Organisation for Economic Co-operation and Development

ENV/CBC/MONO(2022)38

Unclassified English - Or. English

24 February 2023

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

Cancels & replaces the same document of 17 November 2022

Guidance Document on Laboratory Product Performance Testing Methods for Bed Bug Biocide Products

Series on Testing and Assessment
No. 371

Series on Biocides
No. 19

JT03513069

OECD Environment, Health and Safety Publications

Series on Testing and Assessment no. 371

Series on Biocides no. 19

GUIDANCE DOCUMENT ON LABORATORY PRODUCT PERFORMANCE TESTING METHODS FOR BED BUG BIOCIDE PRODUCTS

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2022

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 38 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in twelve different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; Safety of Manufactured Nanomaterials;** and **Adverse Outcome Pathways**. More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

Also published in the Series on Biocides link
and in the Series on Testing and Assessment link

For this and many other Environment,
Health and Safety publications, consult the OECD's
World Wide Web site www.oecd.org/chemicalsafety/

or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2, rue André-Pascal
75775 Paris cedex 16
France
Fax: (33-1) 44 30 61 80
E-mail: ehscont@oecd.org

Foreword

This guidance document provides recommendations for the design and execution of laboratory studies to evaluate the performance of biocidal products intended to be effective against bed bugs.

This document originates from the activities of the OECD Working Party on Biocides (WPB) and was also included in the Test Guidelines Programme as project 5.16 in 2015.

The WPB performed its initial review from 2018-2021, after which the WPB approved the document in May 2022 to be forwarded to the Working Group of National Coordinators of the Test Guidelines Programme (WNT) for its subsequent review and approval. The review round by the WNT took place in June-July 2022, with approval of the guidance document on 21 July 2022. The Chemicals and Biotechnology Committee agreed to its declassification on 22 August 2022.

This document is published under the responsibility of the Chemicals and Biotechnology Committee.

This report has been produced with the financial assistance of the European Union. The views expressed herein can in no way be taken to reflect the official opinion of the European Union.

Table of contents

Guidance Document on Laboratory Product Performance Testing Methods for Bed Bug Biocide Products

1. Scope of Guidance Document

This guidance provides recommendations for the design and execution of laboratory studies to evaluate the performance of biocidal products in any formulation such as a liquid, aerosol, fog, or impregnated fabric intended to repel, attract, and/or kill bed bugs (*Cimex lectularius*). It does not apply to repellent products applied to human skin. The guidance is based upon the American Laboratory Product Performance Testing Methods for Bed Bug Pesticide Products (US EPA 2017, OCSPP 810.3900) and incorporates information from laboratory efficacy testing standards for biocidal products against bed bugs in the framework of the German Infectious Diseases Protection Act (18, 46). Investigators should ensure research is conducted in compliance with any applicable laws or regulations, which are independent of and additional to those cited in this guidance.

2. Definitions

The following definitions are of special importance in understanding this guidance document. They apply only in the context of this guidance and are not intended to be more generally applicable.

1. A bed bug ***attractant*** is a substance that causes bed bugs to make oriented movements towards its source.
2. A ***biocide*** is a product with an active substance or with a formulation containing one or more active substances intended to destroy, deter or attract, render harmless, or prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means.
3. ***Behavioural resistance*** refers to the avoidance by arthropods of a toxin through detection or recognition (22).
4. A product that ***controls bed bugs*** demonstrates that the insecticide application shows a sufficient efficacy (e.g. mortality and/or knockdown/morbidity followed by mortality and/or residual efficacy). See also the definition for "residual efficacy."
5. ***Crossing*** is the act of passage by a bed bug from an untreated surface to a treated surface or over a treated surface to another untreated surface.
6. The $\boldsymbol{EC_{50}}$ of a dose response curve represents the effective concentration of an insecticide needed to knockdown 50% of the test population, after a specified exposure time.
7. The $\boldsymbol{EC_{90}}$ of a dose response curve represents the effective concentration of an insecticide needed to knockdown 90% of the test population, after a specified exposure time.
8. A ***fumigant*** is an insecticide that is applied in the gaseous state or that forms a gas. Fumigants may be used to kill bed bugs indoors or in containers.
9. A bed bug ***harborage*** is a sheltered area or refuge for bed bugs.
10. A bed bug ***host*** is a mammal (especially humans) or bird that bed bugs bite to obtain blood for their survival and reproduction.
11. ***Host-seeking*** of bed bugs is the behaviour of bed bugs actively seeking a host.
12. An ***insecticide*** is a substance that kills insect pests.
13. An ***Insect Growth Regulator (IGR)*** is an insecticide that inhibits the maturation of a bed bug through its life cycle.
14. Bed bug ***knockdown*** refers to a bed bug that is rendered incapable of coordinated movement or unable to right itself following exposure to a biocidal product. In contrast to "moribund", bed bugs considered as knocked down have the potential to recover during evaluation observations.
15. $\boldsymbol{LD_{50}}$ is a measure of lethality of a given toxicant calculated as the dose of toxicant needed to kill 50% of a test population.

16. ***LD_{90}*** is a measure of lethality of a given toxicant calculated as the dose of toxicant needed to kill 90% of a test population.
17. ***Moribund*** refers to bed bugs that are on their backs with only slight muscle twitching of the extremities. Bed bugs exhibiting this behaviour may not be considered dead. In contrast to "knockdown", bed bugs considered as moribund will not recover.
18. ***Mortality*** refers to bed bug death. A dead bed bug is a bed bug that does not move, even when poked or probed.
19. An ***ovicidal product*** is an insecticide product that kills bed bug eggs.
20. ***Insecticide resistance*** is a heritable decrease in the susceptibility of a pest strain or population to a given insecticide. This change is revealed in the repeated failure of a product to achieve the expected level of mortality when used according to the label directions for that pest species (22).
21. A ***positive control*** is a treatment with a well-known effective biocide.
22. A ***repellent*** is a substance, causing bed bugs to avoid a treated substrate (e.g. disrupting the host-seeking or shelter-seeking behaviour of bed bugs).
23. ***Residual efficacy*** refers to a surface or space treated with a biocidal product continuing to provide the intended biocidal effect at an acceptable level for an extended length of time after application. The product's residues should be effective for at least 24 hours post application.
24. ***Resistance Ratio (RR)*** is a quantitative expression of the resistance of a bed bug strain to a specific active ingredient or product formulation. A resistance ratio (e.g. RR50) is calculated by dividing a quantitative measure of the lethality of a given insecticide (e.g. LD50 value) for a bed bug strain of unknown level of resistance by the corresponding measure of lethality for a strain known to be susceptible to the given insecticide. A resistance ratio equal to or greater than 100 is characteristic of a resistance strain. It has to be acknowledged, that practical bed bug control may fail even in bed bug populations with a resistance ratio < 100.
25. A ***resistant bed bug*** population/strain refers to bed bugs that survive an insecticide dose known to kill or control bed bugs.
26. A ***susceptible bed bug*** population/strain refers to bed bugs that exhibit mortality when exposed to an appropriate dose of an effective insecticide for bed bugs. Ideally, susceptible bed bugs will not have a history of exposure to the biocidal mode of action being tested.

3. Development of protocols for bed bug studies

The first major stage of bed bug product testing is the development of a study protocol. General considerations in developing a study protocol for bed bug studies include scientific design of the study, data collection, data analysis, and reporting. Each of these topics is discussed in more detail in the sub-sections below.

3.1. Scientific design of research.

To be scientifically justified, the development of a new test protocol should address an important research or testing issue that cannot be answered by existing data. In addition, the new test design should be likely to provide a definitive answer to the research question. A detailed description of the experimental design and bed bug rearing (h) should be included.

a. **Objectives.** In the case of claims that products kill and/or knockdown bed bugs, the objective of bed bug product performance testing is to determine that a product application made at the proposed label claim kills or knocks down the bed bugs. For claims that products attract or repel bed bugs, the objective of product performance testing is to determine the ability of a product to encourage or deter bed bugs to or from a pre-determined locale. For "control bed bugs" claims, the objective is to determine that the insecticide application shows a sufficient efficacy (e.g. mortality and/or knockdown/morbidity followed by mortality) and/or residual efficacy. In all cases the scientific objective should be stated clearly, and all treated bed bugs should be compared to control bed bugs that have received no treatment (5, 12, 18, 21, 29).

