Guidance Document on Laboratory and Simulated-use Testing the Efficacy of Baits and Repellents against Tropical Ants for Indoor Use

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1. Scope of Guidance Document

(1) This guidance provides recommendations for the design and execution of simulated-use studies to evaluate the efficacy of baits (claim "nest kill") against tropical ants. Furthermore, this guidance deals with laboratory and simulated-use testing of tropical ant repellents (e.g. claim "reduction or prevention of invading ants in houses or sensitive areas"). The recommendations in this document refer to products for control in indoor environments.

(2) The guidance incorporates information from published and unpublished laboratory efficacy testing studies of the German Environment Agency for bait and repellent products against tropical ants. 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. An ***attractant*** is a substance that lures ants to a specific point, e.g. a trap, or increases the palatability of a bait.
2. New colonies can be founded by ***budding***, which means that no nuptial flights are needed and parts of one nest move to another site to establish a new nest.
3. A ***colony*** is the sum of all nests or budding portions of a (super)colony in an infested object (e.g. building, apartment complex), where a pest control operation can be expected to eradicate all ants. A colony is thus defined as a distinct local population of ants.
4. A product that ***controls ants*** ("nest kill") demonstrates that the insecticide application shows sufficient efficacy leading to the death of all members of the ant colony (including brood, queens).
5. ***Crossing*** is the act of passage by an ant in repellent tests of the treated surface or untreated control surface of the bridge.
6. ***Mortality*** refers to the death of individual ants and/or the death of the whole colony. A ***dead ant*** is an ant that does not move, even when poked or probed. Dead brood is dried out in contrast to live brood which is recognizable by the shiny surface of the brood.
7. A ***nest*** is a site where a part of an unicolonial ant species with queens and brood are located. Workers bring food into this nest and take care of the queens and brood.
8. A ***repellent*** is a substance that causes ants to avoid a treated substrate (e.g. disrupting the foraging path).
9. ***Polygynous*** ant species have more than one and up to thousands of queens per colony.
10. ***Residual efficacy*** refers to a surface or space treated with a repellent product continuing to provide the intended repellent effect at an effective level for an extended length of time after application.
11. ***Tramp ants*** are ant species which have a high ability to be moved from place to place, mainly transferred by trade or other human activities. Tramp ants are typically characterized by polygyny, reproduction by budding, close association with humans, multiple colony sites and no nest mate recognition (unicolonialism).
12. ***Unicolonial*** ants have lost their ability for nest mate recognition. Ants move between different nests in contrast to ***multicolonial*** ant species in which worker ants would attack members from another colony.

3. Introduction

The following tropical and invasive ant species are considered candidates for testing as they are species of potential importance by causing inconvenience in buildings in Europe, North and South America, Australia and Asia:

Pharaoh Ant, *Monomorium pharaonis*

Ghost Ant, *Tapinoma melanocephalum*

Argentine Ant, *Linepithema humile*

Of these, the Pharaoh Ant and the Ghost Ant are the most important pest species regarding human health since they can transmit various pathogens, such as *Salmonella* sp., *Escherichia coli*, *Staphylococcus* sp. and some fungi. Information on the distribution of these pests and their public health importance is abundantly available. Especially in hospitals these ant species can cause serious health problems, because ants were found in sterile areas where they can transfer the pathogens (Beatson 1972, Zarzuela et al. 2004, Kim et al. 2005, Moreira et al. 2005, Zarzuela et al. 2007, Wetterer 2009, 2010, Lima et al. 2013). The Argentine Ant is known as an invasive species and important pest in many areas around the world (Wetterer et al. 2009), and also has the potential as mechanical vector for pathogens (Fowler 1993, Lima et al. 2013).

The three mentioned ant species are tramp ants, which are mainly transferred by trade and/or human movement. After leaving their place of origin and spreading in other parts of the world changes in behavior and biology occurred. They evolved polygyny, and nuptial flights are not executed as queens mate inside the nest and new colonies are founded by budding. Furthermore, these ant species evolved unicolonialism, i.e. the ants have lost their ability of nest mate recognition and therefore move between nests without being attacked, which is normally the case in multicolonial species (which are not in scope of this guidance document). In households, they nest nearly everywhere such as cracks and crevices or potted plants and move their nests quickly when being disturbed.

