.158.w&rgn=div6 (last accessed 06/27/2016).

Part 158.2060 Subpart U[56]) Tier 1 non-target insect testing (OCSPP 880.4350[57]) is required for all use sites (except indoor use sites) *"depending on pesticide mode of action, method and timing of application and results of any available efficacy data. Typically, honeybee acute toxicity testing (850.3020) satisfies this requirement, however, additional non-target insect species"* (OCSPP 850.3040[58]; a Tier 3 study) *"may have to be tested if necessary to address issues raised by use patterns and potential exposure of important non-target insect species (e.g. endangered species)."* The honey bee toxicity of foliar residues study (OCSPP 850.3030[59]) is required on a case-by-case basis, dependent upon the route of exposure. In addition, a honey bee acute oral toxicity as shown in **Table 2** may be necessary, on a case-by-case basis, if the active ingredient is systemic within treated plants and is likely to result in residues in pollen or nectar. The honey bee larvae acute oral toxicity testing as shown in **Table 2** may be necessary on a case-by-case basis depending upon the route of exposure to honey bee larvae.

Additional non-guideline chronic toxicity testing is not considered necessary due to the nature of most biochemical pesticide active ingredients which are applied at relatively low rates and are non-persistent in the environment. Chronic oral toxicity and chronic contact toxicity studies may be considered in the future, on a case-by-case basis, should a new biochemical pesticide active ingredient be demonstrated to be persistent, or if chronic exposure resulting from repeated applications would indicate the necessity for such studies.

5.3 Microbial Pesticides

Pollinators may be exposed to microbial pesticides through both contact and oral routes, although the importance of each route to toxicity or pathogenicity varies among active ingredients. Current microbial pesticide data requirements that are specific to pollinators are described in 40 CFR Part 158 Subpart V[60], and include Tier 1 honey bee testing (OCSPP 885.4380[61]) and Tier 3 simulated or actual field testing with insect pollinators (OCSPP 850.3040[62]). Tier 1 testing with honey bees is intended to examine the potential for both toxic and pathogenic effects, and is required for all aquatic and terrestrial food/feed and non-food uses, forestry uses, and outdoor residential uses. Tier 3 testing is conditionally required depending on effects observed in testing at lower tiers.

The Microbial Pesticides Branch (MPB) of the Biopesticides and Pollution Prevention Division (BPPD) recognizes the importance of considering additional pollinator data requirements with the goal of ensuring consistency within OPP and improvements to pollinator testing and risk assessment. Additional attention to bee effects testing is necessary for microbial pesticides due to their unique nature and modes of action.

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[56] CFR40. 2016. Title 40 (Protection of Environment), Part 158 (Data Requirements for Pesticides), Subpart U (Biochemical Pesticides), §158.2060 (Biochemical pesticides nontarget organisms and environmental fate data requirements table). http://www.ecfr.gov/cgi-bin/retrieveECFR?gp=&SID=fc0257afaa2feb94488f8f1c4af6d3e6&n=pt40.24.158&r=PART&ty=HTML#sp40.24.158.u (last accessed 06/27/2016).

[57] USEPA. 1996. Biochemicals Test Guidelines. OPPTS 880.4350. Nontarget Insect Testing. Office of Chemical Safety and Pollution Prevention formerly the Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-96-285. February 1996. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-880-biochemicals-test-guidelines (last accessed 06/27/2016).

[58] *Ibid* USEPA 2012*c*

[59] *Ibid* USEPA 2012*b*

[60] CFR40. 2016. Title 40 (Protection of Environment), Part 158 (Data Requirements for Pesticides), Subpart V (Microbial Pesticides) §158.2150 (Microbial pesticides nontaget organism and environmental fate data requirements table) http://www.ecfr.gov/cgi-bin/retrieveECFR?gp=&SID=fc0257afaa2feb94488f8f1c4af6d3e6&n=pt40.24.158&r=PART&ty=HTML#se40.24.158_12150 (last accessed 06/27/2016)

[61] USEPA. 1996. Microbial Pesticides Test Guidelines OPPTS 885.4380. Honey Bee Testing, Tier I. Office of Chemical Safety and Pollution Prevention formerly the Office of Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-96-337. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-885-microbial-pesticide-test-guidelines (last accessed 06/27/2016).

[62] *Ibid* USEPA 2012*c*

The MPB is considering these data needs and will develop future guidance for microbial pesticide pollinator data requirements.

5.4 Plant-Incorporated Protectants

Pollinator data requirements for Plant-Incorporated Protectants (PIPs) are currently determined on a case-by-case basis. The MPB does not anticipate changes to the current approach for PIPs at this time.

Appendix 1. Tier 3 Field Study Design Considerations

Tier 3 studies conducted under full-field conditions where bees are free foraging are intended to address specific uncertainties/risks that have been identified in lower-tier studies. The design of these studies will depend on the specific questions that need to be answered; therefore, it is not possible to define a single study design or specific design elements that must be incorporated into every full-field study. Below are elements that the risk assessor should consider; however, these are not intended to be prescriptive. Some of these study design elements have also been identified in the EFSA guidance[63] document. It is incumbent on the chemical team to ultimately identify the study design elements that should be considered by the pesticide registrant/applicant in developing a study protocol that is responsive to the Tier 3 study requirement.

Full-Field Pollinator Study Design Considerations

Application Conditions

- Maximum application rate
- Minimum reapplication interval
- Maximum number of applications
- Use of formulated end-product
- Application method
    - Foliar
    - Soil treatment
    - Seed treatment
    - Combination
- Suitable weather
    - Avoid applications when rain and/or high winds are predicted.
- Season

Test crop

- Attractive test bees
- Long bloom period to address concerns identified at lower tiers
- Large area to ensure majority of foraging on test crop
- Follow standard [local] agriculture practices

Colonies

- Package bees/new equipment to limit incidence of disease; if older colonies are used, they should be as pest/disease free as possible.
    - Colonies should not be used if they have received any chemical treatments within last 4 weeks. Colonies suspected of having American foulbrood should not be utilized. Other disease treatments should be reported.
    - Beekeeper standard practice for maintain colony health during study
        - All treatments must be uniform across study colonies.

[63] *Ibid* EFSA. 2013.