   Test materials and treatments. Product performance should be tested using the end-use formulation and application rates as registered or as proposed for use. Test materials should be stored at ambient temperature and humidity for at least one day before use.

b. **Product treatments for product performance tests:**

   i. **Products that target bed bug nymphs and adults.** Testing of products that target bed bug nymphs and adults should be conducted with both, bed bug nymphs and adults. Preferably, when nymphs are tested, the last two nymphal stages (J4 or J5) should be used.

   ii. **Products that target bed bug development.** Testing of products that target bed bug development should be conducted with mixed nymphal stages or eggs as appropriate. A blood meal should be available to provide the nourishment needed for bed bugs to moult from one nymphal stage to the next. The blood meal should be identified.

   iii. **Products that target bed bug eggs (ovicidal products).** Testing of products that target bed bug eggs should be conducted with the egg life stage only.

c. **Dose determination.** The test dose in bed bug product performance studies is the lowest application rate from a proposed product label. The dose will differ according to the surface type (sorptive/non-sorptive). The rate should be reported using metric system measurements, as mg or ml of test chemical/cm^2 for surface area treatments or volumetrically as ml of test chemical/m^3 for space

spray, total release aerosols, and fumigant treatments. The amount of active ingredient tested per unit area or volume should also be given.

d. **Testing conditions.** During bed bug product performance testing the temperature should be kept at 22 ± 4°C (unless otherwise justified, e. g. realistic use in cold conditions), with a relative humidity of 40-60%, and a photoperiod ranging from L:D 12:12 h to L:D 16:8 h. The temperature during the test should be kept as constant as possible because changes can affect the performance of the product treatments.

e. **Choice of endpoints and measures.** Endpoints chosen for the study should be appropriate for the specific objectives of proposed research and likely to provide a robust answer to the research question. Generally, the endpoints tested will be bed bug knockdown, morbidity, kill, repellency, or attraction tested at the claimed labelled application rate and will determine whether or not a product is efficacious. The selected endpoint should be included into the protocol. Bed bugs should be removed and placed into a clean container within a maximum of 4 hours or, in accordance with the claim, after onset of exposure to the biocide application.

   The exception in the product evaluation is the simulated-use test with a test duration of 24 h (Section 4.3) and the simulated-use test for repellents with a test duration of at least 8 h (Section 4.4.).

   Besides product evaluation, laboratory studies can be done for resistance ratio determination and characterization of bed bug strain susceptibility where bed bugs should be exposed to the treated filter paper in each treatment for 24 hours (Section 4.1.).

   For knockdown evaluation, observations should be made within a few minutes post exposure. For mortality evaluation, observations should be reported at 24, 48, 72, and 96 hours up to 8 days post-exposure (or according to the claim) unless all bed bugs die or negative control mortality exceeds 10% (18). Data will not be acceptable if control mortality exceeds 10%. Observations of mortality occurring after 96 hours should be justified based on the mode of action and application type. Survival of bed bugs beyond 8 days in negative control replicates does not justify making observations after 8 days. The number of dead, knocked down, moribund and live bed bugs in each replicate should be recorded. A count on mortality should be separate from a count on knockdown and morbidity. The percentage of bed bugs killed, moribund and knocked down, exclusively, for each treatment at each test interval should be recorded. Confidence limits around the reported mean or median values should be reported. Evaluation of speed of kill or additional knockdown evaluations should include more observations on the first day of the test (e.g. observations 2, 4 and 6 hours post exposure) to record the data needed to support the desired claims. If a test recommends that a bed bug should be contained during exposure to an insecticide, a bed bug should not be confined to a treated surface for more than 4 hours or in accordance with the claim. For the evaluation of products containing attractants or repellents, the endpoint is the minimum efficacy on the label claim, e.g. percentage of repelled or attracted bed bugs in a given time, respectively.

f. **Test organisms.** Testing should be conducted with a demonstrably susceptible laboratory strain of the common bed bug, *Cimex lectularius.* If *Cimex hemipterus* is claimed, this species should be tested. Tests should be conducted

with both, male and female bed bugs, in a balanced sex ratio. If strains collected in the field are tested, their susceptibility to the given insecticide has to be evidenced by bioassays and/or molecular methods before testing. Testing should occur no later than the second lab-reared generation. If more generations are needed to produce sufficient numbers of bed bugs for testing, it should be indicated in the study report. Bed bugs should be blood fed and should be tested preferably seven days after the last blood meal. Deviations of five to ten days after the last blood meal are possible.

g. **Representative sampling.**

   i. **Sample size.** The sample should be large enough (with a minimum of 50 bed bugs) to likely yield a definitive answer to the research question being addressed, and its size should be justified statistically, taking into account the specific characteristics of the proposed research and the necessary accuracy and precision of the results.

   ii. **Replication.** A minimum of five replicates of ten bed bugs each and balanced (equal number of treated and control replicates) experimental designs are recommended for most studies. Exceptions will be noted in the guidance that follows in this document.

   Other factors that may affect sample size and replication are the number of treatments, the experimental design, and the heterogeneity of the sample bed bug population (e.g. developmental stage, sex, insecticide susceptibility) and the environment (different habitat population densities). The protocol should fully describe how sample size and replication were determined.

h. **Bed bug rearing, handling, and maintenance.** When applicable, a description of the bed bug laboratory colony rearing practices should be included. Collection details and maintenance procedures for field-collected strains should be described. Bed bug feeding can be conducted on animal as well as on human hosts or alternatively artificially. Although bed bugs can be fed on small mammals like rabbits, for animal welfare reasons *in vitro* feeding methods should be preferred (1, 28). Different membrane feeding systems can be used to offer the blood meal (1, 28). In addition, a Hemotek® system can be used. In this case, a flat blood reservoir should be preferably used, in which erythrocytes do not sink to the bottom of the feeder where bed bugs cannot reach them. Different membranes like double stretched parafilm, collagen or silicone can be used. Before feeding, bed bugs should be placed in a jar closed with gauze or mesh screening which bed bugs can pierce but cannot escape.

i. **Negative control.** A negative control should be included in all testing. The number of negative control replicates should equal the number of replicates for each treatment. When appropriate, a negative control is typically treated with diluent only or receives no treatment at all.

j. **Positive controls.** A positive control is not necessary. An appropriate positive control, if available, is recommended for determining a resistance ratio.

k. **Statistical analysis plan.** Protocols should include a full description, explanation, and justification for the statistical methods proposed to analyse both resistance ratio determinations and product performance test results, taking into account the specific study objectives and variables. A statistician should be consulted when developing test protocols. Protocols should explicitly describe the model to be used and demonstrate whether or not assumptions

underlying the model can be met for all proposed analyses. Restrictions on randomisation of any testing components should be documented clearly and should be accounted for correctly in the statistical analyses. Generally, generalized linear models (GLMs) (25) are recommended to fit models directly to non-normal (e.g., binomial – which describe much of the collected product performance data sets) data using a probit link or logit link function. GLMs do not involve transforming the response variable, thereby allowing the data to remain on the original scale of measurement. Generalized linear mixed-models (GLMM) (13, 14, 19, 21) may also be appropriate. Software for analysis using GLMs or GLMMs is available in many widely sold statistical analysis packages. If survival analyses (27), such as the Kaplan- Meier Estimator, are used, provide justification for use of the median value to characterise product performance and demonstrate that the underlying assumptions of these analyses have been met. Other analysis including assumption of the normal distribution should be described and justified (2, 9, 37, 40, 44).

l. **Quality assurance /Quality control (QA/QC) 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 OECD Principles of Good Laboratory Practice (GLP) and Compliance Monitoring (ENV/MC/CHEM(98)17).

m. **Quality Assurance (QA) oversight.** Product performance testing is subject to the OECD Principles of Good Laboratory Practice and Compliance Monitoring (GLP) (ENV/MC/CHEM(98)17). The GLP states that each testing facility should include an independent Quality Assurance (QA) unit and that the QA unit monitors execution of each protocol and documents its conduct in accordance with the GLP (ENV/MC/CHEM(98)17). The QA unit will inspect each study at intervals adequate to ensure the integrity of the study and maintain written and properly signed records of each periodic inspection.

n. **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.

o. **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.

