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Office of Chemical Safety and Pollution Prevention (7101)
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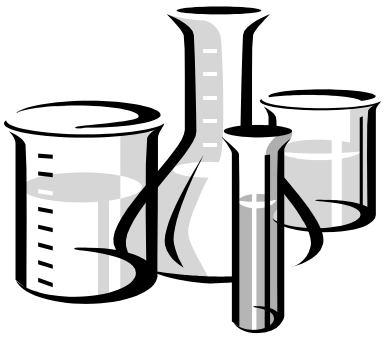
Product Performance Test Guidelines

OCSPP 810.3100: Treatments for Imported Fire Ants



NOTICE

This guideline is one of a series of test guidelines established by the Office of Chemical Safety and Pollution Prevention (OCSPP) [formerly the Office of Prevention, Pesticides and Toxic Substances (OPPTS) prior to April 22, 2010], United States Environmental Protection Agency (US EPA) for use in testing pesticides and chemical substances to develop data for submission to the agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a), referred to hereinafter as the harmonized test guidelines.

The OCSPP test guidelines serve as a compendium of accepted scientific methodologies for research intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting appropriate tests, and is also used by EPA, the public, and the companies that are required to submit data under FIFRA. These guidelines are not binding on either EPA or any outside parties, and the EPA may depart from them where circumstances warrant and without prior notice. The methods described in these guidelines are strongly recommended for generating the data that are the subject of the guidelines, but EPA recognizes that departures may sometimes be appropriate. You may propose alternatives to the methods described in these guidelines, with supporting rationale. The agency will assess them for appropriateness on a case-by-case basis.

For additional information about the harmonized test guidelines and to access the guidelines electronically, please go to *https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances*. You may also access the guidelines in *http://www.regulations.gov* grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, EPA-HQ-OPPT-2009-0576, and EPA-HQ-OPP-2011-1017. ***EPA-HQ-OPP-2017-0693*** is the docket number for this guideline.

The contents of this document do not have the force and effect of law and are not meant to bind the public in any way. This document is intended only to provide clarity to the public regarding existing requirements under the law or agency policies.

(a) Introduction.

a. Scope. This guideline provides recommendations for the design and execution of laboratory and field studies, the results of which, when combined, are used to evaluate the performance of pesticide products for the treatment of ants in the *Solenopsis saevissima* complex, which includes in part, for the purposes of the guideline, only *S. invicta*, *S. richteri*, and their hybrids in connection with registration of pesticide products under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*). This guidance applies to products in any formulation, such as a liquid, aerosol, granular or bait, if intended to be applied for control of imported fire ant colonies of the above listed species. This guideline does not apply to those products exempt from FIFRA Registration under 40 CFR 152.25 or to product performance testing described in other agency guidelines. For example, tests for additional formulations of products targeting fire ant individuals (rather than colonies), such as direct spray testing and indoor/outdoor residual applications targeting foragers, can be found in the Product Performance Test Guidelines OCSPP 810.3500: Premises Guideline for appropriate testing. This guideline revises OCSPP Test Guideline 810.3100: Soil Treatments for Imported Fire Ants (March 1998).

b. Purpose. This guideline provides laboratory and field study methods, the results of which, when combined, are used to evaluate product performance of pesticides against imported fire ant colonies of *S. invicta*, *S. richteri*, and their hybrids and includes statistical analysis and reporting recommendations.

(b) Organization of the OCSPP 810.3100 Guideline.

a. (a) Introduction;

(b) Organization of the of the OCSPP 810.3100 Guideline;

(c) Definitions;

(d) Development of protocols for efficacy studies;

(e) Review of protocols for efficacy studies;

(f) Execution of efficacy studies;

(g) Reporting of completed efficacy studies to the agency;

(h) Retention of records;

(i) Specific guidance for field studies for testing area-applied pesticide products;

(j) Specific guidance for field studies for testing mound-applied pesticide products;

(k) Specific guidance for laboratory studies for testing bait products;

(l) Specific guidance for laboratory studies for testing products other than baits;

(m) Specific guidance for laboratory studies for testing Insect Growth Regulator (IGR) products;

(n) References.

b. General Considerations. Any protocol and/or study developed using this guidance must meet

the provisions set forth in several statutes and regulations, including, but not limited to, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA, 7 U.S.C. 136, *et seq*.) under which EPA regulates pesticides. This guideline does not supersede or overrule the regulations governing research conducted with human subjects such as those contained in 40 CFR Part 26, or any other Agency regulations. To the extent there are any unintended conflicts between this guideline and any EPA regulation, the regulation at issue governs.

i. **Good Laboratory Practice Standards.** Good Laboratory Practice (GLP) Standards set forth in 40 CFR § 160 apply to laboratory studies evaluating pesticide product performance. Part 158 specifies that "applicants must adhere to the good laboratory practice (GLP) standards described in 40 CFR § 160 when conducting studies" [40 CFR § 158.70(b)]. However, studies that do not comply with GLP standards may nonetheless be considered if, in the Agency's judgment, the design and conduct of the study provide results that are scientifically reliable. 40 CFR §160.12(b) states that with any submitted research data "[a] statement describing in detail all differences between the practices used in the study and those required by this part" must be submitted to aid in making that determination.

ii. **State requirements.** Investigators and Sponsors should ensure research is conducted in compliance with any applicable state laws or regulations, which are independent of and additional to those cited in this guideline.