- Queen-right (healthy queen present); sister queens for each replicate.
- Acclimation period: 2 months minimum to establish representative age distribution in newly established hives;
- Homogeneous colony strength, brood pattern as close as possible
- If existing colonies are to be used; broad spectrum residue analysis in hive products (honey/nectar, pollen, wax); must document low incidence of diseases/parasites.
- Size of the colonies may vary depending on the focus of the study and when the study is initiated. Typically, each hive should at least 10,000 bees to cover 10 frames and include at least 5 brood frames. Excessive food storage should be avoided.
- Colonies can be positioned in plots when test crops are blooming enough to minimize test bees foraging on plants other than the test crop, *e.g.*, 20-25% bloom

Study Design Considerations

Historically there has been difficulty in controlling the extent to which the free-foraging bees utilize the treated crop or that treatment groups cross-over (*i.e.*, return to colonies other than their own). Sufficiently large field plots, if feasible, will overcome the cross-over issue between plots and problems of insuring exposure to treated crops due to competing vegetation.

- Mean honey bee foraging distance 1.5 – 3 km with extreme distances of 10 km; average surface area range 7 – 100 km^2 (Medrzycki *et al*. 2013[64]).
    - EFSA 2013[65] recommends minimum of 2 ha to provide sufficient flowers and support exclusive foraging; Medrzycki *et al*. 2013 recommends minimum of 5 ha.
- Suitable crop that is representative of actual use; good source of both pollen and nectar, (*e.g.*, phacelia (*Phacelia tanacetifolia*), canola/oilseed rape/mustard (*Brassica napus*, *Brassica rapa, Sinapis alba, Brassica juncea, or Brassica nigra*), buckwheat (*Fagopyrum esculentum*))
    - Pollinator-attractive
- Account for crops/alternative forage within 3 km of colonies.

Distance of treated crop from other nectar producing plants is essential to insure exposure and must be documented.

- Pollen traps should be used to demonstrate extent to which bees have foraged on treated crop.
- Pollen identification (palynological analysis) may be used to insure origin of pollen
- Pollen/nectar collection for residue analyses
    - Collected by bees and sampled using pollen traps (corbicular pollen)
    - Collected directly from plants
    - Sampling of nectar forager honey stomachs
    - Sampling comb pollen/nectar

Minimum number of replicate colonies: 6 - 10 per treatment (Medrzycki *et al*. 2013); the number of replicates per treatment will depend on the targeted magnitude of effects and desired statistical power.

[64] Mwsezycki, P. H. Giffard, P. Aupinel, L. P. Belzunces, M-P. Chauzat, C. Classen, M. E. Colin, T. Dupont, V. Girolami, R. Johnson, Y. LeConte, J. Lückmann, M. Marzaro, J. Pistorius, C. Porrini, A. Schur, F. Sgolastra, N. S. Delso, J van der Steen, K. Wallner, C. Alaux, D. G. Biron, N. Blot, G. Bogo, J-L Burnet, F. Delbac, M. Diogon, H. El Alaoui, B. Provost, S. Tosi and C. Vidau. 2013. Standard methods for toxicology research in *Apis mellifera.* Journal of Apicultural Research 52(4): http://www.coloss.org/beebook/I/introduction (last accessed 06/27/2106).
[65] *Ibid* EFSA 2013

Study duration should assess at least two brood cycles (42 days) to ensure brood is exposed to residues stored in the colony (EFSA 2013).

Measurement Endpoints: depend on the risk hypothesis tested and the nature of uncertainties identified in lower-tier tests. Possible measurement endpoints may include.

Adult Forage Bees

- Adult bee survival/longevity
- Adult bee foraging activity (visual counts of returning foragers; mark-and-recapture; calibrate Dead Bee Dead Zone traps)
- Queen status over the course of the exposure

Colony health (disease/pest incidence)

Colony Strength

- Brood (quantify number of eggs; larvae, capped cells, pollen, honey/nectar cells)
- Monitoring of brood in a minimum of two staggered cohorts, mid-way and late in the exposure period
- Adult longevity: measured by using 30 newly-emerged adult bees from each colony (minimum n=6 colonies/treatment) in a controlled laboratory cage experiment monitoring daily mortality
- Newly-emerged bee weights

Other potential endpoints include the following:

Overwintering Success

Fitness measure: Pathogen challenge (*e.g.*, Nosema exposure) newly emerged bees

Assess the ability of colonies to re-queen themselves by removing all queens and determining the success of each colony in rearing a replacement queen.

Documenting Exposure

- Residue analyses in pollen/nectar
- Residue analyses in bee carcasses
- Residue analyses in wax
- Foliar Residue analysis
- Measure total residues of concern (parent + degradate(s))
- Pollen source (palynology) to ensure bees have been foraging on target crop.

Suitable control bees (residue analyses to demonstrate lack of exposure). Utility of mark-and-recapture to document drift of bees from treated colonies.

Appendix 2. Data Justification Tables for Non-Codified Exposure and Effects Studies with Bees

The following data justification tables should be considered by OPP risk assessors when recommending additional bee exposure and effects studies in support of pesticide review actions (*e.g.,* Problem Formulation documents under Registration Review).

Study Title: Tier 1 Honey bee Adult Acute Oral Toxicity
Rationale for Requiring the Data
Terrestrial invertebrates are likely to be impacted if exposed to pesticides in various use settings. With eusocial bees, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to the hive. Therefore, potential acute effects to adult honey bees and other insect pollinators from oral exposure to some pesticides could exist. Currently available toxicity studies do not address possible effects of oral exposure on adult terrestrial insect survival. Because of the potential for pollen and nectar to be contaminated with pesticide residues, and subsequently brought back to the hive, it is important to determine the acute oral toxicity of this compound to adult honey bees and other insect pollinators. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate test species. The guidance discusses Tier 1 laboratory-based acute oral toxicity studies of individual adult bees as a critical component of the screening-level risk assessment process for examining potential adverse effects from specific routes of exposure. The guidance can be found at: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Additional guidance on the honey bee oral toxicity test design can be found in OECD Test Guideline 213 (http://www.oecd-ilibrary.org/docserver/download/9721301e.pdf?expires=1423074617&id=id&accname=guest&checksum=2F0764FCB4DCF01D32382952A2E995C3)
Practical Utility of the Data
How will the data be used? The Tier 1 acute oral toxicity data on adult honey bees serve as a foundation for the screening-level assessment of potential risk non-target organisms such as federally listed threatened or endangered and non-listed terrestrial invertebrate insects, including pollinators, from acute oral exposures to pesticides. The data will be used to reduce uncertainties associated with the risk assessment for terrestrial invertebrates and will improve EPA's understanding of the potential direct and indirect effects on a broad range of taxa. This study will also provide information with which to compare whether oral toxicity estimates differ from contact toxicity estimates obtained from other Tier 1 studies. If acute oral effects data for adult honey bees are not available, risks to terrestrial insects from acute oral exposure will be assumed. **How could the data impact the Agency's future decision-making?** The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect listed threatened or endangered species and/or modify their designated critical habitat. Without these data, EPA may need to presume risk, which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in use restrictions.