3.2. Data collection and reporting

Study protocols should provide for collection and reporting of data covering all aspects of the research including the following elements:

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.

b. **Approved or proposed label directions for use:** A copy of the proposed or approved product label should be included.

c. **Study objective(s):** The purpose of the study should be stated.

d. **Testing conditions:** Information on temperature, relative humidity, ambient light and photoperiod, and air flow (where applicable) should be reported.

e. **Testing system, including but not limited to:**

- Bed bug species tested, including identification of strains of susceptible and field bed bug populations, where bed bug strains were collected/obtained; development stage, age, and sex of bed bugs; and methods for preparation of bed bugs for test (feeding/starving), and when appropriate, the blood meal should be identified (20, 35, 42).
- Bed bug rearing, handling, and maintenance.
- Description of test chemical (i.e. product, % active ingredient, and formulation to be tested). Negative control should also be described.
- Description of the experimental unit.
- Treatment application rate and method of application (rate should be consistent with label instructions).
- Number of product treatments.
- Number of negative control replicates.
- Number of positive control replicates (where applicable).
- Number of replicates per treatment.
- Number of bed bugs per replicate for each treatment.
- Length of time for bed bug exposure period to each treatment.

f. **Data/Results reporting.** Report the following information:

i. **Protocol with amendments and study deviations from the protocol.** A copy of the study protocol should be included with amendments and deviations. Amendments and deviations should be justified, and described together with their impact on the validity of the study.

ii. **Data and endpoints.** Knockdown, morbidity and mortality values should be corrected for negative control knockdown, morbidity and/or mortality with Abbott's Formula or the equivalent. Endpoints should be reported as observed throughout the test, though total and percent knockdown, morbidity and mortality should be reported at the final evaluation.

iii. **Amount of product applied.** The product rate should be reported using metric system measurements, as mg or ml of test chemical/cm2 for surface area treatments or volumetrically as ml of test chemical/m3 for space spray, total release aerosols, and fumigant treatments for each replicate. The amount of active ingredient tested per unit area or volume should also be given.

iv. **Report the following other data:**

- Test results on all aspects of the research.
- Copies of all raw data.
- Certification of the test chemical's identity and origin.
- Description of each product treatment and the negative controls.

- Note: when (national) guidelines are used, those should be referred to in the report.

v. **Data analysis.** The report should include the statistical analysis plan. Refer to Section 3.1.l for recommendations on data analyses.

vi. **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.

vii. **Storage and Retention of Records and Materials.** The record-keeping provisions of OECD Principles of Good Laboratory Practice and Compliance Monitoring (GLP) (ENV/MC/CHEM(98)17) apply to records of any study conducted under the Good Laboratory Practices rule, in compliance with any applicable state laws or regulations.

4. Specific Guidance

4.1. Specific guidance for laboratory studies for resistance ratio determination and characterization of bed bug strain susceptibility.

Response ratio determination, commonly known as a "Resistance Ratio," is useful in characterising the magnitude of susceptibility or resistance to insecticides used in bed bug control. Different bioassays are suitable for resistance ratio determination of bed bug strain susceptibility (e.g. 39). This guidance describes the filter contact bioassays designed to determine the resistance ratio (37, 46).

4.1.1. Study objective.

To estimate the susceptibility and magnitude of resistance of bed bug strains to insecticides used in product testing.

4.1.2. Materials and methods

Filter contact bioassay in petri dishes

a) **Experimental units.** Place a piece of white filter paper on the bottom of a 6 or 10 cm glass petri dish and secure a screen over the top of the petri dish. An insecticide concentration should be applied to filter paper in each replicate at a volume that saturates the paper, generally at least 200 µl. Allow paper to dry before exposing the bed bugs. Prepare an equal number of negative control dishes with paper treated with the diluent only.

b) **Number of treatments.** Five concentrations of the active ingredient should be prepared with the appropriate diluent. Active ingredient concentrations should be prepared based on a logarithmic scale, i.e. 0.0001%, 0.001%, 0.01%, 0.1%, and 1.0%. Other concentrations may be used based on previous knowledge of bed bug susceptibility to the insecticide being tested but a justification should be provided (3, 7, 8, 17, 19, 31, 36, 43, 51, 53). If a product contains a synergist, use only the insecticide component with a solution concentration based on the active ingredient, not the synergist (33).

c) **Number of replicates.** Ten replicates per concentration with ten bed bugs each are recommended. It is recommended to test male bed bugs only, because results on females with sublethal concentrations are often more variable due to traumatic insemination.

Filter contact bioassay in 24-well cell culture plates.

Since bed bugs show strong aggregation behaviour, it is often practical to place test individuals in separate chambers.

a) **Experimental units.** Place pieces (ø 1.6 cm) of white filter paper on the bottom of 24-well cell culture plates. An insecticide concentration should be applied to filter papers in each replicate at a volume that saturates the paper, generally at least 50 µl. Allow paper to dry completely (preferably in a laboratory fume hood) before exposing the bed bugs.

b) **Number of treatments.** Five concentrations of the active ingredient should be prepared with the appropriate diluent. Active ingredient concentrations should be prepared based on a logarithmic scale, i.e. 0.0001%, 0.001%, 0.01%, 0.1%, and 1.0%. Other concentrations may be used based on previous knowledge of bed bug susceptibility to the insecticide being tested but a justification should be provided (3, 7, 8, 17, 19, 31, 36, 43, 51, 53). If a product contains a synergist, use only the insecticide component with a solution concentration based on the active ingredient, not the synergist (33).

c) **Number of replicates.** Five replicates of separate 24-well plates per concentration should be conducted. On each of the five separate plates it is recommended to place 18 individual bed bugs on treated filter papers and 6 bed bugs as negative controls (number of bed bugs tested per concentration: n=90 treated bed bugs and n=30 as negative controls, number of bed bugs tested in total: n=450 treated bed bugs and n=150 as negative controls. It is recommended to test male bed bugs only, because results on females with sublethal concentrations are often more variable due to traumatic insemination.

The following specifications find application with all bioassays.

a) **Bed bug exposure to treatments.** Bed bugs should be exposed to the treated filter paper in each treatment for 24 hours.

b) **Negative control.** The negative control should be treated with the diluent for insecticide solution preparation.

c) **Positive control.** An appropriate positive control, if available, should be used.

d) **Lethal dose (LD) or effective concentration (EC) values.** An analysis using GLMs is recommended to determine the LD or EC values for each bed bug strain tested. Use of a logit or probit analysis should be justified (45).

e) **Resistance ratios (RR).** Resistance ratios should be calculated and reported as follows:

LD or EC (lab or field strain)/LD or EC (susceptible strain) = RR

For example: LD_{50} (lab or field strain)/LD_{50} (susceptible strain) = RR_{50}

LD_{90} (lab or field strain)/LD_{90} (susceptible strain) = RR_{90}

4.1.3. Reporting results.

Refer to Section 3.2.f. of this guidance for guidance in reporting results and the data analysis. Report the resistance ratio values for each strain for each insecticide tested and the associated data analysis (30).

4.1.4. Efficacy evaluation.

Immediately after the 24 h exposure period efficacy evaluation should be performed. If further observations are necessary, transfer bed bugs to clean, untreated petri dishes or 24-well cell culture plates and evaluation observations should be reported as described under Section 3.1.e. A resistance ratio equal to or greater than 100 is characteristic of a resistant strain. It has to be acknowledged, that practical bed bug control may fail even in bed bug populations with a resistance ratio < 100.

4.1.5. Study conclusions.

Summarise and discuss the study outcome.

4.2. Specific guidance for laboratory studies for forced exposure (no-choice) residual surface treatments.

Different bioassays are suitable for determination of the residual product performance with a forced exposure (no-choice) test design (e.g. 18).

4.2.1. Study objective.

To determine the residual product performance of an application made to different sorptive and non-sorptive surfaces in a forced exposure (no-choice) test (18).