(c) Definitions. The following definitions are of special importance in understanding this guideline. They apply only in the context of this guideline and are not intended to be more generally applicable.

a. ***Active mound***; see ***Mound***.

b. ***Application rate*** refers to the amount of product applied per unit area or volume (e.g., oz/ft^2).

c. ***Brood*** refers to the immature members of a colony collectively, including eggs, larvae, and pupae (Torre-Bueno 1989).

d. ***Colony*** refers to a group of related individuals, which constructs nests and rears offspring in a cooperative manner (Torre-Bueno 1989); mature colonies contain brood.

e. ***Control*** refers to when a product kills a fire ant colony for a specified duration of time in field studies (e.g., ≥ 30 days) and is supported by evidentiary data from laboratory tests for product performance specifically against the workers, queen and in some cases, brood.

f. ***Field study*** refers to a scientific investigation that occurs in the listed fire ant's typical habitat or dwelling space.

g. ***Food lure*** refers to a food item, such as hot dog pieces, used to attract foraging ants.

h. ***Forager*** refers to, in social insects, a member of the worker caste that gathers food and brings it back to the colony (Torre-Bueno 1989).

i. ***Method of application*** refers to the way a pesticide can be delivered (applied) to a pest or site. Examples of application methods include dusts, watered-in granular products, and bait.

j. ***Monogyne*** refers to colonies containing a single fertile queen.

k. ***Mortality*** refers to ant death. A dead ant is an ant that does not move, even when poked or probed.

l. ***Mound*** refers to the above-ground and below-ground structure constructed for thermoregulation

of a colony; an active mound contains all or part of a colony. A mound is considered active when at least 20 workers exit the mound immediately following disturbance (Oi & Oi 2006).

m. ***Negative control*** refers to the group of specimens in an experiment that receive no treatment or a treatment with the diluent only or are given a placebo bait; no response is expected.

n. ***Photoperiod*** refers to the relative amount of time during the day in which it is light or dark (Triplehorn et al. 2005)

o. ***Polygyne*** refers to colonies containing multiple fertile queens.

p. ***Product performance testing*** refers to scientific studies that are designed to test the effectiveness of a pesticide product against the specific target pest.

q. ***Queen*** refers to a mated, egg-laying female member of the reproductive caste.

r. ***Social form*** refers to the number of egg laying queens in a colony; a monogyne colony has a single fertile queen and a polygyne colony has multiple fertile queens. Polygyne infestations may establish twice the density of monogyne infestations (Porter 1992).

(d) Development of protocols for efficacy studies. Testing pesticides for efficacy against listed fire ants begins with development of a study protocol that includes both laboratory and field components. General considerations in developing a study protocol for efficacy studies include scientific design of the study, data collection, data analysis, and reporting. The product should be tested in a manner that reflects intended use and that will provide data to support product performance. Both field and laboratory tests should be used to adequately address efficacy. Laboratory tests provide evidentiary data for product performance specifically against the workers, queen and in some cases, brood. Each of these topics is discussed in more detail in the sub-sections below. Additional study-specific considerations can be found in Sections (i) through (m).

a. Scientific design of study. The experimental methods should be likely to provide a definitive answer to the research question and should include a detailed description of the experimental design, addressing topics i. through viii., given directly below.

i. Objectives. For products that kill listed fire ant colonies, the objective of product performance testing is to determine efficacy at the lowest proposed label rate that kills/controls a colony. Typically, if a product kills a colony, this includes controls claims. In all cases, the scientific objective and intended label claims should be stated clearly and all treated colonies/mounds/nests should be compared to negative control colonies/mounds/nests that have received no treatment, placebo bait treatment or a diluent-only treatment.

ii. Test materials and treatments. End-use formulations should be tested using the lowest labeled application rates for use on listed fire ant colonies. Test materials should be stored at ambient temperature and humidity for at least one day before use.

1. Products that are area-applied (i.e., applied to an area vs. individual mounds) should be tested using assessments of foragers when field testing and workers, queen and brood for the laboratory portion of the testing. Refer to section (i) vi. 1-3 for specific guidance for area-applied field study endpoints. Refer to section (k) through (m) for specific guidance for laboratory studies.

2. Products that are mound-applied should be tested using assessments of mounds when field testing and workers, queen and brood for the laboratory portion of the testing. Refer to section (j) vi. 1-2 for specific guidance for mound-applied field

study endpoints. Refer to section (k) through (m) for specific guidance for laboratory studies.

3. Products that target ant development should be tested using brood, queen, and workers.

iii. **Application rate determination.** The test rate in product performance studies is the lowest application rate on the target pest from a proposed product label, typically expressed as amount of product per unit area for surface area treatments (e.g., 1 lb/500 ft^2). Rates may also be expressed as amount of product applied per mound (including a maximum amount per acre, if applicable). The amount of active ingredient applied per unit area should also be reported. Label rate should match the rate tested in the efficacy study. The application device used in testing should be the same as the proposed or registered product (e.g., to support use of a bait station, the bait product should be tested in a bait station).While rates should be reported in units according to the U.S. traditional systems of weights and measures, units may additionally be reported using metric system measurements. The method to measure test rate can vary among studies; however, the following are common methods of measurement:

1. Measure the amount of test product (e.g., granular bait or dust) applied per mound. To calculate the amount applied per unit area of the test plot containing the treated mounds, multiply the amount applied per mound by the total number of treated mounds on a plot, divided by the area of the plot.

2. Measure the amount of test product added to a bait station. To calculate the total amount applied per unit area of the test plot containing the bait stations, multiply the amount applied per bait station by the total number of bait stations on a plot, divided by the area of the plot.

3. Measure the amount of test product, such as a liquid or granular product, in its container before and after application, and divide the difference by the unit area treated.

iv. **Testing conditions.** During product performance testing in the laboratory, testing may be conducted at ambient temperature and relative humidity (RH) in the testing arena as long as conditions are optimal for foraging behavior, such as 82°-91° F (Banks et al. 1981). Conditions that are too cool or dry will inhibit normal colony activity. In addition, temperature and RH within the nest cell should also be maintained at conditions optimal for brood development, such as ≥ 65 % RH and 82°-91° F (Williams 1989). Also, laboratory colonies should be provided time to acclimate to the photoperiod, as photoperiod is known to affect foraging behavior (Lei et al. 2019). Environmental conditions for laboratory testing, including temperature, humidity, and photoperiod during testing, should be supported by cited literature. The temperature during the test should be kept as constant as possible because changes can affect the performance of the product treatments. A food and water source should be provided for all test organisms throughout the study.