Study Title: Tier 1 Honey bee Larvae Acute Oral Toxicity
Rationale for Requiring the Data
Terrestrial invertebrates are likely to be impacted if exposed to pesticides in various use settings. With eusocial bees, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to the hive where developing larvae and pupae may be exposed. Therefore, potential adverse effects to developing bees could result from exposure to pesticide residues. Available toxicity studies do not address possible effects on brood (larvae and pupae) survival/development. Because of the potential for pollen and nectar to be contaminated with pesticide residues, and subsequently brought back to the hive, it is important to determine the acute toxicity of this compound to bee brood. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate test species. The guidance discusses Tier 1 laboratory-based acute toxicity studies of individual honey bee larvae as a critical component of the screening-level risk assessment process for examining potential risks from specific routes of exposure. The guidance be found at: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Additional guidance on larval honey bee toxicity test design can be found in OECD Test Guideline 237 (http://www.oecd-ilibrary.org/docserver/download/9713171e.pdf?expires=1422485600&id=id&accname=guest&checksum=D8E07C2B1DF77BF096C3B29F55BF86A7).
Practical Utility of the Data
How will the data be used? The Tier 1 acute toxicity data on honey bee larvae serve as a foundation for the screening-level assessment of potential risk to non-target organisms including federally listed threatened or endangered and non-listed terrestrial invertebrates, including pollinators, and/or modify their designated critical habitat from acute exposures to pesticides. The data will be used to reduce uncertainties associated with the risk assessment for terrestrial invertebrates and will improve EPA's understanding of the potential effects on terrestrial species and whether there is a differential sensitivity of larval bees relative to adult bees. If acute effects data for larvae are not available, risks to terrestrial insects from acute exposure will be assumed. **How could the data impact the Agency's future decision-making?** The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect listed threatened or endangered species and/or modify their designated critical habitat. Without these data, EPA may need to presume risk which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in use restrictions.

Study Title: Tier 1 Honey Bee Adult Chronic Oral Toxicity
Rationale for Requiring the Data
Terrestrial invertebrates are likely to be impacted if exposed to pesticides in various use settings. With eusocial bees, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to the hive. Therefore, potential chronic effects to adult honey bees and other pollinators from oral exposure to some pesticides could exist. Currently available toxicity studies do not address possible lethal and sublethal effects of chronic oral exposure on adult terrestrial invertebrates and will assist in determining whether the sensitivity of adult bees differs from that of earlier life stages. Because of the potential for pollen and nectar to be contaminated with pesticide residues, and subsequently brought back to the hive, it is important to determine the chronic oral toxicity of this compound to adult honey bees and other pollinators. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate test species. The guidance discusses Tier 1 laboratory-based chronic oral toxicity studies of individual adult honey bees as a critical component of the screening-level risk assessment process for examining potential risks from specific routes of exposure. The guidance can be found at: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Although study design elements for the chronic 10-day oral toxicity test with honey bees are similar to the OECD TG 213 acute oral toxicity test (http://www.oecd-ilibrary.org/docserver/download/9721301e.pdf?expires=1422484908&id=id&accname=guest&checksum=C38495D2A570AC2216CFB1F223D24AA7), EPA requires that the proposed protocol for this study be submitted for review and approval by EPA prior to initiating the test.
Practical Utility of the Data
How will the data be used? The Tier 1 chronic toxicity data on adult bees serve as a foundation for the screening-level assessment of potential risk to non-target organisms including federally listed threatened or endangered species and non-listed terrestrial invertebrates, including pollinators, from chronic oral exposures to pesticides. The data will be used to reduce uncertainties associated with the risk assessment for terrestrial invertebrates and will improve EPA's understanding of the potential direct and indirect lethal and sublethal effects on a broad range of terrestrial species, particularly insect pollinators and to determine whether adult toxicity differs substantially from other life stages evaluated in other Tier 1 tests. If chronic oral effects data for adults are not available, risks to terrestrial insects from chronic exposure will be assumed. **How could the data impact the Agency's future decision-making?** The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect listed threatened or endangered species and/or their designated critical habitat. Without these data, EPA may need to presume risk which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in use restrictions.

Study Title: Tier 1 Honey Bee Larvae Chronic Oral Toxicity
Rationale for Requiring the Data
Terrestrial invertebrates are likely to be impacted if exposed to pesticides in various use settings. For eusocial bees, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to the hive where larvae and pupae may be exposed. Therefore, potential effects to developing bees could result from chronic exposure to pesticide residues. Available toxicity studies do not address possible chronic effects on brood (larvae and pupae) survival. Because of the potential for pollen and nectar to be contaminated with pesticide residues, and subsequently brought back to the hive, it is important to determine chronic larval/pupal toxicity and whether adult emergence is adversely affected. This study will provide information on whether honey bee larvae differ in sensitivity from adult bees following chronic exposure. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate test species. The guidance discusses Tier 1 laboratory-based chronic toxicity studies of individual honey bee larvae as a critical component of the screening-level risk assessment process for examining potential risks from specific routes of exposure. The guidance can be found at: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Additional information on larval honey bee toxicity repeat exposure test design can be found in the OECD draft guidance (http://www.oecd.org/env/ehs/testing/Draft_GD_honeybees_rep_exp_for_2nd_CR_25_November_2013.pdf). Although study design elements for the chronic 21-day toxicity test with honey bee larvae have been drafted, EPA requires that the proposed protocol for this study be submitted for review and approval by EPA prior to initiating the test.
Practical Utility of the Data
How will the data be used? The Tier 1 chronic toxicity data on bee larvae serve as a foundation for the screening-level assessment of potential risk to non-target organisms including federally listed threatened or endangered and non-listed terrestrial invertebrates, including insect pollinators, from chronic exposures to pesticides. These data will be used to reduce uncertainties associated with the risk assessment for terrestrial invertebrates and will improve EPA's understanding of the potential direct and indirect lethal and sublethal effects on a broad range of terrestrial species, particularly insect pollinators. These data will also assist in determining whether early life stages of the bee differ in their sensitivity to pesticides relative to adults. If chronic effects data for larvae are not available, risks to terrestrial insects from chronic exposure will be assumed. **How could the data impact the Agency's future decision-making?** The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect listed threatened or endangered species and/or modify their designated critical habitat. Without these data, EPA may need to presume risk which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in use restrictions.