4.2.2. Materials and methods

a) **Experimental units.** The surfaces to be treated with the product should have sorptive and non- sorptive properties that are representative of where bed bugs are found. For example: unpainted/unfinished plywood; wallpaper, 100% cotton sheeting stretched over a cardboard panel, commercial linoleum tile; laminate or tiles. Surfaces should be pre-cut to 10 x 10 cm or larger panels (18). Cotton sheet replicates should be affixed to the top of the panel to provide a flat, rigid surface for treatment. An application should be made to each surface. An equal number of negative control replicates should be established with the same surfaces. Petri dishes can be used as container for the surfaces. A glass ring on the surfaces can prevent bed bugs from escaping.

b) **Application of product dilutions.** The claimed application rate for bed bug control should be applied on each panel. A metered bench top sprayer is preferred as the delivery device to ensure consistent application volume and even distribution of spray particles. Generally, panels are sprayed from a distance of 30 cm above the panel surface. Use of other heights should be justified. Panels should be stored and exposed to ambient conditions at the test site to age residues. Panels should be fully dried before exposing bed bugs. Measure the volume of spray applied and calculate the weight of the active ingredient(s) delivered. The quantity of product per square meter should be determined.

c) **Ready-to-use product application.** Application to panel surfaces should be made at the rate equivalent to the amount of product to be sprayed per unit area as directed by the label. Generally, panels are sprayed from a distance of 30 cm above the panel surface. Use of other heights should be justified. In product performance testing, the amount of product delivered by a ready-to-use spray product (aerosol or pump-spray) is described as the amount of product sprayed per second or as number of pumps per unit area and should be determined before treatments can be made. To determine the quantity sprayed per second, spray five panels of each surface type for three seconds each for each treatment. The product container should be weighed before and after each spray and the difference recorded. The mean value of the five replicates should be determined and that result divided by three to determine the average amount of product applied per second of spraying. The same procedure should be conducted to evaluate dust product formulations except that application should be made from a height of 15 cm or as directed by

the product label. Other modifications to the protocol needed to apply dusts should be described and justified.

d) **Replication.** A replicate should consist of a treated or untreated panel, each with 10 to 12 bed bugs per each life stage confined to the panel. A minimum of 5 replicates should be used for each product treatment and negative control. For each bed bug strain tested at each exposure time, prepare 15 treated (3 surfaces x 5 replicates per surface) and 15 untreated panels. Three hundred to 360 bed bugs are necessary for each exposure time tested per strain (including the negative control specimens).

e) **Bed bug exposure to product treatments.** Bed bugs should be exposed to treated panels for no more than 4 hours or in accordance with the claim. After the exposure period, transfer the bed bugs to a clean, untreated container for further observation and evaluation. After an initial test 24 hours post application, the insecticide residues should be tested regularly until the end of residual period claimed. Treated panels should be retested though bed bugs should not be reused.

f) **Positive control.** A positive control is not necessary.

4.2.3. Reporting results.

Refer to Section 3.2.f. of this guidance for guidance on reporting results and data analysis.

4.2.4. Efficacy evaluation.

Efficacy is usually considered sufficient if – at the end of the test, in each life stage tested, – 100% mortality rate, is achieved.

4.2.5. Study conclusions.

Summarize and discuss study outcomes for residual control of bed bugs.

4.3. Specific guidance for laboratory studies to determine the product performance of insecticide products under practical use situation.

Different bioassays are suitable for determination of the residual product performance with a simulated-use test design. This guidance describes the efficacy evaluation of residual surface treatments in a test system designed to mimic a practical use situation (46).

4.3.1. Study objective.

To determine the residual product performance of an application made to different sorptive and non-sorptive surfaces in a simulated-use test.

4.3.2. Materials and methods

a) **Experimental units.** The test arena should consist of three closed chambers joined with round or rectangular connectors. In the first chamber, a sealed harborage should be provided (harborage chamber). The design of the harborage should enable the bed bugs to leave easily, e.g. a pocket made of towel paper and tape opened with scissors. After a minimum of 1 h of acclimatisation, the harborage should be opened. In the chamber connected to the harborage chamber, the treated surface or textile is placed (insecticide chamber). The insecticide chamber is

connected to a third chamber containing a carbon dioxide (CO_2) source and a heat source (host chamber). The end of the last connector should protrude approximately 10 cm into the host chamber. A collecting vessel should be placed under the open end of the connecting tube. The collecting vessel should contain filter paper as a harborage. Bed bugs which have crossed the surface then fall into the vessel, which prevents bed bugs from escaping the test arena. Bed bugs should be removed 24 h post exposure or according to the claim for determination of efficacy. Bed bugs that have stayed in the towel paper pocket are excluded from evaluation.

Distance from the harborage to host chamber should be between 50 cm and 80 cm and the amount of CO_2 should be then adjusted to 0.75 l/min. If deviating distances are used, the amount of CO_2 has to be adjusted. A suction pump reaching in at the top of the harborage chamber prevents an intoxication of the bed bugs due to CO_2. It should be used a CO_2 flow rate that guarantees a minimum of 80% bed bugs leaving the harborage. Inside the host chamber, temperature should be adjusted to 37°C ± 2°C. Connectors between the three chambers should be about 15 cm to 20 cm. Connectors should be lined with material (e.g. masking tape or paper) which is not slippery for bed bugs. Inner walls of the collection vessel should be treated with a substance which prevents bed bugs from escaping. The surfaces to be treated with the product should have sorptive and non-sorptive properties: unpainted/unfinished plywood; wallpaper, 100% cotton sheeting stretched over a cardboard panel, commercial linoleum tile; laminate or tiles. Surfaces should be pre-cut to 10 x 10 cm or larger panels (18). Cotton sheet replicates should be affixed to the top of the panel to provide a flat, rigid surface for treatment. An application should be made to each surface. An equal number of negative control replicates should be established with the same surfaces.

b) **Application of product dilutions.** The claimed application rate for bed bug control should be applied on each panel. A metered bench top sprayer is preferred as the delivery device to ensure consistent application volume and even distribution of spray particles. Generally, panels are sprayed from a distance of 30 cm above the panel surface. Use of other heights should be justified. Panels should be stored and exposed to ambient conditions at the test site to age residues. Panels should be fully dried before exposing bed bugs. Measure the volume of spray applied and calculate the weight of the active ingredient(s) delivered.

c) **Ready-to-use product application.** Application to panel surfaces should be made at rates equivalent to the amount of product to be sprayed per unit area as directed by the label. Generally, panels are sprayed from a distance of 30 cm above the panel surface. Use of other heights should be justified. In product performance testing, the amount of product delivered by a ready-to-use spray product (aerosol or pump-spray) is described as the amount of product sprayed per second or per number of pumps per unit area and should be determined before treatments can be made. To determine the quantity sprayed per second, spray five panels of each surface type for three seconds each for each treatment. The product container should be weighed before and after each spray and the difference recorded. The mean value of the five replicates should be determined and that result divided by three to determine the average amount of product applied per second of spraying. The same procedure should be conducted to evaluate dust product formulations except that application should be made from a height of 15 cm or as directed by the product label. Other modifications to the protocol needed to apply dusts should be described and justified.

d) **Replication.** Tests should be conducted with 50 to 100 bed bugs (equal sex ratio and per each life stage) in minimum five treated and negative control replicates per surface type.

e) **Positive control.** A positive control is not necessary.

f) **Additional testing conditions.** Within the exposure period, darkness or red light is obligatory.

4.3.3. Reporting results.

Refer to Section 3.2.f. of this guidance for guidance on reporting results and data analysis.

4.3.4. Efficacy evaluation.

Efficacy is usually considered sufficient if – in each life stage tested – 100% mortality rate after 24 h exposure and until the end of residual period claimed, corrected according to Abbott is achieved.

4.3.5. Study conclusions.

Summarize and discuss study outcomes for residual control of bed bugs.

4.4. Specific guidance for laboratory studies to determine the product performance of a repellent or attractant product.

Different bioassays are suitable to determine the efficacy of a repellent or an attractant (11, 24, 38, 41, 49, 50). The following guidance describes approaches that may be used to assess whether a product is a repellent or attractant.