Field studies should be conducted in weather conditions that are realistic for use. Extreme weather (temperature, wind and/or precipitation) should be avoided. Recommendations for field study test durations differ based on product application (i.e., mound vs area-applied) and whether an IGR is included in the product formulation. Field study duration for a mound applied product, where the colony within the mound presumably receives the full labeled rate, typically follows a ≥ 30-day duration. Area-applied products typically follow a ≥ 60-day duration because an application is used to treat all colonies within an area.

Colonies within the treated area may collect different amounts of the applied product due to variability in sizes of the colonies and their ability to monopolize foraging territory. Therefore, the treatment effect may take longer to be apparent and/or may dissipate quicker (i.e., shorter residual effect) in colonies that did not acquire a lethal dose. Likewise, for products containing an IGR, a ≥ 60-day duration is recommended to observe an effect unless a longer duration is needed for specific claims, such as colony mortality or a 6-month control claim.

v. **Choice of endpoints.** Study endpoints should be appropriate for the specific objectives of the proposed research and likely to provide a robust answer to the research question. Endpoints such as mortality should be evaluated at the lowest labeled application rate for listed fire ant colonies/mounds/nests. The endpoint selected should be claimed on the proposed label. The following are examples of commonly used endpoints; see specific study sections (i) through (m) for additional information or variations.

    1. **Mortality.** Observations of mortality should be reported based on recommendations provided in the specific study design. The number of dead worker ants and queens in each replicate should be recorded separately at each time point tested, as practically possible. A mortality count should include only dead ants.

    2. **Number of active mounds.** Numbers of active mounds on plots should be reported based on recommendations provided in the specific study design. The number of active mounds in each replicate should be recorded separately at each time point tested. To determine if mounds are active, the surface of each mound should be scratched with a pair of forceps or similar object. Alternatively, a narrow probe, similar to a stem of a field flag (approximately 0.12”), can be inserted into the mound from 6”– 8” deep.

    3. **Forager numbers.** Numbers of foragers collected at food lures should be reported based on recommendations provided in the specific study design. The number of foragers collected in each replicate should be recorded separately at each time point tested.

    4. **Presence/absence of brood.** Observations of presence/absence of brood should be reported for all colonies/mounds/nests based on recommendations provided in the specific study design.

vi. **Test organisms.** Testing should be conducted with adult listed fire ants unless the product is intended to target ant development and/or immature stages. All sources of fire ants should be listed in the study methods along with species.

vii. **Representative sampling**.

    1. **Replication.** The protocol should fully describe how sample size and replication were determined, and a power versus sample size analysis should be performed to support the proposed sample size. Factors that may affect sample size and replication are the number of treatments, experimental design, the environment (e.g., different habitat population densities), and heterogeneity in the sample pest population (e.g., social form). For example, areas known to contain the polygyne social form may have twice the density of mounds compared to the monogyne social form (Porter 1992).

    2. **Rearing, handling, and maintenance of listed fire ants.** When applicable, a

description of the laboratory colony rearing practices should be included. Collection details and maintenance procedures for field-collected fire ants should be described.

3. **Negative control.** In most studies, a negative control should be included. The number of control replicates should equal the number of replicates for each treatment. When appropriate, a negative control is typically treated with a placebo bait, diluent only or receives no treatment at all (i.e., untreated control).

viii. **Quality assurance (QA) plan.** Protocols should provide for periodic quality assurance inspections that are adequate to ensure the integrity of the study and consistency with the provisions of EPA's GLP regulations (40 CFR §160).

b. Data collection and reporting. Study protocols should provide for collection and reporting of data covering all aspects of the research including those discussed in section (g) of this guideline. GLP regulations specify that each study protocol should provide for collecting and reporting all elements provisioned by the GLP regulation at 40 CFR §160.120.

c. Data analysis. Protocols should include a full description and explanation for the statistical methods proposed to analyze product performance test results, taking into account the specific study objectives and variables. A statistician may be consulted regarding the sample size vs. power of the study design and the statistical methods for data analysis when developing test protocols. Analysis of data is recommended to determine if the mortality rate of the group treated with the product differs from the negative control mortality and if any within treatment effects were significant. Ninety-five percent confidence intervals should be reported around any estimated statistic. Protocols should explicitly describe the statistical model to be used and demonstrate that the assumptions underlying the model can be met for all proposed analyses. Restrictions on randomization of any testing components should be clearly documented and should be correctly accounted for in the statistical analyses. Generally, generalized linear models (GLMs) are recommended to fit models directly to non-normal (e.g., binomial – which describes many of the collected product performance data sets) data using an appropriate link function. GLMs do not involve transforming the response variable, thereby allowing data to remain on the original scale of measurement. Generalized linear mixed-models (GLMM) may also be appropriate for correlated data. Software for analysis using GLMs or GLMMs is available in many widely sold statistical packages. If survival analyses (e.g., Kaplan-Meier Estimator) are used, justification should be provided for use of the median value to characterize product performance and demonstrate that the underlying assumptions of these analyses have been met. One-way analysis of variance (ANOVA) or mixed-effects models can be used if their assumptions, such as normality, are justified.

(e) Review of protocols for efficacy studies. Protocols proposing novel testing methods (i.e., non-guideline testing methods) or testing of other species of ants for which the Agency requires data to support product performance should be submitted to EPA for review before the study begins.