Study Title: Tier 2 Semi-field Testing for Pollinators (tunnel studies)
Rationale for Requiring the Data
Tier 2 studies are conditional on the outcome of the screening-level assessment where acute and/or chronic risk levels of concern have been exceeded for terrestrial invertebrates. Terrestrial invertebrates are likely to be impacted if exposed to pesticides in various use settings. For eusocial bees, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to the hive and may adversely affect developing brood (egg, larvae, and pupae) and adult bees. Screening-level (Tier 1) studies of individual bees do not address possible effects and/or exposure to pesticide residues at the colony-level. Because of the potential for pollen and nectar to be contaminated with pesticide residues, and subsequently brought back to the hive, it is important to determine whether bee colonies may be negatively affected under relatively controlled exposure conditions of a semi-field study. In addition to providing effects data, these studies can provide data on pesticide residues in pollen/nectar of treated plants. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate test species. This guidance describes the tiered testing process and can be found at: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance, Additional information on honey bee colony studies under semi-field conditions can be found in the OECD Guidance 75 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282007%2922&doclanguage=en). Due to the complexities of this study, EPA requires that the proposed protocol for this study be submitted for review EPA prior to initiating the test.
Practical Utility of the Data
How will the data be used? Tier 2 colony-level data will be used to assess potential risk to non-target organisms including listed and non-listed terrestrial social invertebrate species and to determine whether effects observed in the screening-level (Tier 1) laboratory-based studies of individual bees are evident in colony-level studies under semi-field conditions. The Tier 2 semi-field test of whole colonies is a relatively controlled study, *i.e.*, bees are confined to a specific area that is designed to represent potential field-level exposure and account for hive dynamics, which are not achievable from other pollinator studies. This study will be used to determine whether adverse effects to insect pollinators at the whole colony level, may result for the use of pesticides and will help to refine risk estimates derived in the screening-level risk assessment for beneficial terrestrial invertebrates. Measured residues in pollen/nectar can also be used to refine risk estimates derived from model-based or default values in the screening-level assessment. **How could the data impact the Agency's future decision-making?** The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect federally listed threatened or endangered species or their designated critical habitat. Without these data, EPA may need to presume risk which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in significant use restrictions.

Study Title: Tier 2 Semi-field Testing for Pollinators (colony feeding studies)
Rationale for Requiring the Data
For eusocial bees, pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to the hive and may adversely affect developing brood (egg, larvae, and pupae) and adult bees. Tier 2 feeding studies are conditional on the outcome of the screening-level assessment where acute and/or chronic risk levels of concern have been exceeded for terrestrial invertebrates based on Tier 1 studies of individual bees. Feeding studies utilize free foraging bee colonies that are "dosed" with specific quantities of test material and represent a means of ensuring exposure to the test material through spiked pollen and/or sugar solutions fed to the colony while still allowing the bees to forage freely. Since bee colonies are not confined to enclosures, colonies can be exposed for longer duration periods without subjecting the bees to stress that typically results from Tier 2 tunnel studies. Available toxicity studies of individual bees (Tier 1) conducted to support screening-level assessments do not address possible effects and/or exposure to pesticide residues at the colony-level. It is therefore important to determine whether bee colonies may be negatively affected where bees are free foraging and have the option to collect/consume alternative forage items beyond the spiked food. Since multiple dose levels can be more readily tested, feeding studies can help to define dose-response relationships at the whole colony level. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate. This guidance describes the tiered testing process and can be found at:: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Additional information on honey bee colony feeding studies can be found in the EPPO Guidance 170 (www.nationalbeeunit.com/downloadDocument.cfm?id=191). Although study design elements for the feeding study are available through the EPPO Guidance 170, EPA requires that the proposed protocol for this study be submitted for review and approval by EPA prior to initiating the test.
Practical Utility of the Data
How will the data be used? Tier 2 colony feeding data will be used to assess potential risk to non-target organisms including listed and non-listed terrestrial social invertebrate species. The colony feeding study is designed to represent potential field-level exposure and account for hive dynamics using longer duration exposure periods than are possible in Tier 2 tunnel studies. This study will be used to determine whether potential adverse effects to insect pollinators at the whole colony level when bees are able to forage naturally beyond the spiked food. Results from the feeding study will help to refine the screening-level risk assessment for beneficial terrestrial invertebrates that were based on Tier 1 studies on individual bees. Since feeding studies can help to define a dose-response relationship at the colony level, the studies can provide a means of determining exposure thresholds below which the likelihood of adverse effects on colonies may be low. **How could the data impact the Agency's future decision-making?** The Tier 2 colony-level data will be used to refine screening-level risk estimates derived using Tier 1 laboratory-based data on individual bees. The Tier 2 data will help to inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect federally listed threatened or endangered species or their designated critical habitat. Without these data, EPA may need to presume risk which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in significant use restrictions.

Study Title: Tier 3 Field Testing for Pollinators
Rationale for Requiring the Data
Tier 3 studies are conditional on the outcome of the screening-level assessment (Tier 1) where acute and/or chronic risk levels of concern have been exceeded for terrestrial invertebrates and where Tier 2 studies either under semi-field tunnel conditions and/or feeding studies have indicated potential adverse effects at the colony level. Available toxicity studies from lower-tier studies do not address possible effects and/or exposure to pesticide residues at the colony-level under actual pesticide use conditions and where specific uncertainties regarding the likelihood of exposure and/or effects remain. Full-field studies also provide an opportunity to measure residues in pollen and nectar as well as various matrices (beebread, honey, wax) within the colony to obtain a more realistic understanding of exposure. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate. This guidance describes the tiered testing process and can be found at:: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Additional information on honey bee colony studies under full-field conditions can be found in the OCSPP 850.3040; useful guidance is also available through OCSPP 850.2500 (Field Testing of Terrestrial Wildlife; http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines) Although design elements for the full-field colony-level study are available through the 850.3040 and 850.2500, EPA requires that the proposed protocol for this study be submitted for review and approval by EPA prior to initiating the test; the protocol should attempt to address specific uncertainties identified in lower-tier studies.
Practical Utility of the Data
How will the data be used? Tier 3 colony-level data will be used to further characterize potential risk to non-target organisms including listed and non-listed terrestrial social invertebrate species and to refine screening-level risk estimates that were based on individual bee responses. The semi-field test is a controlled study that is designed to represent potential field-level exposure under relatively controlled conditions and account for hive dynamics, which are not achievable from lower-tier pollinator studies. This study will be used to determine whether adverse effects to insect pollinators at the whole colony level, may result for the use of pesticides and will help to refine the screening-level risk estimates for beneficial terrestrial invertebrates. This study will also be used to determine whether more refined (Tier 3) studies are needed to characterize risk. **How could the data impact the Agency's future decision-making?** The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect federally listed threatened or endangered species or their designated critical habitat. Without these data, EPA may need to presume risk which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in significant use restrictions.