4.4.1. Study objective.

To determine if a biocide product repels or attracts bed bugs.

4.4.2. Materials and methods

Repellents.

Simulated-use tests for volatile repellents and impregnated fabric.

a) The test arena described by (46) should consist of three closed chambers joined with round or rectangular connectors. In the first chamber, a sealed harborage should be provided (harborage chamber). The design of the harborage should enable the bed bugs to leave easily e.g. a pocket made of towel paper and tape opened with scissors. After a minimum of 1 h of acclimatisation the harborage should be opened. In the chamber connected to the harborage chamber, the treated surface or textile is placed (treated chamber). The treated chamber is connected to a third chamber containing a CO_2 source and a heat source (host chamber). The end of the last connector should protrude approximately 10 cm into the host chamber. A collecting vessel should be placed under the open end of the connecting tube. The collecting vessel should contain filter paper as a harborage. Bed bugs which might have crossed the surface then fall into the vessel, which prevents bed bugs from escaping the test arena. Bed bugs should be removed 8 h post exposure or according to the claim for determination of efficacy. Bed bugs that have stayed

in the towel paper pocket are excluded from evaluation. Bed bugs that have stayed in the first chamber outside the towel paper pocket are considered as "repelled", while specimen that are found on the treated surface or have fallen into the vessel are considered as "not repelled". Non-insecticidal efficacy should be demonstrated with bed bugs that have been in contact with the freshly applied product. Mortality of the insects should be monitored at the end of the test until 8 days post exposure.

Distance from the harborage to host chamber should be between 50 cm and 80 cm and the amount of CO_2 should be then adjusted to 0.75 l/min. If deviating distances are used, the amount of CO_2 has to be adjusted. A suction pump reaching in at the top of the harborage chamber prevents an intoxication of the bed bugs due to CO_2. It should be used a CO_2 flow rate that guarantees a minimum of 80% bed bugs leaving the harbourage. Inside the host chamber, temperature should be adjusted to 37°C ± 2°C. Connectors between the three chambers should be about 15 cm to 20 cm. Size of the treated surface should be no larger than 20 cm. Connectors should be lined with material (e.g. masking tape or paper) which is not slippery for bed bugs. Inner walls of the collection vessel should be treated with a substance which prevents bed bugs from escaping.

b) The test arena described by (47) consists of a plastic tray (80 by 75 by 5 cm) and a small bed or imitations with four legs (e.g. a stool or small table) placed in the center. Onto the simulated bed, a CO_2 and additionally a heat source should be placed to mimic a human host. Under each leg a bed bug interceptor (i.e. a double-walled bed bug trap, where the insects are being trapped between both walls, which form a ring around the bed leg) should be placed as collection vessel. The repellent product should be applied on the outer wall of the interceptor according to the label claim. If the test surface should be larger, the interceptors should be placed onto the treated surfaces. A minimum of 100 ml/min of CO_2 should be released on the top of the bed. A harborage should be placed in the center of the plastic tray right under the bed. For acclimatisation, the harborage has to be closed. After a minimum of 1 h of acclimatisation, the harborage should be opened. The design of the harborage should enable the bed bugs to leave easily, e. g. a pocket made of towel paper and tape opened with scissors. Test arena should be lined with material (e.g. paper and masking tape) which enables normal bed bug movements. Inner walls of the tray should be treated with a substance which prevents bed bugs from escaping.

c) **Replication.** Tests should be conducted with 50 to 100 bed bugs (equal sex ratio) in minimum five treated and negative control replicates. The exposure period should be according to the label claim, but at least 8 h (to cover the natural bed bug activity time over night).

d) **Positive control.** A positive control is not necessary.

e) **Additional testing conditions.** Testing under darkness or red light is obligatory.

Laboratory choice tests for volatile repellents.

a) A petri dish assay with a treated and untreated filter paper similar to the test setup of (47) may be considered.

b) Also a version of the still-air olfactometer (10) as modified by (48) may be considered. The still-air model should be adapted to provide a source such as CO_2 and heat to mimic a human host in the presence and absence of repellent as alternative choices in the same arena. The negative control should consist of the

same arrangement in a separate arena provided with only CO_2 and heat and with no repellent.

c) A Y-tube assay (15) may also be considered.

d) **Replication.** Ten bed bugs per replicate for a total of five replicates per trial and negative control or 50 replications of one bed bug each per trial and negative control should be used. Justify the choice of individuals or groups of ten.

e) **Positive control.** A positive control is not necessary.

f) **Additional testing conditions.** Testing under red light is recommended.

Laboratory choice tests for impregnated fabric.

Untreated and impregnated fabric should be tested together. Bed bugs should be placed onto the untreated fabric, and their movement both towards or away from the treated fabric should be observed and measured. A negative control arena with untreated fabric only should also be included in the study (e.g. 11, 16, 23).

Attractants.

Choice tests are recommended for testing the product performance of attractant products (48, 49). Depending on its use pattern (whether the product is intended to attract bed bugs towards a harborage or "pull" them away from human hosts), the product performance of an attractant product may be determined by comparing its effect on bed bugs to that of bed bug aggregation cues or host cues such as CO_2 and heat. Therefore, in this experiment either host cues such as CO_2 and heat or bed bug aggregation cues deposited in a harborage should be presented as an alternative to the attractant formulation in the same arena. Observations of bed bug location should be recorded at the end of the exposure period. The exposure period should be according to the label claim, but at least 8 hours.

a) **Replication.** Ten bed bugs per replicate for a total of five replicates per trial and negative control or 50 replications of one bed bug each per trial and negative control should be used. Justify the choice of individuals or groups of ten.

b) **Positive control.** A positive control is not necessary.

c) **Additional testing condition.** Testing in the dark under red light is recommended (49).

4.4.3. Reporting results.

Refer to Section 3.2.f. of this guidance for guidance on reporting results and data analysis. Analysing data from replicates with one bed bug or replicates with ten bed bugs is likely to differ and should be justified.

a) **Percent repellency.** For repellent testing, report the number of bed bugs that avoided the host mimic and the ones that did not. Calculate percent repellency corrected for negative control results. The exposure period should be according to the label claim, but at least 8 h.

b) **Percent attractancy.** For attractant testing, report the number of bed bugs that were attracted to the attractant and the number that were not. Calculate the percentage of bed bugs found at each location at 15 minutes post exposure and until the end of the claimed period in treated choice and negative control arenas.

c) **Mortality, morbidity and knockdown.** Non-insecticidal efficacy should be demonstrated with bed bugs that have been in contact with the freshly applied

product. Mortality of the insects should be monitored at the end of the test until 8 days post exposure. All raw data should be reported.

4.4.4. Efficacy evaluation.

a) **Repellents.** For the claim "prevents bed bug bites" or "prevents the spreading of bed bugs" 100 % repellency are required in each life stage tested.

   The exposure period should be according to the label claim, but at least 8 h (to cover the natural bed bug activity time over night).

b) **Attractants.** The efficacy of an attractant is usually considered sufficient when at least 80% of the test individuals were attracted compared to the negative control within the test period or according to the claim, from the beginning and until the end of the claimed efficacy period.

   The percentage of bed bugs found at each location at 15 minutes post exposure and until the end of the claimed period has to be reported in treated choice and negative control arenas.

4.4.5. Study conclusions.

Describe the product performance of the product treatment. Include a discussion on negative control results, and the adequacy of the host or aggregation cues used in the study.

4.5. Specific guidance for laboratory studies for testing indoor insecticide total release aerosols, space sprays, and insecticide vapour strip products.

This guidance applies to testing total release aerosols and space sprays including misters, hand-held aerosol products and vaporizing strips on surfaces used for indoor applications to control bed bugs. Different bioassays are suitable to determine the efficacy these treatments.

4.5.1. Study objective.

To determine the performance of insecticide products intended for total release aerosols, space sprays, and vapour strip treatments against bed bugs.