(f) Execution of efficacy studies.

a. Execution of protocol. In cases where a protocol has been submitted to EPA for review, testing should be initiated when the EPA review is complete and as applicable, EPA comments should be incorporated into the revised protocol.

b. QA oversight. Product performance testing is subject to GLP regulations at 40 CFR §160. GLP regulations state that each testing facility should include an independent QA unit. The QA unit monitors and documents execution of each protocol in accordance with the GLP regulations (40 CFR §160.35). The QA unit should inspect each study at intervals adequate to ensure the

integrity of the study and maintain written and properly signed records of each periodic inspection. Please see (b) b. i. above for the discussion of the use of GLP laboratory methods when conducting pest product performance studies.

c. **Protocol amendments.** Amendments are planned changes to the protocol and should be made before the study is executed. All amendments to the protocol should be noted in the written report to the agency.

d. **Deviations from protocol.** Even when executing the best-designed and most comprehensive protocols, unanticipated deviations from the protocol may occur. All such deviations from the protocol and their impact on the research should be fully reported in the study report submitted to EPA (40 CFR §160.185).

(g) Reporting of completed efficacy studies to the agency.

a. **Study identification.** Title, identifying study number(s), sponsor, study director, investigators, name and location of the testing facility, and dates of the study should be reported. If tests are conducted outside the U.S., the relevance of the study for U.S. regulatory purposes should be justified in the study report.

b. **Study objective(s).** The purpose of the study should be stated, including whether the study is the laboratory or field component of the data set.

c. **Testing conditions.** Information on temperature, RH, ambient light and photoperiod (L:D), and air flow (where applicable) should be reported.

d. **Testing system.** Testing system information, including but not limited to the following, should be reported:

   i. Identification of field populations, if applicable; where colonies were collected/obtained; development stage; and methods for preparation of fire ants for testing (e.g., feeding).

   ii. Rearing, handling, and maintenance of fire ants.

   iii. Description of test substance (i.e., product, % active ingredient, and formulation to be tested).

   iv. Description of the negative control including the application, if applicable.

   v. Description of the experimental unit.

   vi. Treatment application rate and method of application (rate and method of application should be consistent with label instructions).

   vii. Number of product treatments if greater than one.

   viii. Number of replicates per treatment.

   ix. Number of individuals or mounds/plots per replicate for each treatment including controls.

   x. Length of ant exposure to each treatment.

   xi. Endpoints and time intervals of endpoint recordings.

e. **Data/results reporting.**

   i. **Raw data.** Include legible copies of all raw data.

   ii. **Results summary.** Report summary test results on all aspects of research. The amount of

product applied, and active ingredient delivered per replicate should be reported. See sections (i) through (m) for more details.

iii. **Data analysis**. Provide a copy of the statistical analysis plan and results from statistical analysis. Refer to Section (d) c. for recommendations on data analyses, unless otherwise indicated in a study-specific data analysis and reporting section.

f. **Study conclusions.** The report should include a discussion of the study results and conclusions based on treatment endpoints. Conclusions should state why and how the study results do or do not support the tested hypothesis and label claims.

g. **Protocol with amendments and study deviations from the protocol**. A copy of the study protocol should be included with amendments and deviations. Deviations should be justified and described together with their impact on the validity of the study. The study should align with the protocol.

(h) Retention of records. The record-keeping provisions of 40 CFR §160.190 and §160.195 apply to records of any study conducted under the GLP rule.

(i) Specific guidance for field studies for testing area-applied pesticide products.

a. **Study objective:** The field studies described in this section are designed to determine product performance of area-applied pesticide products, such as, but not limited to, broadcast baits, bait stations, and products other than baits, against listed fire ants in a colony/mound/nest. In addition, laboratory tests should be conducted in conjunction with field tests: for baits (see section (k)); for products other than baits (see section (l)); and for products containing an IGR (see section (m)).

b. **Materials and methods.**

i. **Site selection.** A minimum of two sites should be selected from within the range of the listed imported fire ants and should not have been exposed to pesticide treatment for at least one year prior to the study. A site refers to a contiguous stretch of land; therefore, a minimum of two separated (i.e., non-contiguous) stretches of land should be used for the study.

ii. **Experimental units.** The treatment should be applied to a plot within a site. Plots may be any size, so long as all plots within all sites are the same size, and should contain a minimum density of active fire ant mounds equivalent to 20 active mounds per acre (no less than 10 active mounds per plot) to ensure sufficient numbers of foraging fire ants are present for testing of area-applied treatments (Drees et al. 2013). All plots within a site should be separated from each other by at least 56 ft or more (Colby et al. 2008, Stringer et al. 2011). Plots within a site should be as similar as possible with respect to density of active fire ant mounds, relative numbers of active foragers, and environmental conditions such as hydrology and vegetation cover.

iii. **Number of replicates per treatment.** Depending on the results of power vs. sample size analysis, the study should include a minimum of four plots per treatment per site. The design should be balanced with an equal number of treated and negative control plots per site.

iv. **Application method.** The lowest application rate to be supported on the product label should be applied using methods consistent with the product label. If bait stations are used, the number of deployed bait stations and the amount of bait in each station should

correspond to the lowest number of stations per unit area proposed on the label. Bait station products should be tested as they would be deployed by a consumer.

v. **Ant exposure to product treatments.** Area-applied product studies should be conducted for a minimum of 60 days post-application at each site. A longer study duration may be needed based on desired label claims and the product's mode of action.

vi. **Data collection and endpoints.** All sampling should be conducted when soil temperatures are between 65 – 97 °F (Porter & Tschinkel 1987, Helms & Vinson 2005, Drees et al. 2007). A pre-treatment sampling of mounds and forager activity should be conducted 1-7 days prior to treatment application. The number of active mounds should be used to establish homogeneity among plots on a site (see Snedecor and Cochran (1989) for guidance on randomization of field trials based on number of mounds). Sampling should be conducted over a minimum of 60 days with a minimum of four sample collections, with the final sample occurring on the last day (i.e., day 60) or soon thereafter if all samples cannot be collected on the last day.