Study Title: Field trial of residues in pollen and nectar
Rationale for Requiring the Data
Terrestrial invertebrates are likely to be impacted if exposed to pesticides residues in various use settings. Pesticide residues may be transferred to pollen and/or nectar of treated plants and subsequently brought back to hive all life stages may be exposed. For some pesticides, the quantification of pollinator-relevant residues in treated flowering plants is needed, since pollinators will be exposed to residues from either current or prior season applications (due to the potential for residues to accumulate in plants and trees). Residues in edible/transportable-to-hive parts of treated trees and plants, including (where appropriate), but not limited to, guttation water, sap/resins, whole plant tissue (*e.g.,* leaves, stems), as well as blooming, pollen-shedding, and nectar producing parts (*i.e.*, flowers and, if present, extra-floral nectaries) of plants may inform the potential for risk. Studies should be designed to provide residue data for crops and application methods of concern. The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate. This can be found at: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Since residue studies are intended to provide exposure data in multiple matrices and under specific application conditions, EPA requires that the protocol is submitted for review and approval by EPA prior to initiation of the study.
Practical Utility of the Data
How will the data be used? Measured residue data will be used to refine conservative estimates of pesticide exposure and reduce uncertainties associated with the Tier 1 exposure assessment by providing direct measurements of pesticide concentrations resulting from actual use settings. Measured residues may provide a more realistic understanding of exposure through contact or ingestion with which to calculate risk quotients for individual bees as well as to characterize exposure to the colony. If measured residue data are not available, risk estimates for terrestrial insects will be based on model generated or default values used to support the screening-level assessment. **How could the data impact the Agency's future decision-making?** The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect federally listed threatened or endangered species or their designated critical habitat. Without these data, EPA will have to rely on conservative estimates of exposure which may limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in use restrictions.

Study Title: Tier 1 Pollinator Acute Vapor Exposure Toxicity (modification of acute contact toxicity test)

Rationale for Requiring the Data

Pesticide chemicals can come in the form of solids, liquids or gases. Some pesticides are highly volatile or are gases (*e.g.*, fumigants). Conducting toxicity testing based on contact and ingestion routes such as might occur with liquid or solid pesticides is not appropriate for evaluating the toxicity of highly volatile compounds or gases. If environmentally-relevant concentrations are possible, such as may be the case for most pesticides used as fumigants, evaluation of the impact on non-target species, such as terrestrial invertebrates including pollinators, provides valuable information for mitigating that risk in the use labeling. Therefore, to assess the toxicity of highly volatile pesticides and gases to terrestrial invertebrates, an acute vapor exposure toxicity study is appropriate *in lieu* of the toxicity testing through other delivery methods.

The Office of Pesticide Programs has made available a guidance regarding ecological testing for bees using the honey bee as a surrogate. These can be found at: http://www2.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance. Design elements from Tier 1 laboratory-based studies of individual adult bees (OCSPP 850.3020 and OECD TG 213, OECD TG 214) and larval bees (OECD TG 237 as well as draft OECD guidance on chronic larval bee toxicity testing) may also provide useful information. EPA requires that the proposed protocol for the inhalation study be submitted to EPA for review and approval prior to initiating the study.

Practical Utility of the Data

How will the data be used?

Tier 1 data on individual bees serve as a foundations for the screening-level risk assessment process used to determine the potential for a pesticide (in the form of a gas/vapor) to affect non-target terrestrial invertebrates, including pollinators, in their environment. These data will be used to reduce uncertainties associated with the risk assessment for terrestrial invertebrates and will improve EPA's understanding of the potential effects on terrestrial species. If inhalation toxicity data are not available, risks to terrestrial insects from vapor exposure will be assumed.

How could the data impact the Agency's future decision-making?

The data will inform the determination required under FIFRA or the ESA as to whether continued registration of a pesticide is likely to result in unreasonable adverse effects to non-target species or is likely to adversely affect federally listed threatened or endangered species or their designated critical habitat. Without these data, EPA may need to presume risk which will limit the flexibility of pesticide products to comply with FIFRA and the ESA, and could result in use restrictions.

Appendix 3. Laboratory Larval Study Design Elements

Laboratory-based studies with larval bees are described in the OECD Test Guideline 237[66]; however, this design is for single dose studies where bees are euthanized after Day 9. An OECD draft guidance[67] has been developed for repeat dose exposure where the study is conducted for 21 days and is intended to extend through adult bee emergence. Bees are fed treated diets from Day 3 through Day 6 and mortalities are recorded from Day 4 to Day 8, Day 15 and Day 22. The study provides a NOAEC and/or EC_{50} for adult emergence on Day 22 as well. Study conditions described in the draft guidance do not differ substantially from OECD TG 237; however, some contract labs have had difficulty in achieving control mortality rates of less than 20%. High mortality rates may in some cases result from contamination of the individual test wells where fungi and/or bacteria overtake the well. In the development of brood, the digestive tract of the larvae is not complete until roughly Day 9; once the digestive tract is complete, the organism will defecate and this process is typically considered the initiation of pupation. The excrement may be the source of contamination that increases mortality levels in these tests.

Minor modifications in the study design have been effective in reducing control mortality and are typically intended to reduce the likelihood of well contamination. Protocols submitted for review and approval by EPA should describe measures taken to reduce the likelihood of contamination in culture wells. One option is to transfer larvae to clean culture wells on Day 9. During the transfer, individual test organisms can be carefully cleaned with sterile physiological saline and gently blotted dry.

Concerns regarding diet preparation for chemicals with limited solubility and/or high sorption characteristics should be resolved through discussions with the registrant during protocol review.

Additional study designs for evaluating the effects of chronic exposure on bee larval development and which extend through adult emergence are under development. Similar to evaluating data for other taxa, risk assessors should consider the strengths and weaknesses of alternative study designs, *i.e.*, non-guideline studies, in providing data to evaluate potential effects.