4.5.2. Materials and methods

a) **Experimental unit.** Testing should be conducted in a Peet-Grady chamber with a volume of 6.12 cubic meters (1.83 x 1.83 x 1.83 m) or greater (52). The chamber should have a window for observation. The wall, ceiling, and floor of the room/chamber may be lined with plastic or other suitable materials to facilitate cleaning. Test doses for aerosols and mister products should be delivered by an automatic dispenser calibrated for the proper droplet size and application rate. At the end of each replicate, the air in the chamber should be exhausted and any surface residues washed off. Surfaces should be clean and dry before the next test. Alternative product application methods may be considered, but should be described and justified. Vaporizing strips should be hung from the ceiling in the center of the room/chamber or applied according to label directions.

b) **Replication.** Tests should be conducted with in minimum five treated and five negative control replicates per each exposure period. One half of the replicates will

be in the chamber treated with the insecticide product while the other half will be the negative control.

c) **Cage Placement.** For each exposure period, twelve cages from each strain should be used. Appropriate shelters, such as stacked egg cartons should be added to each cage to serve as a harborage. Allot six cages to the product treatment and place in the chamber, while the other six should be kept outside the chamber as a negative control for each exposure period. Ten bed bugs (equal sex ratio) should be transferred to each cage. In the chamber use balanced replicates representing three heights (two at floor level, two at mid wall and two at ceiling level).

d) **Bed bug exposure to the treatments.** The chamber should be sealed and the product application made. After the application is made, the test cages should be left in place for two hours or according to the claim and removed after the insecticide has been evacuated from the chamber. Knockdown, morbidity and mortality should be recorded at two hours after application or according to the claim. Bed bugs from each cage should be transferred to a clean, untreated container after the assessments are made, but no later than 4 hours post treatment or in accordance with the claim. Vaporizing strips should be assessed in a similar manner following 24 hours of exposure.

e) **Negative control.** Negative control replicates should be placed in the lab under the same abiotic conditions as the treatments. Negative control replicates should be untreated because treating with diluent is impractical.

f) **Positive control.** A positive control is not necessary.

4.5.3. Reporting results.

Refer to Section 3.2.f of this guidance for guidance on reporting results and data analysis. In addition, the following information should be reported.

a) **Data and endpoints.** Mortality data from the treated group should be corrected for negative control mortality with Abbott's Formula or the equivalent.

b) **Mortality, morbidity and knockdown.** Report the number and percentage of bed bugs killed, moribund and knockdown exclusively for each treatment replicate at each observation interval. Report mean mortality data for each strain in each treatment at each height level and all heights combined as corrected arithmetic mean values. Confidence limits around the mean values should be reported.

c) **Data analysis.** The analysis should consider the effect of the treatment cage height and bed bug strain effects on product performance.

4.5.4. Efficacy evaluation.

Efficacy is usually considered sufficient if – at the end of the test, in each life stage tested – 100% mortality rate, corrected according to Abbott is achieved.

4.5.5. Study conclusions.

Discuss the mortality (total and percent) for each replicate and treatment.

4.6. Specific guidance for laboratory studies for direct application testing of insecticide products.

Different bioassays are suitable for determination of a direct effect against bed bugs.

4.6.1. Study objective.

To determine the product performance of direct application of insecticide product formulations against bed bugs.

4.6.2. Materials and methods

a) **Experimental unit.** Testing should be conducted with caged bed bugs. Typically, a test cage unit is a 0.4 l squat plastic cup with a screened bottom that has the inside lined with a lubricant to prevent bed bug escape. Other cage designs are acceptable provided the spray does not pool in the cage after spraying. A metered bench top sprayer is preferred as the delivery device to ensure consistent application volume and even distribution of spray particles. Applications should be made at the claimed label rate and should be made from 30 cm above the test cage.

b) **Replication.** Tests should be conducted with in minimum five treated and five negative control replicates each with 10 bed bugs.

c) **Bed bug exposure to the treatments.** Bed bugs should be transferred to clean containers (e.g. petri dishes) in less than 4 hours or in accordance with the claim after product application. Containers should be stored under ambient test site conditions.

d) **Positive control.** A positive control is not necessary.

4.6.3. Reporting results.

See Section 3.2.f. of this guidance for guidance on results reporting and data analysis.

4.6.4. Efficacy evaluation.

Efficacy is usually considered sufficient if – at the end of the test, in each life stage tested, – 100% mortality rate, is achieved.

4.6.5. Study conclusions.

Discuss knockdown, morbidity and mortality (total and percent) of the product treatment.

4.7. Specific guidance for laboratory studies for testing ovicidal products.

Different bioassays are suitable for determination of an ovicidal effect against bed bug eggs.

4.7.1. Study objectives.

To determine the product performance of insecticide products intended for use as ovicides.

4.7.2. Materials and methods

Treatments.

I. **Direct application.** Testing should be conducted with bed bug eggs laid on filter paper the night before the test. Therefore, gravid female bed bugs of the same age should be separated from the colony, allowing them to lay eggs in a separate chamber on a filter paper surface. For females, the same feeding conditions/blood source like in the rearing should be used. Tissue culture plates or small petri dishes are recommended as test containers. Pieces of egg-laden filter paper should be cut into pieces that fit into the depressions on the plate or in a petri dish. Twenty eggs should be allotted to each depression or well. A metered bench top sprayer is preferred as the spray device to ensure consistent application volume and even distribution of spray particles. Applications should be made at the claimed label rate and should be made from 30 cm above the test surface (or as directed on the label). For a dust formulation, application should be made at the claimed label rate from a height of 15 cm or less (or as directed on the label). Record the weight of formulation applied and the weight of active ingredient delivered. An equal number of negative control eggs should be included in the study design on the same type of plates.

II. **Contact with residual surface application.** Gravid female bed bugs of the same age should be separated from the colony, allowing them to lay eggs in a separate chamber on a filter paper surface. For females the same feeding conditions/blood source like in the rearing should be used and the eggs used should be collected every day to ensure they are the same age. A petri dish is recommended as the test container. The surfaces to be treated with the product should have sorptive and non- sorptive properties and should be representative of where bed bugs are found. For example: unpainted/unfinished plywood; wallpaper, 100% cotton sheeting stretched over a cardboard panel, commercial linoleum tile; laminate or tiles. Surfaces should be treated with the product formulation at the claimed label rate and the residues should be aged for the desired time. Another piece of the respective surface should be left untreated to serve as the negative control. One piece should be placed into each dish. Collect eggs from the filter paper in the gravid female chamber carefully without damaging the eggshell and transfer 20 eggs to each treated and untreated surface in the petri dishes.

a) **Replication.** A minimum of ten treated and ten untreated replicates each with 20 eggs should be tested.

b) **Exposure time.** Plates or dishes should be stored in the laboratory. Eggs should be exposed continuously for 14-30 days or according to the claim. Observations for mortality and hatching should be made every 24 hours for up to 30 days. Eggs should be examined microscopically to determine if egg hatch has taken place, and the number of unhatched and hatched eggs should be recorded from the treated and negative control groups.

c) **Positive control.** A positive control is not necessary.

4.7.3. Reporting results.

See Section 3.2.f. of this guidance for guidance on results reporting and data analysis.

4.7.4. Data and endpoints.

Mortality data from the treated group should be corrected for negative control mortality with Abbott's Formula or the equivalent.

Egg mortality.

Report observations every 24 hours for a minimum of 14 but no more than 30 days post-exposure. The percentage of unhatched and hatched eggs for each treatment at each observation interval should be reported.

4.7.5. Efficacy evaluation.

Efficacy is usually considered sufficient if – at the end of the test – 100% mortality rate, is achieved.

4.7.6. Study conclusions.

Discuss ovicidal performance of the product formulation treatment.

4.8. Specific guidance for laboratory studies for fumigant products against all bed bug life stages.

Different bioassays are suitable for determination of an effect by fumigant products against all bed bug life stages (e.g. 6).

4.8.1. Study objective.

To determine the product performance of a fumigant in the laboratory against all bed bug life stages.