The primary endpoint for measuring efficacy of area-applied products in this design focuses on forager numbers. The number and timing of sampling intervals for foragers should be based on the pesticide's mode of action and desired label claims. If uncertain about suitable establishment of plots and/or sampling regime, consult the Agency for appropriate intervals. Ideally, data should be collected simultaneously from treated and control plots.

1. **Sampling of foragers.** The number of foragers present in the plot at each sampling interval should be estimated. This may be accomplished using an attractive food lure (e.g., hot dog pieces) and vials. Place a minimum of 9 vials containing the attractive food lure equidistant from the plot edge and each other within the plot for an established sampling period, approximately 30 minutes to 1 hour. Vials should be placed in contact with the soil and provided with some degree of shading from direct sunlight, such as use of semi-opaque vials (Vogt et al. 2003). Sampling of foragers may be conducted at 50% of the plot area. At the end of the sampling period, cap the vials. The number of fire ants in each vial should be counted and reported for the plot.

2. **Number of active mounds**. The number of active mounds per plot should be used to establish homogeneity among plots on a site. A mound is considered active when at least 20 workers exit the mound immediately following disturbance (Oi & Oi 2006). To determine if mounds on each plot are active, the surface of each mound should be scratched with a pair of forceps or similar object. Alternatively, a narrow probe, similar to a stem of a field flag (approximately 0.12"), can be inserted into the mound from 6"– 8" deep. It is optional to compare numbers of active mounds pre- and post-treatment for specific mound claims, such as "no new mounds." In this case, at each sampling interval, all mounds in each plot per site should be assessed for activity.

3. **Insect growth regulators (IGRs).** For products containing an IGR, an additional sampling assessment to confirm the presence of brood in all mounds in each plot should be conducted. To assess for the presence of brood during a single pre- and post-treatment sampling of each mound, using a small-headed shovel or hand

trowel, a small portion of the mound (about the size of a fist) should be turned over to observe brood. Record whether brood is present or absent in all mounds in each plot. The post-treatment sample should occur on the last day (i.e., day 60) or soon thereafter if all samples cannot be collected on the last day.

c. Data analysis and reporting. Refer to Section (g) of this guideline for guidance on data/result reporting. In addition, the following information should be included in the completed efficacy studies to the agency:

i. Test Conditions. For each assessment, report the date, time, temperature, and weather conditions.

ii. Treatment data. The amount of product applied, expressed as weight of product (and active ingredient) per unit area, should be reported for each replicate. If reapplication or rebaiting occurred, specify methods and thresholds for reapplication/rebaiting.

For bait stations, the amount of bait per bait station and the duration of baiting events needed to achieve product efficacy should be specified. The number of bait stations per unit area and location of bait stations, including distance from the mound/colony and other bait stations, should be reported. Bait removal by the test species as the difference in pre- and post-weights and accounting for water loss/gain should be documented.

iii. Forager numbers. For each assessment, the date, time, temperature, and weather conditions (e.g., precipitation) should be reported. Numbers of fire ants per vial should be recorded. The statistical analysis should consider the random effect of the plot and the correlation of data collected from the same plot at different time points. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of fire ants. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

iv. Number of active mounds. For each assessment, the date, time, temperature, and weather conditions (e.g., precipitation) should be reported. The number of active and inactive mounds per plot per treatment within a site should be reported for each assessment. If product application is intended to prevent new mounds from forming on treated plots, then the number of all active mounds per plot per treatment within a site should be reported for each assessment. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of active mounds, where the random effect of plot and the correlation of data collected from the same plot at different time points are accounted for in the model. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

v. Presence/absence of brood if using an IGR. For each assessment, the date, time, temperature, and weather conditions should be reported. The number of mounds with and without brood per plot per treatment within a site should be reported for each of the pre- and post-assessments. If product application is intended to prevent new mounds from forming on plots with treated mounds, then brood assessments should be conducted on all new mounds per plot per treatment within a site at each assessment. This establishes the first or pre-assessment for each of the new mounds identified. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of mounds with brood, where the random effect of plot and correlation of data collected from the same

plot at different time points are accounted for in the model. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

d. Study conclusions. Summarize study outcomes for area applied product testing against imported fire ants in a colony/mound/nest and discuss their implications for product labeling.

(j) Specific guidance for field studies for testing mound-applied pesticide products.

a. Study objective: The field studies described in this section are designed to determine the product performance of mound-applied pesticide products, such as but not limited to baits, dusts, and injectable aerosols, against listed fire ants in a colony/mound/nest. In addition, laboratory tests should be conducted in conjunction with field tests: for baits (see section (k)); for products other than baits (see section (l)); and for products containing an IGR (see section (m)).

b. Materials and methods.

i. Site selection. A minimum of two sites should be selected from within the range of listed imported fire ants and should not have been exposed to pesticide treatment for at least one year prior to the study. A site refers to a contiguous stretch of land; therefore, a minimum of two separated (i.e., non-contiguous) stretches of land should be used for the study.

ii. Experimental units. The treatment should be applied to individual mounds on designated plots within a site. For this field test, a plot is simply the area containing at least 10 active mounds. Treatment is applied to each active mound within a plot. Plots may be any size. All plots within a site should be separated from each other by at least 56 ft or more (Colby et al. 2008, Stringer et al. 2011). Plots within a site should be as similar as possible with respect to density of active fire ant mounds and environmental conditions such as hydrology and vegetation cover.

iii. Number of replicates per treatment. Depending on the results of power vs. sample size analysis, the study should include a minimum of four plots per treatment per site. The design should be balanced with an equal number of treated and negative control plots per site.

iv. Application method. The lowest application rate to be supported on the product label should be applied using methods consistent with the product label. Note: the cumulative mound application rate should not exceed the maximum label rate per acre.