[66] *Ibid* OECD 2013.
[67] OECD 2013*b*. OECD Draft Guidance Document Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure. http://www.oecd.org/env/ehs/testing/Draft_GD_honeybees_rep_exp_for_2nd_CR_25_November_2013.pdf

Appendix 4. Tier 2 Feeding Study Design Considerations

Study Overview:
The honey bee colony feeding study is designed to evaluate the effects of long-term exposure to various concentrations of a pesticide in a food source for honey bees. The study is intended as a Tier 2 study consistent with the overall tiered approach of pollinator risk assessment as identified in the 2013 White Paper and the 2014 Guidance on Assessing Pesticide Risks to Bees. Measurement endpoints relate to colony strength, specifically in terms of brood abundance, adult population size, and the amount of food stores (both pollen and nectar) within the hives. These measurement endpoints are also intended to provide information on the concentrations of the pesticide that affect whole colonies due to their long-term exposure to a pesticide in food sources for honey bees. Although not intended to be prescriptive, the study design considerations described here are intended to help ensure that a no observed adverse effect level (NOAEL) can be established and used in conjunction with field residue studies to qualitatively characterize the potential toxicity of a pesticide to honey bees based on corresponding application rates to specific crops. Past study designs have included in-hive feeders with untreated sucrose solutions (control hives) or pesticide-spiked sucrose solutions (treatment hives) over a 6-week period. Colony condition assessments are observed prior to and during exposure period as well as after overwintering.

Pesticide Treatments:
Past feeding study designs have included feeding spiked sucrose solutions and/or spiked pollen. Depending on the nature of the pesticide exposure and toxicity, one or both of these dietary media may be used for pesticide exposure. Generally, pesticide treatments should include a negative control and at least five test concentrations. Selected concentrations should bracket the Tier 1 estimates for residues in pollen and nectar, take into consideration measured residue data from field studies and incident reports, and include the lowest concentration where sublethal effects were noted in acute or chronic toxicity studies with honey bees. Treatment levels should be chosen to ensure that a NOAEL and lowest observed adverse effect level (LOAEL) are obtained.

The study design should make every effort to minimize variability between the exposure levels for each of the hives (see potential sources of variability below). It is important to confirm the test material exposure levels in the food prior to replenishment, as well as confirm test material stability during the study. The volume/mass of the new and old treatment solutions/pollen paddies at replenishment should also be reported.

Initial Hive Conditions:
At initiation, each hive should consist of an appropriate number of bees to ensure that the colony would be sizable enough to have overwintering success (*e.g.*, 10,000 bees (3 lb package)). The size of the initial colony will vary based on geographic location; therefore it is important to follow local beekeeping practices. Each hive should consist of one hive box with an initial 8-frames, and an empty box above for the feeder. After the exposure phase of the study, hive boxes should be expanded (*i.e.,* adding more frames or boxes) as appropriate to facilitate food storage and colony growth.

It is expected that the use of new colonies (*i.e*., single box) earlier in the season and new hive equipment would minimize the infestation levels of diseases and parasites. The new hive equipment (*e.g*., plastic foundation) would minimize exposure to other contaminants as well. The study should report levels of pests

(*e.g.*, Varroa mite; small hive beetle; wax moths) and disease (*e.g.*, fungal; bacterial; viral) levels in the colonies, pre-exposure as well as during the exposure/post-exposure period.

Queen genotype may influence the differential performance of the hives. Colonies should use sister queens to minimize genotypic variability as much as possible. If a queen dies during the pre-exposure period, she should be replaced; however queen replacement is not acceptable during the exposure or post-exposure phases, the hives should be allowed to generate a new queen (*i.e.*, supersede) naturally.

The study design should reduce and equalize the amount of stored food at the start of the exposure period to minimize this source of variability between the hives and ensure that bees are consuming the sucrose solution provided during the experiment. The amount of stored food prior to the exposure period can affect the extent and timing of the exposure to the artificial food source, thus effects on the colony may be delayed due to delayed consumption of treated food.

Hive Maintenance and Apiculture:
All colonies should be maintained as typical for apicultural practice in the relevant region, including the application of antibiotics, pesticide treatments and supplemental food that may be required to maintain colony vitality. Apicultural practices must be clearly described and applied equally across the hives when one hive requires intervention. Interventions have the potential to mask the effects of the pesticide and should be used judiciously. The study report should note when an intervention is applied.

Ideally the hives should be positioned in an area that provide adequate forage outside of the dearth period should not require supplemental feeding. However, during the pre-exposure period, supplemental feeding with both pollen and nectar will provide ample resources for building up the frames with comb and stimulate brood production. In locations with marginal forage habitat (*i.e.*, inadequate supplies of pollen and/or nectar) for honey bees, planting a bee-attractive crop adjacent to the study sites that is known to provide both pollen and nectar (*e.g.*, buckwheat, alfalfa, clover) can also stimulate brood production and hive strength/condition during the pre-exposure and post-exposure periods.

In addition, precautions should be taken to prevent swarming (*e.g.*, adding a box to increase hive size); details of incidents of swarming must be provided in the study report. Robbing screens should be used, where necessary, to reduce the potential for robbing, this is especially important during and after the exposure period.

Test Site Locations and Characteristics:
The study should include a sufficient number of replicates across sites to capture the environmental variability in the geographic region (suggested 12 sites). Each site should contain one group of hives containing the control, treated colonies, and one colony used to identify residues collected during foraging. Hive selection for sites should be grouped (*i.e.*, blocked) by hive strength (*e.g.*, food storage (pollen and honey), adult population size, brood (egg, larval, pupal) abundance), pre-exposure colony assessments will help to normalize the hives to reduce within site variability. It is recommended that a visual layout of each test site is provided to better ascertain the design of the feeding study (*e.g.*, **Figure 1**).

The study should attempt to minimize inter-site differences in habitat and potential foraging sources. This may be done by examining land-use/land-cover maps and selecting sites as equivalent as possible in land use within at least a 3 mile range around the individual sites where bees can be expected to forage. Although

the honey bee foraging range can exceed 3 miles, this radius is assumed to provide a reasonable distance to standardize the land-use/land-cover for each of the sites. The study should include justifications for the selection of sites, specifically related to land-cover types, and the methods used to select various sites. During the conduct of the study, the surrounding area should be adequately characterized in terms of available forage, and potential sources of pesticide contamination. To the extent possible or feasible, the hives should be placed in an environment with minimal pesticide use. For studies designed to mimic exposure through contaminated nectar, the exposure period should occur during a period when alternative sources of floral nectar are low (*i.e*., dearth) at the selected study site to increase honey bees' reliance on the in-hive sucrose solution as their source of nectar. At each site, meteorological data should be recorded, including temperature, humidity, rainfall, *etc*.