4.8.2. Materials and Methods

a) **Experimental unit (34).** Five, 3.8 liter sealed glass containers with tubing capable of delivering and evacuating fumigant in a closed system should be used as the fumigation chambers. Bed bugs should be placed in a separate ventilated glass vial that should be wrapped in mattress padding and placed in the chamber before fumigation.

b) **Product treatment.** Treatment should be at the claimed rate directed by the product label. This rate should be monitored by chemical detection to ensure the target dose was achieved.

c) **Bed bug life stage.** This test may be used to evaluate product performance against all bed bug life stages.

d) **Replication.** Five treated and five negative control replicates of 10 bed bugs each from the same life stage of the same strain should be tested, with the exception of eggs where 20 eggs should be included per each of the ten treated and ten untreated replicates.

e) **Exposure time.** Bed bugs should be exposed for 24 hours or according to the label claim. After fumigation, transfer the bed bugs to clean containers. For experiments with eggs and nymphs, observe for egg hatch and survival of nymphs for 14-30 days.

f) **Environmental conditions.** Replication and treatment should be repeated at 15°C (59° F) and 25°C (77° F).

g) **Negative control.** Bed bugs in the control group should be placed in a separate ventilated glass vial and should be wrapped in mattress padding but placed in an untreated fumigation chamber for the same period of time as the treatment group.

h) **Positive control.** A positive control is not necessary.

4.8.3. Reporting results.

Refer to Section 3.2.f. of this guidance for guidance on reporting results and data analysis. In addition, report the following information.

a) **Amount of product applied.** The amount of product, expressed as weight of product per unit volume, should be reported for each replicate.

b) **Mortality.** Mortality should be reported as number of dead bed bugs and percent mortality as stated in Section 3.1.e.

c) **Egg hatch and survival of emerging nymphs.** Report egg hatch (total and percent) and length of time the emerging nymphs survive.

4.8.4. Efficacy evaluation.

Efficacy is usually considered sufficient if – at the end of the test, in each life stage tested, – 100 % mortality rate, is achieved.

4.8.5. Study conclusions.

Report the application rates at which product performance was achieved.

4.9. Specific guidance for laboratory studies of insect growth regulators (IGRs).

Different bioassays are suitable for determination of an effect by IGRs against bed bugs. This guidance is based on approaches to evaluate hormonal IGRs and chitin synthesis inhibitors against bed bugs (4, 32).

4.9.1. Study objective.

To determine the product performance of an insect growth regulator in the laboratory against bed bugs.

4.9.2. Materials and Methods

a) **Life stages.** Life stages should be evaluated separately and individuals in the population should be the same age. These products may be tested against eggs, nymphs, and adults (potential decreased fecundity and oviposition). If specific stages are claimed, these will have to be tested.

b) **Replication.** A minimum of five treated replicates and five untreated replicates each with a minimum of 10 specimens should be tested for every life stage for every IGR product tested.

c) **Test chemical.** Testing should be performed with the whole product formulated with IGRs in combination with other active ingredients. In addition, testing should

be performed also with the IGR alone to verify its effect. Testing should not be performed with tank mixes (mixtures) containing IGRs.

d) **Direct spray testing.** Testing should be conducted as described in Section 4.6. of this guidance. Evaluation observations should be reported up to 30 days post-exposure or according to the claim.

e) **Confinement to treated surfaces.** Testing should be conducted as described in Section 4.2. of this guidance but evaluated up to 30 days.

f) **Blood meal.** A blood meal is needed for a bed bug to molt from one instar to the next and from the final nymph instar to the adult stage. A source of blood should be available regularly throughout the testing period.

4.9.3. Reporting results.

See Section 3.2.f. of this guidance for guidance on results reporting and data analysis. Additional data reporting is described below.

a) Report any abnormalities in bed bug development including deformities.

b) Report egg hatch success and development of hatching nymphs.

c) Report survivorship of all life stages.

d) If female bed bugs were tested, track egg production, hatching success of eggs, developmental success and survivorship of nymphs.

4.9.4. Efficacy evaluation.

Efficacy is usually considered sufficient if, in each life stage tested, 100% of the bed bugs do not develop to the next instar. In addition, if female bed bugs were tested neither eggs should be produced nor nymphs should hatch.

4.9.5. Study conclusions.

Discuss results and describe whether or not IGR effects impacted bed bug survivorship and development.

5. References

The following publications were consulted for supporting guidance recommendations.

1. Aak, A. and B. A. Rukke. Bed bugs, their blood sources and life history parameters: a comparison of artificial and natural feeding. *Medical and Veterinary Entomology*. 2014. 28: 50-9. doi: 10.1111/mve.12015.
2. Aitchison, J. The Statistical Analysis of Compositional Data. *Chapman and Hall. London*. 1986. 416.
3. Bailer, A. J. and W. W. Piegorsch. Chapter 7 Dose-response modeling and analysis. *Dose Response Modeling in Statistics for Environmental Biology and Toxicology.* Chapman & Hall. London. 1997.
4. Bajomi, D., J. Szilagyi, R. Naylor and L. Takacs. S-Methoprene formulations laboratory tests for efficacy against bed bugs. *Proceedings of the Seventh Annual ICUP*. 2011. 249-256.
5. Barile, J., R. Nauen, G. Nentwig, R. Pospischil and B. Reid. Laboratory and field evaluation of deltamethrin and bendiocarb to control *Cimex lectularius* (Heteroptera: Cimicidae). In: *W. H. Robinson and D. Bajomi (eds). Proceedings of the Sixth International Conference on Urban Pests. OOK-Press Kft., H-8200 Veszprém, Pápai út 37/a*. Hungary. 2008. 105-111.
6. Bonds, J. A. S, M. Greer, J. Coughlin and V. Patel. Caged mosquito bioassay: Studies on cage exposure pathways, effects of mesh on pesticide filtration, and mosquito containment. *American Mosquito Control Association*. 2010. 26(1):50-56.
7. Busvine, J. R. Insecticide resistance in bed-bugs. *Bulletin of the World Health Organization*. 1958. 19: 1041-1052.
8. Busvine, J. R. and J. Lien. Methods for measuring insecticide susceptibility levels in bed-bugs, cone-nosed bugs fleas and lice. *Bulletin of the World Health Organization*. 1961. 24: 502-517.
9. Cochran, W. G. and G. M. Cox. Experimental Designs (Second edition). John Wiley & Sons, Inc., NY. 1992.
10. Dawson, G., Griffiths, D., Janes, N., Mudd, A., Pickett, J., Wadhams and L. & C. Woodcock, C. Identification of an aphid sex pheromone. *Nature*. 1987. 325: 614– 616.
11. Domingue, M. J., M. Kramer and M. F. Feldlaufer. Sexual dimorphism of arrestment and gregariousness in the bed bug *(Cimex lectularius)* in response to cuticular extracts from nymphal exuviae. *Physiological Entomology*. 2010. 35(3):203-213.
12. European Chemicals Agency. Transitional Guidance on the Biocidal Products Regulation. Transitional Guidance on Efficacy Assessment for Product Type 18, Insecticide, Acaricides & other Biocidal Products against Arthropods and Product Type 19, Repellents & Attractants. 2016. 1-111.
13. Faraway, J. J. Extending the Linear Model with R: Generalized Linear, Mixed

Effects and Nonparametric Regression Models. *Chapman and Hall/CRC. Boca Raton*, FL. 2006.