v. Ant exposure to product treatments. Treatments should be applied no less than 24 hours after disturbance of the mounds, unless required for product application. Studies testing mound-applied products should be conducted for a minimum of 30 days at each site. A longer study duration may be needed based on desired label claims and the product's mode of action.

vi. Data collection and endpoints. All sampling should be conducted when soil temperatures are between 65 – 97 °F at a depth of 0.8" (Porter & Tschinkel 1987, Helms & Vinson 2005, Drees et al. 2007). A pre-treatment assessment of mounds should be conducted 1-7 days prior to treatment application. Sampling should be conducted over a minimum of 30 days with a minimum of four sample collections, with the final sample occurring on the last day (i.e., day 30) or soon thereafter if all samples cannot be collected on the last day. The number and timing of sampling intervals should be based on the pesticide's mode of action and desired label claims. If uncertain about a suitable sampling regime, consult the Agency

for appropriate intervals. Ideally, data should be collected simultaneously from treated and control plots.

1. **Number of active mounds.** The number of active mounds per plot is used to establish homogeneity among plots on a site and to compare numbers of active mounds pre- and post-treatment (see Snedecor and Cochran (1989) for guidance on randomization of field trials based on number of mounds). A mound is considered active when at least 20 workers exit the mound immediately following disturbance (Oi and Oi 2006). To determine if mounds on each plot are active, the surface of each mound should be scratched with a pair of forceps or similar object. Alternatively, a narrow probe, similar to a stem of a field flag (approximately 0.12" diameter), can be inserted into the mound from 6"– 8" deep. If product application is intended to prevent new mounds from forming on treated plots, then the number of all active mounds per plot per treatment within a site should be sampled for each assessment.

2. **Insect Growth Regulators (IGRs).** For products containing an IGR, an additional sampling assessment to confirm the presence of brood in all mounds in each plot should be conducted and the duration of the study should be extended to a minimum of 60 days, post-treatment. To assess for the presence of brood during a single pre- and post-treatment sampling of each mound, using a small-headed shovel or hand trowel, a small portion of the mound (about the size of a fist) should be turned over to observe brood. Record whether brood is present or absent in all mounds in each plot. The post-treatment sample should occur on the last day (i.e., day 60) or soon thereafter if all samples cannot be collected on the last day.

c. Data analysis and reporting. Refer to Section (g) of this guideline for guidance on data/result reporting. In addition, the following information should be included in the completed efficacy studies to the agency:

i. Test Conditions. For each assessment, report the date, time, temperature, and weather conditions.

ii. Treatment data. The amount of product applied, expressed as weight of product per unit area, should be reported for each replicate. If reapplication/rebaiting occurred, specify methods and thresholds for reapplication/rebaiting. For bait, specify the duration of baiting events needed to achieve product efficacy.

iii. Number of active mounds. For each assessment, the date, time, temperature, and weather conditions should be reported. The number of active treated mounds per plot per treatment within a site should be reported for each assessment. If product application is intended to prevent new mounds from forming on plots with treated mounds, then the number of all active mounds per plot per treatment within a site should be reported for each assessment. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of active mounds, where the random effect of plot and the correlation of data collected from the same plot at different time points are accounted for in the model. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

iv. Presence/absence of brood if using an IGR. For each assessment, report the date, time, temperature, and weather conditions. The number of treated mounds with/without brood per plot per treatment within a site should be reported for each of the pre- and post-

assessments. If product application is intended to prevent new mounds from forming on plots with treated mounds, then brood assessments should be conducted on all new mounds per plot per treatment within a site at each assessment. This establishes the first or pre-assessment for each of the new mounds identified. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of mounds with brood, where the random effect of plot and the correlation of data collected from the same plot at different time points are accounted for in the model. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence intervals should be adjusted for baseline values and reported for each time point.

d. Study conclusions. Summarize study outcomes for mound applied product testing against imported fire ants in a colony/mound/nest and discuss their implications for product labeling.

(k) Specific guidance for laboratory studies for testing bait products.

a. Study objective: The laboratory studies described in this section are designed to determine product performance of bait products against all life stages of listed fire ants. In addition, field tests should be conducted in conjunction with laboratory tests: for area-applied products (see section (i)) and for mound-applied products (see section (j)).

b. Materials and methods.

i. Experimental units. An experimental unit consists of a single test arena containing fire ant brood, workers, and a queen. The arena should contain a water source for the ants, harborage for a queen and brood, alternative food source, and the bait to be tested. Alternate food source and bait should be placed at opposite ends of the arena from each other and equidistant from the harborage. The test arena should consist of an open-top box where interior vertical surfaces are coated with a non-stick material (e.g., Fluon® or talcum powder) to prevent escape of fire ants. Size of the arena (e.g., 15” L x 12” W) may vary but should allow adequate space for ants to forage. All test arenas used in this study should have the same dimensions.

ii. Number of replicates per treatment. Depending on the results of power vs. sample size analysis, a minimum of five replicates per treatment, with a minimum of 100 workers, a queen, and a recorded and consistent amount of brood per replicate arena, should be tested. Design should be balanced with an equal number of treated and control arenas. All arenas should be held under the same environmental conditions. Laboratory testing may be conducted at ambient temperature and RH in the testing arena as long as conditions are optimal for foraging behavior, such as, 82° - 91° F (Banks et al. 1981). Conditions that are too cool or dry will inhibit normal colony activity. In addition, temperature and RH within the nest cell should also be maintained at conditions optimal for brood development, such as ≥ 65 % RH and 82° - 91° F (Williams 1989). Also, laboratory colonies should be provided time to acclimate to the photoperiod, as photoperiod is known to affect foraging behavior (Lei et al. 2019). Environmental conditions for laboratory testing, including temperature, humidity, and photoperiod during testing, should be supported by cited literature.