Within Site Hive Placement, Orientation and Treatment Levels:
Prior to study initiation, colonies should be ranked according to colony strength. At each site, hives should be grouped with similar colony strength to minimize confounding influence of hive strength within a site. The orientation of the hives may also contribute to inter-hive variability. Hives that face the north would be exposed to the least amount of solar radiation. Temperature affects the ability of a bee to fly such that cold temperatures can inhibit the flight of forager bees. Different hive orientations may affect the productivity of hives and introduce variability into the study. Conversely, different hive orientations minimize the opportunity for bees to mistake their hive for another hive (*i.e.*, drift). The hives may therefore be arranged in a semi-circle and treatments would be randomly assigned a position on that semi-circle at the first site. The order of the hives by treatment would then incrementally rotate from site to site, as in **Figure 1**. If the study design can ensure that drift between hives would be negligible if all of the hives have the same orientation, then all hives could face the same direction to minimize the effect of aspect on colony performance.

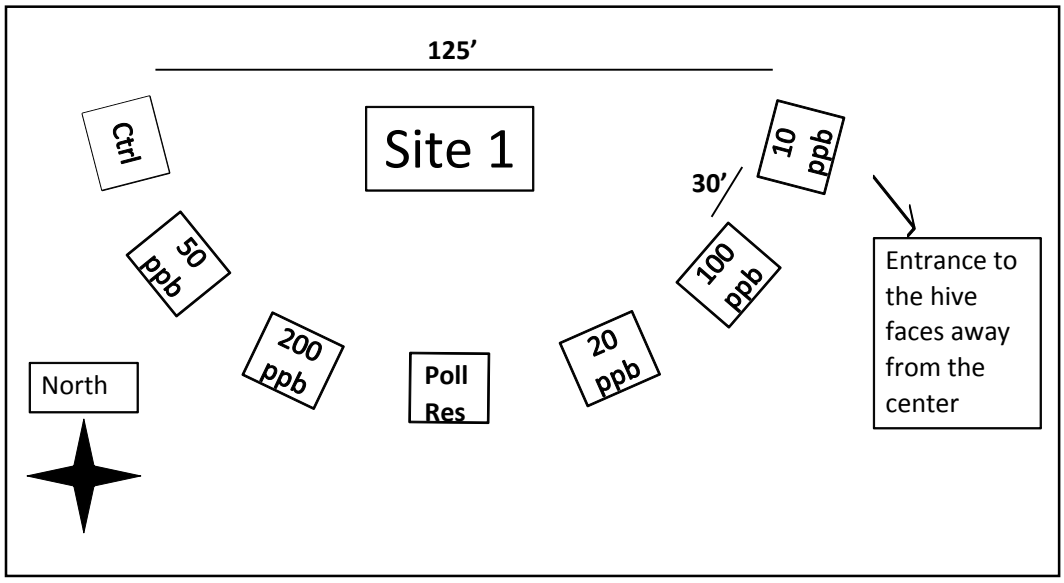

Figure 1. Potential layout of the hives within site locations where the treatments are randomly assigned a position within the shape of semi-circle, with the center of the semi-circle oriented to the South. The hive marked "Poll Res" is the designated hive for evaluating the pollen diversity and residues that bees are foraging over the course of the study. At the next site, the treatment location is rotated one space counter-clockwise. Note

that the selected site locations may not necessarily move in a North-South direction, but rather sites locations are selected based on land-use/land-cover.

In-Hive Observations:

Colony Condition Assessments (CCA's) should assess all of the frames in each colony for food storage (pollen and honey), presence/absence of queens, adult population size, brood (egg, larval, pupal) abundance, brood termination rate, compensation index, and brood index. Where possible, the study design should rely on digital image analysis or specifically state why a different method will be used. Digital imagery can provide a more reliable estimate of frame area beyond acetate sheets (EFSA report[68]), and can reduce the amount of time the hive is open. The study design should provide a clear indication of the measurement standard operating procedures for the collection of measurement endpoints considering that more one person may be collecting measurements. The study directors should record and report any behavioral observations using a standardized approach when performing the CCA's. The protocol and final study report should clearly describe the measurement unit and mechanism for assessing each assessment endpoint and colony descriptor (*e.g.*, strength of the adult population) used in the study. Furthermore, steps to monitor queen replacement (supersedure) by the colony, such as utilizing marked queens, should also be conducted. Care should be taken to ensure that any re-marking of queens during the study does not result in adverse effects on the queen or colony.

CCA's should be made during the pre-exposure (3 or more assessments), the exposure period (assessments every 2 weeks), the post-exposure period prior to overwintering (2 assessments, including once just before overwintering), and post-overwintering (2 assessments). No assessments should take place during the over-wintering period. The exact timing of the pre- and post-overwintering assessments will depend upon the weather and geographic location. To reduce the potential impact of colony assessments on the hive, assessments should be conducted at the same time as normal beekeeping practices that would open the hives.

Residue Analysis:

The study design should include an additional hive at each location to collect pollen samples for residue analysis of contaminants (including the test material) that may enter the hives through freely foraged food sources. Samples for residue analysis from hive matrices should be taken to monitor residues of the pesticide, relevant metabolites, and other residues of concern in capped honey, royal jelly, corbicular pollen, and comb pollen at a minimum of four time points during the study. The study report should clarify if contaminated hive matrices were detected and from which sites the residues were found. Details should be provided in the final protocol as to the analytical sampling scheme for the various exposure media.

Reducing Sources of Variability:

To the extent possible, it is important to control the sources of variability in the feeding study. Some sources of variability include, but are not limited to, the following:

[68] EFSA Panel on Plant Protection Products and their Residues (PPR); Scientific Opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). EFSA Journal 2012; 10(5) 2668. [275 pp.] doi:10.2903/j.efsa.2012.2668. Available online: http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/2668.pdf (last accessed 06/27/16).

- Inter-site differences in microclimate, habitat (percent crop, forest, residential within the foraging range of the colony), and available forage
- Pre-exposure variability or inadequate equalization of hive strength in hive parameters of adult bees, stored honey, stored pollen, capped brood, and open brood
- Infection/infestation by diseases or parasites
- Typical apicultural practices
- Orientation of the hives and consequent differences in aspect as it relates to microclimate
- Differences in queen genotype as it relates to colony phenotype across the colonies
- Variability in data collection techniques, especially when the data are collected based on visual estimation of area of frame that is covered and by multiple people
- Variation in pesticide residue levels in hives within treatment groups
- Amount of stored food in each hive immediately prior to exposure
- Differences in consumption rates of the supplied sucrose solution
- Exposure to external sources of contaminants via collection of pollen and nectar outside of the hives
- Variability in hive maintenance by beekeepers

Given the many potential sources of variability in the measurement endpoints related to a study at the colony level with honey bees in a field setting, each of these sources of variability can individually or collectively affect the interpretation of the study results. It is recommended that the study address these and other potential sources of variability and attempt to minimize them in the feeding study design.