14. Gbur, E. E., W. W. Stroup, K. S. McCarter, S. Durham, L. J. Young, M. Christman, M. West and M. Kramer. Analysis of Generalized Linear Mixed models in the Agricultural and Natural Resources Sciences. *American Society of Agronomy*. Madison, WI. 2012.
15. Geier, M. and J. Boeckh. A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odors. *Entomologia Experimentalis Et. Applicata*. 1999. 92: 9-19.
16. González-Morales, M. A., M. Terán and A. Romero. Behavioral responses of the common Bed Bug to essential oil constituents. *Insects*. 2021.12, 184.
17. Gratz, N. A survey of bed-bug insecticide resistance in Israel. Bulletin of the *World Health Organization*. 1959. 20: 835-840.
18. Habedank B., Snelinski B., Reinsch M. and J. Klasen. Advanced method for evaluation of the residual efficacy of products to control *Cimex lectularius* (Hemiptera: Cimicidae). Robinson W.H. and de Carvalho Campos A.E. (eds.): *Proceedings of the Seventh International Conference on Urban Pests, Instituto Biologico, Sao Paulo, SP Brasil*. 2011. 271-276.
19. Halliday, W.R. and K. P. Burnham. Choosing the optimal diagnostic dose for monitoring insecticide resistance. *Journal of Economic Entomology*. 1990. 83(4): 1151-1159
20. Harlan, H. J., M. K. Faulde, and G. J. Baumann. Bedbugs. In: X. Bonnefoy, H. Kampen, and K. Sweeney (eds.) Public Health Significance of Urban Pests. *World Health Organization*. Copenhagen, Denmark. 2008. 131-151.
21. Health and Safety Executive (HSE), Biocides and Pesticides Unit (BPU). Guidelines on the efficacy data for approval of non-agricultural pesticide products: insecticides and acaricides. Merseyside, UK. 2004.
22. Insecticide Resistance Action Committee (IRAC). 2016. Resistance Definition. Available on-line at: http://www.irac-online.org/about/resistance/
23. Krüger A., E. Schmolz, and A. Vander Pan. Methods for Testing Repellents Against Bed Bugs (Hemiptera: Cimicidae). *Journal of Economic Entomology*. 2021. 114(1), 265–273
24. Levinson, H. Z. and A. R. Bar Ilan. Assembling and alerting scents produced by the bedbug *Cimex lectularius. Experientia*. 1970. 27:102-103.
25. McCullagh, P. and J. A. Nelder. Generalized Linear Models (Second edition). *Chapman and Hall*, NY. 1989.
26. Miller, R. G., Jr. Survival Analysis. *John Wiley & Sons, Inc*. NY. 1981.
27. Miller, D. M. and M. L. Fisher. Bed bug (Hemiptera: Cimicidae) response to fumigation using sulfuryl fluoride. IW. H. Robinson and D. Bajomi (eds). *Proceedings of the Sixth International Conference on Urban Pests. OOK-Press Kft., H-8200 Veszprém, Pápai út 37/a, Hungary*. 2008. 123-127.
28. Montes, C., Cuadrillero, C. and D. Vilella. Maintenance of a Laboratory Colony of Cimex *lectularius* (Hemiptera: Cimicidae) Using an Artificial Feeding Technique. *Journal of Medical Entomology*. 2002. 39: 675–679. doi: 10.1603/0022-2585-39.4.675.

29. Moore, D. J. and D. M. Miller. Laboratory evaluations of insecticide product efficacy for control of *Cimex lectularius*. *Journal of Economic Entomology*. 2006. 99(6): 2080-2086.

30. Morgan, B. J. T. (editor). Statistics in Toxicology. Oxford University Press. NY. 1996.

31. Myamba, J., C. A. Maxwell, A. Sidi and C. F. Curtis. Pyrethroid resistance in tropical bedbugs, *Cimex hemipterus*, associated with use of treated bed nets. *Medical and Veterinary Entomology.* 2002. 16:448-451.

32. Naylor, R. D. Bajomi and C. Boase. Efficacy of (s)-Methoprene against *Cimex lectularius* (Hemiptera: Cimicidae) in: *W. H. Robinson and D. Bajomi (eds). Proceedings of the Sixth International Conference on Urban Pests.* OOK-Press Kft., H-8200 Veszprém, Pápai út 37/a, Hungary. 2008.

33. Pennetier, C., V. Corbel**,** P. Boko, A. Odjo, R. N'Guessan**,** B. Lapied and J-M Hougard. Synergy between repellents and non-pyrethroid insecticides strongly extends the efficacy of treated nets against *Anopheles gambiae*. *Malaria Journal 6:38.* 2007. doi:10.1186/1475-2875-6-38.

34. Phillips, T. W., M. J. Atkins, E. Thomas, J. Demarck and C. Wang. Fumigation of bed bugs (Hemiptera: Cimicidae): effective application rates for sulfuryl fluoride. *Journal of Economic Entomology*. 2014. 107(4): 1582-1589.

35. Reinhardt, K. and M. T. Siva-Jothy. The biology of bed bugs (Cimicidae). *Annual Review of Entomology.* 2007. 52, 351-374.

36. Robertson, J. L., R. M. Russell, H. K. Preisler and N. E. Savin (eds). Bioassays with Arthropods. Second edition. CRC Press. Boca Raton, FL. 2007.

37. Romero, A., M. F. Potter, D.A. Potter and K. F. Haynes. Insecticide resistance in the bed bug: A factor in the pest's sudden resurgence? *Journal of Medical Entomology.* 2007. 44(2): 175-178.

38. Romero, A., M. F. Potter and K. F. Haynes. Behavioral response of the bed bug to insecticide residues. *Journal of Medical Entomology.* 2009. 46(1): 51-57.

39. Romero, A. and T. D. Anderson. High levels of resistance in the common Bed Bug, *Cimex lectularius* (Hemiptera:Cimicidae), to Neonicotinoid Insecticides. *Journal of Medical Entomology*. 2016. 53(3): 727-731.

40. Schabenberger, O. and F. J. Pierce. Contemporary Statistical Models for the Plant and Soil Sciences. *CRC Press. NY, NY.* 2002.

41. Singh, N., Wang C. and R. Cooper. Effect of trap design, chemical lure, carbon dioxide release rate, and source of carbon dioxide on efficacy of bed bug monitors. *Journal of Economic Entomology*. 2013. 105 (4): 1802-1811.

42. Siva-Jothy, M. T. Trauma, disease and collateral damage: conflict in cimicids. *Philosophical Transactions of the Royal Society of London Biological Sciences*. 2006. 361:269-275.

43. Steelman, C. D., A. L. Szalanski, R. Trout, J. A. McKern, C. Solorzano and J. W. Austin. Susceptibility of the bed bug *Cimex lectularius* L. (Heteroptera: Cimicidae) collected in poultry production facilities to selected insecticides. *Journal of Agricultural and Urban Entomology*. 2008. 25(1): 41–51.

44. Stuart, A., and Ord and K. Kendall's Advanced Theory of Statistics Sixth Edition Volume 1. Distribution Theory. *Wiley. London*. 1998. 351.

45. Throne, J. E., D. K. Weaver and J. E. Baker. Probit analysis: Assessing goodness-of- fit based on back-transformation and residuals. *Journal of Economic Entomology*. 1995. 88(5), 1513-1516.

46. Vander Pan, A., E. Schmolz, J. Krücken and C. Kuhn. A Novel Simulated-Use Test for Determining the Efficacy of Insecticides Against Bed Bugs (Hemiptera: Cimicidae). *Journal of Economic Entomology*. 2019. 112(5), 2345–2353

47. Wang, C., Lü, L., Zhang, A. and C. Liu. Repellency of Selected Chemicals Against the Bed Bug (Hemiptera: Cimicidae). *Journal of Economic Entomology*. 2013. 2522-2529.

48. Weeks, E. N. I., J. G. Logan, M. A. Birkett, J. A. Pickett and M. M. Cameron. Tracking bed bugs (*Cimex lectularius*): a study of the effect of physiological and extrinsic factors on the response to bed bug-derived volatiles. *The Journal of Experimental Biology*. 2013. 216: 460-469. doi:10.1242/jeb.074930

49. Weeks, E. N. I., J. G. Logan, S. A. Gezan, C. M. Woodcock, M. A. Birkett, Pickett J. A. and M. M. Cameron. A bioassay for studying behavioural responses of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae) to bed bug-derived volatiles. *Bulletin of Entomological Research*. 2011. 101: 1–8. doi: 10.1017/S0007485309990599.

50. White, G. B. Chapter 2: Terminology of Insect Repellents. pp. In: *M. Debboun, S. P. Frances, and D. Strickman (eds). Insect Repellents: Principles, Methods, and Uses.* CRC Press, Boca Raton, FL. 2007. 31-47.

51. World Health Organization Pesticide Evaluation Scheme (WHOPES). Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticide on treated surfaces. *World Health Organization*. Geneva, Switzerland. 1998.

52. WHOPES. 2009. Guidelines for efficacy testing of household insecticide products – Mosquito coils, vaporizer mats, liquid vaporizers, ambient emanators and aerosols. *World Health Organization*. Geneva, Switzerland.

53. WHO Vector Control and Biology Division. 1982. VI. Bed bugs. WHO/VBC/82.857, 9 pp. *World Health Organization*, Geneva, Switzerland.