iii. Application method. Treatment should be product specific and applied in the manner and duration as directed by the product label. This study design should consist of a choice test to demonstrate acceptability of the bait product and the alternate food source should be palatable, established in the literature, and consistent across trials. The alternate food source should be available *ad libitum* to the fire ants throughout duration of the study. Control arenas should receive the alternate food source only.

iv. **Ant exposure to product treatments.** Fire ants should be acclimated to the arenas for a minimum of 72 hours, during which time they are provided with the alternative food source and water. Dead individuals should be removed prior to treatment. Uneaten food should be removed and replaced with a fresh food source immediately prior to initiation of the study. Tests should be conducted for a maximum of 14 days post-treatment.

v. **Data collection and endpoints.** The total amount of treated bait used, expressed as weight of product per unit area, should be documented for each replicate. The number of workers per replicate should be reported. Mortality counts should be conducted at intervals ≤ 48 hours through the duration of the study. Dead individuals should be removed and counted. Control groups should be assessed in the same manner as those receiving treatment. Following the final assessment, all nest arenas should be frozen to determine the number of surviving individuals and for calculation of the total number of workers.

c. **Data analysis and reporting.** Refer to Section (g) of this guideline for guidance on data/result reporting. The amount of time for test colonies to acclimate to environmental conditions in the laboratory prior to the start of the test should be reported. Control mortality should not exceed 25% for workers and there should be no queen mortality. In addition, the following information should be included in the completed efficacy studies to the agency:

i. **Reduction in worker numbers.** Mortality counts of workers should be reported and generalized linear mixed effect models for binomial distribution should be used to analyze the proportion of number of surviving workers at each time point (= number of surviving workers/total workers = (total workers – cumulative mortality counts of workers at the time point)/total workers). The model should take into account the random effect of replicate and the correlation of data collected from same replicate at different time points. Survival proportion per treatment group and survival proportion ratio between treatment and control groups should be calculated with 95% confidence limits per assessment.

ii. **Queen mortality.** Mortality counts of queen should be reported and mortality rate should be calculated with 95% confidence limits across replicates per assessment using generalized linear models or generalized linear mixed effect models for Poisson distribution.

d. **Study conclusions.** Summarize study outcomes for bait products against imported fire ant colonies (i.e., workers and queen) and discuss their implications for product labeling.

(l) Specific guidance for laboratory studies for testing products other than baits.

a. **Study objective:** The laboratory studies described in this section are designed to determine product performance of products, other than baits or premises treatments (e.g., soil and mulch amendments) against all life stages of listed fire ants. In addition, field tests should be conducted in conjunction with laboratory tests: for area-applied products (see section (i)) and for mound-applied products (see section (j)).

b. **Materials and methods.**

i. **Experimental units.** An experimental unit consists of an individual nest arena containing fire ant workers, a queen, plus brood affixed to two foraging arenas via plastic tubing (e.g., Tygon® tubing) or suitable bridge (Choe & Rust 2008; Knight & Rust 1990). Arenas should consist of an open-top container where interior vertical surfaces are coated with a non-stick material to prevent escape of fire ants. The nest arena should contain water and harborage for the colony. Food should be available in the nest arena during the acclimation

period, then removed immediately prior to test initiation. One foraging arena should contain substrate (e.g., sand, soil) treated with the product and the other foraging arena should contain an equal amount of untreated substrate or substrate treated with diluent-only. All foraging arenas used in the study should have the same dimensions; the length of the bridges or tubing connecting nest arena to the foraging arenas should be equal. The food sources in the foraging arenas should be palatable, established in the literature, consistent across all trials, and should be available *ad libitum* to the fire ants through the duration of the study. Foraging arenas should contain equal amounts of food placed at the far end of each arena, so ants travel through the substrate. Testing begins when tubes or bridges from the foraging arenas are connected to the nest arena.

Alternative nest- and foraging-arena designs may be considered if laboratory space is a concern. Arena design may consist of a larger nest arena (e.g., 24" L x 16" W) within which two separate foraging arenas (e.g., nursery pots 4" L x 4" W x 3.5" H) may be placed (Costa et al. 2005). The nest arena should consist of an open-top box where interior vertical surfaces are coated with a non-stick material to prevent escape of fire ants. The nest arena should contain water and harborage for the colony. Food should be available in the nest arenas during the acclimation period, then removed immediately prior to test initiation. The foraging arenas (e.g., nursery pots) should each be filled with treated or untreated substrate, each with equal amounts of food provided *ad libitum* and placed in the center of the substrate in order to ensure the fire ants cross over the substrate to access food. Foraging arenas should be placed at equal distances away from the harborage within the nest arena. Testing begins when the foraging arenas are placed into the nest arena.

ii. **Number of replicates per treatment.** Depending on the results of power vs. sample size analysis, a minimum of five replicates per treatment, with a minimum of 100 workers, a queen, and a recorded and consistent amount of brood per replicate arena, should be tested. Design should be balanced with an equal number of treated and control arenas. Laboratory testing may be conducted at ambient temperature and RH in the testing arena as long as conditions are optimal for foraging behavior, such as, 82° - 91° F (Banks et al. 1981). Conditions that are too cool or dry will inhibit normal colony activity. In addition, temperature and RH within the nest cell should also be maintained at conditions optimal for brood development, such as, ≥ 65 % RH and 82° - 91° F (Williams 1989). Also, laboratory colonies should be provided time to acclimate to the photoperiod, as photoperiod is known to affect foraging behavior (Lei et al. 2019). Environmental conditions for laboratory testing, including temperature, humidity, and photoperiod during testing, should be supported by cited literature.

iii. **Application method.** Treatment should be product specific and applied at the lowest labeled rate and in the manner as directed by the product label. Testing should be conducted on substrates (e.g., soil) aged to the maximum residual time point specified on the label. Aging of substrates should occur outdoors or indoors using simulated temperature, rain, and sunlight. This study should consist of a choice test of two foraging arenas: one containing treated substrate (e.g., sand, soil or impregnated material) and the other containing untreated substrate.

iv. **Ant exposure to product treatments.** Fire ants should be acclimated to the arenas for a minimum of 72 hours, during which time they are provided with the alternative food source and water. Uneaten food and dead individuals should be removed prior to treatment. Foraging arenas should contain a layer of substrate that completely covers the floor of the arena. The depth of the substrate layer should be consistent and equal in all foraging arenas.