Appendix 5. Residue Field Study Design Considerations

Objectives

These studies can be designed to meet one or more of the following objectives:

1) To quantify pesticide parent (and degradates of concern) residues in pollen and nectar of crops to which the assessed pesticide has been applied;
2) To determine the extent of year-to-year pesticide "carryover" in pollen and nectar;
3) To estimate dissipation rates of pesticides in pollen and nectar following application; and
4) To quantify pesticide (and degradates of concern) residues in leaves/flowers of the treated crop over time and related these to concentrations in pollen and in nectar.

Due to the differing objectives of such residue studies, protocols should be submitted and reviewed by EFED prior to the conduct of the residue study.

Expected utility of data

While it may be difficult to refine estimates of contact exposure, dietary exposure estimates can be refined. Studies of pesticide concentrations in nectar and/or pollen may be used to further characterize pesticide (and degradates of concern) exposures to bees and in doing so, provide a means of refining screening-level dietary-based RQs.

Residue data may be collected in multiple plant matrices (*e.g.*, foliage, intact flowers or associated structures, pollen and/or nectar). For systemic pesticides, data describing pesticide concentrations over time (*i.e.*, uptake and decline curves) in leaves of treated crops are useful because they may allow EPA to expand its understanding of the relationship between pesticide concentrations in leaves and in pollen and in nectar. For example, if a reliable relationship between pesticide concentrations in pollen and nectar and leaves can be established, data available from magnitude of residue studies (*e.g.*, residues in foliage and edible fruits in studies already submitted to EPA) may be useful in characterizing exposures to bees and may serve as protective estimates of exposure in pollen/nectar. Another example of a potential use of the uptake and decline in leaves may allow EPA to evaluate impacts of changes to application timing (*e.g.*, pre-application intervals) on residue levels in pollen and nectar. These studies may also be useful in determining the potential for year to year accumulation in pesticide concentrations in pollen and nectar or accumulation in bee-relevant matrices of rotational crops.

Site selection and replication

To the extent possible, for selected crop groupings, sites should be selected such that soils and regions are representative of where the crop is grown and where the pesticide is used. A minimum of three separate study sites are desired; soil type could be important if the pesticide is systemic and used as a soil application or seed treatment. Site-specific factors that may lead to variability in pollen, nectar and leaf concentrations of a pesticide should be considered when selecting sites. Potential sources of variability among sites containing the same crop include soil characteristics and weather. The selected sites should not have prior uses of the assessed pesticide for prior to the study. However, in situations where prior treatments may have occurred, the duration between a prior treatment and the study initiation should consider the half-life of the parent compound and the potential presence of degradates in the soil. Each site should include at

least 3 replicates, represented by separate plots within the same field (block). The number of plants sampled within a replicate should be clearly described in the protocol. Control (reference) treatments may be included, but are not necessary in these residue study designs if assessing background contamination is not a concern.

Pesticide application

The protocol should specify the treatment frequency and application method. Applications should be at maximum label rate, maximum number of applications, and minimal reapplication interval consistent with the product label. If the label allows for pre-bloom application, the study design should include pre-bloom application.

Sample collection

A single sample may be collected from multiple plants within the same plot, thus a sample would represent a composite. Minimally, the following plant matrices are recommended for collection: pollen, leaves, floral and extra-floral nectar. When nectar or pollen for a species is not feasible given the biology of the species, whole flowers or flower parts may be collected to represent pollen (*e.g.*, anthers) and nectar concentrations. Prior to conducting the study, justification for using these surrogate structures should be provided in the protocol submission.

Depending on the species of plant, pollen and/or nectar samples may be readily collected directly from the plant as discussed above; however, it may also be appropriate to use bees to sample these matrices where pollen is in turn collected using pollen traps while nectar is collected from the honey stomach of returning forager bees. The use of hive matrices (*e.g.*, bee bread, pollen stores, and stored nectar) are not recommended as they may not be representative of the current exposure period, and may incorporate dilution and/or degradation.

When information on dissipation rates are desired there are additional sampling considerations. The pesticide residue levels in plant tissues are expected to rise to a certain level and then decline over time. The frequency of leaf, pollen, or nectar residue sampling should minimally include 4 different samples, and across a time frame sufficient to define the pattern of residue uptake and decline over time (*e.g.*, enough to establish the DT_{50}). For dissipation in leaves, it is recommended that sampling of leaves begin on the day of application, followed by regular sampling after application. If the crop is a perennial or a biennial species and a clear declining trend in concentrations is not observed during the first year, sampling should continue into the second year (no additional application of the active ingredient is needed the second year).

With respect to leaf sampling, the selection of individual leaves should consider the hypothesis being tested. If a compound accumulates in leaves (*i.e.*, xylem only transported compounds), sampling the youngest leaves will provide a measure of current transport; whereas, sampling oldest leaves will account for a longer period of transport of the chemicals as well as any degradation occurring. Where applicable, based on the plant form (*e.g.,* trees, palms, or vines) a composite of leaf samples should be taken from across the lower, middle, and upper portion of the individual plant, and for large leaves isolating a region of leaf tissue (*e.g.*, terminal leaflet of a compound leaf, cross section at middle of a large leaf) to reduce bias in the sampling regime.

For soil-applied pesticides, it is recommended that residues also be quantified in soils prior to and after pesticide application. If information on year-to-year carryover is of concern, then multi-year sampling of soil and/or bee-relevant matrices should be conducted.

Detection limits

Analysis for the parent compound as well as major degradates is recommended. Appropriate environmental chemistry methods (ECM) with independent laboratory validation (ILV) should be provided to support the analysis method that demonstrate adequate limits of detection (LOD) and limits of quantification (LOQ). Ecological effects studies should be considered in the process of selecting analytical methods to obtain appropriate levels of detection and quantification for the studies. In addition, when reporting residue data, it should be clearly stated that the residues for pollen and nectar are based on fresh weight and those for leaves are in terms of dry weight.

Data Reporting

Residue data are not typically normally distributed and outliers are common. Box and whisker plots may be a means of visualizing data. The protocol should specify how residue levels below the level of quantification (LOQ) are captured in the distribution (*e.g.*, ½LOQ or utilize the LOQ). All raw data should be submitted electronically (*e.g.*, in spreadsheet format) to facilitate data analysis and interpretation.