Testing begins when tubes or bridges from the foraging arenas are connected to the nest arena. It is important to note that once foraging arenas are attached to the nest arena, ants may mound the substrate. Therefore, to avoid disrupting colony activity, measures should be in place during the initial setup that will prevent ants from escaping test arenas. Equal amounts of food should be available for the ants to forage at the far end of each the foraging arenas from the entrance. Control colonies should also have access to two foraging arenas, both containing untreated substrate only. Tests should be conducted for a maximum of 14 days post-treatment.

v. **Data collection and endpoints.** The total number of workers per replicate should be collected. Mortality should be evaluated at intervals ≤48 hours through the duration of the study. Dead individuals should be removed and counted. Controls should be assessed in the same manner as those receiving treatment. Following the final assessment, all nest arenas should be frozen to determine the number of surviving individuals and for calculation of the total number of workers per replicate.

c. **Data analysis and reporting.** Refer to Section (g) of this guideline for guidance on data/result reporting. The amount of time for test colonies to acclimate to environmental conditions in the laboratory prior to the start of the test should be reported. Control mortality should not exceed 25% for workers and there should be no queen mortality. In addition, the following information should be included in the completed efficacy studies to the Agency:

i. **Reduction in worker numbers.** Mortality counts of workers should be reported and generalized linear mixed effect models for binomial distribution should be used to analyze the proportion of number of surviving workers (= number of surviving workers/total workers = (total workers – cumulative mortality counts of workers at the time point)/total workers). The model should take into account the random effect of replicate and the correlation of data collected from the same replicate at different time points. Survival proportion per treatment group and survival proportion ratio between treatment and control groups should be calculated with 95% confidence limits per assessment.

ii. **Queen mortality.** Mortality counts of queen should be reported and mortality rate should be calculated with 95% confidence limits across replicates per assessment using generalized linear models or generalized linear mixed effects models for Poisson distribution.

d. **Study conclusions.** Summarize study outcomes for tested products (i.e., other than baits) against imported fire ant colonies (i.e., workers and queen) and discuss their implications for product labeling.

(m) Specific guidance for laboratory studies for testing IGR products.

a. **Study objectives:** The laboratory studies described in this section are designed to determine product performance of IGR products against immature life stages of listed fire ants. In addition, field tests should be conducted in conjunction with laboratory tests: for area-applied products (see section (i)) and for mound-applied products (see section (j)).

b. **Materials and methods.**

i. **Experimental units.** Guidance for setting up experimental units to test products containing an IGR may be found in (k) b. i. of this guideline for baits or (l) b. i. for products other than baits. However, the size of the nest arena should be larger (e.g., 24” L x 16” W) to allow adequate space for testing the larger colony size. In addition, for an IGR laboratory study,

harborage in the nest arenas should contain a covered plastic Petri dish measuring 6 in (150 mm) in diameter and filled 0.24 in deep with plaster. After the plaster has set, one or more access holes (0.04”- 0.08” diameter) should be soldered into the side of the dish, just above the plaster, to allow access into the nest cell. Transparent yellow acetate paper affixed over the lid will filter ambient light allowing for observation of brood inside the nest cell with minimal disturbance (Williams 1990).

ii. **Number of replicates per treatment.** Depending on the results of a power vs. sample size analysis, a minimum of five replicates per treatment with 200 - 1,000 workers, a queen, and a minimum of 0.34 oz of brood per replicate arena should be tested (Banks et al. 1983). Specific methods establishing larger colonies in the laboratory for extended studies are explained in Banks et al. 1981. Additional guidance for setting up replicates to test products containing an IGR may be found in (k) b. ii. of this guideline for baits or (l) b. ii. for products other than baits.

iii. **Application method.** Guidance for application of test products can be found in (k) b. iii. of this guideline for baits or (l) b. iii. for products other than baits.

iv. **Ant exposure to product treatments.** Fire ants should be acclimated to the nest arenas for a minimum of 72 hours and provided with food and water prior to being granted access to the foraging arenas. Tests with IGRs should be conducted for a minimum of 30 days post-treatment. Additional guidance for ant exposure to products may be found in (k) b. iv. of this guideline for baits or (l) b. iv. for products other than baits.

v. **Data collection and endpoints.** For IGRs, data should be collected at intervals ≥ 7days through the duration of the study. A minimum of three time points for collection of data should include the following: presence or absence of brood, changes in caste structure and/or deformities of the brood, or presence of dead brood in the colony midden (trash pile). If the test product contains additional active ingredients that are not IGRs, then data collection corresponding to (k) b. v. for baits or (l) b. v. for products other than baits should be conducted. Controls should be assessed in the same manner as those receiving treatment.

c. **Data analysis and reporting.** Refer to Section (g) of this guideline for guidance on data/result reporting. In addition, the following information should be included in the completed efficacy studies to the agency:

i. **Brood.** For treated and control replicates, presence or absence of brood, changes in caste structure and/or deformities of the brood, and any dead brood observed should be reported.

ii. **Additional reporting.** If the test product contains additional active ingredients that are not IGRs, then results and data analysis corresponding to (k) c. for baits or (l) c. for products other than baits should be conducted.

d. **Study conclusions.** Summarize study outcomes for IGR products against imported fire ant brood and discuss implications for product labeling.

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