4. Development of protocols for ant studies

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

(1) **Scientific design of research.** The protocol should include a detailed description of the experimental design.

a) **Objectives.** In the case of claims that products control ants (“nest kill”), the objective of bait product performance testing is to determine that a product application made at the proposed label rate kills all ants of a test colony (including brood and queens). For products that claim “repel ants”, the objective of product performance testing is to determine the ability of a product to keep ants away from a specific area. In all cases the scientific objective

should be stated clearly and all treated ants should be compared to control ant colonies that have received no treatment.

b) **Test materials and treatments.** Product performance should be tested using the end-use formulation and application rates as registered or as proposed for use.

c) **Dose determination.** The test dose in ant product performance studies is the lowest application rate from a proposed product label. The rate should be reported using metric system measurements, as mg of test substance/cm^2 of test surface for surface area treatments. The amount of active ingredient tested per unit area or time should also be given. If the product is applied as a bait, the entire bait, including the bait box if applicable, should be tested, not only the product which is contained in the bait. The application rate of bait products should be in accordance with the proposed product label (e.g. mg of bait or number of bait boxes/ant colony or ant trail) (for details see chapter 6.13).

d) **Residual efficacy** and aged product (in case of long shelf-life i.e. more than 2 years) should be tested.

e) **Testing conditions.** Room temperature should be maintained constant around 24 ± 3°C and relative humidity at 55 ± 10%. Conditions should be recorded during the test procedure and a photoperiod ranging from 12 hours of light and 12 hours of darkness to 16 hours of light and 8 hours of darkness. The temperature during the test should be kept as constant as possible because changes can affect the ant behavior.

f) **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 ant nest kill or efficacy of the repellent product according to the label claim. The endpoint selected should be included into the protocol.

g) **Test organisms.** Due to the specificity of bait products, only effects against colonies of tropical ant species e.g. *Monomorium pharaonis*, *Tapinoma melanocephalum* or *Linepithema humile* that have been tested can be claimed on the product label. Repellent product efficacy testing should be conducted for products claiming "against tropical ants" with exotic species e.g. *M. pharaonis*, *T. melanocephalum* or *L. humile* or in case for a general claim "against ants" with endemic species, e.g. in Europe *Lasius niger*.

h) **Sample size.** The sample should be large enough 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.

i) **Replication.** A minimum of five replicates (equal number of treated and control replicates) for all studies are recommended.

j) **Ant colony rearing, handling, and maintenance.** When applicable, a description of the ant laboratory colony rearing practices should be included. Collection details and maintenance procedures for field-collected strains should be described.

k) **Statistical analysis plan (if applicable).** Protocols should include a full description, explanation, and justification for the statistical methods proposed to analyze product performance test results, taking into account the specific

study objectives and variables.

l) **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.

m) **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.

(2) **Data Collection and Reporting.** Study protocols should include details on data 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 as well as a batch number.

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

d) **Test organisms:** Scientific name, strain, health status of the colony (absence/presence of parasites like mites etc.), source, method of rearing and handling including description of food and its components as well as date of preparation should be stated.

e) **Testing conditions:** Information on temperature, relative humidity, ambient light and photoperiod should be reported.

f) **Testing system, including but not limited to:**

i. Description of test substance (i.e., product, % active ingredient, and formulation to be tested). Negative control should also be described.

ii. Description of the experimental unit.

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

iv. Duration and conditions of acclimatization period.

v. Number of product treatments.

vi. Number of negative control replicates.

vii. Number of replicates per treatment.

viii. Length of time for ant exposure period to each treatment.

g) **Health status of test organisms.** Although only healthy ants are introduced in the tests, the insecticide or repellent may have side effects on their health status. Symptoms observed in test organisms (abdominal swelling, changes in body color, odor, mobility and/or behavior) as well as mortality in repellent tests should be described.

h) **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.** Endpoints should be reported as observed throughout the test.

iii. **Amount of product applied.** Report the amount of product, expressed as weight of product applied to each replicate. Report the quantity of active ingredient applied.

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

v. **Data analysis.** The report should include the statistical analysis if applicable.

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.

5. Baiting

(1) Foraging ants store food in their crops to feed other colony members in the nest by regurgitation. This behavior is known as trophallaxis and is an important component of brood care in the majority of the ant families of Myrmicinae, Dolichoderinae and Formicinae. Other colony members such as worker ants and queens are fed, as well. One worker ant can feed up to 12 further worker ants which then again feed up to 12 worker ants by themselves in a short period (Markin 1970). Treatments with baits can take advantage of this behavior since by it, the toxicant can be distributed throughout the colony. However, as a consequence the insecticides need to be efficient over a wide dose range as trophallaxis may dilute a toxicant to sub-lethal doses (Rust et al. 2004). A delayed mode of action of the insecticide is essential since foragers should reach the nest before death to distribute the insecticide as well as to recruit other foragers to the bait. This is the reason why the application of residual high-toxicity spray insecticides only eliminates a small number of ants that forage on the surface; it is not suitable to eradicate an entire infestation, as not all colony members can be affected this way.

(2) Bait products are available as gel, granular and liquid baits and can be applied as crack and crevice application, spot applications or in bait stations.

(3) An ant control bait usually consists of a mixture of four components: (i) an insecticide, which should have a delayed effect to ensure that the worker ants can transfer the insecticide throughout the colony via trophallaxis; (ii) an attractant to recruit other

foraging ants; (iii) a palatable matrix responsible for the physical structure of the bait (i.e. its particle size); and (iv) other materials added for specific purposes, such as emulsifiers, preservatives and/or antimicrobial agents.

(4) In practice, application of baits in bait stations is considered the most suitable delivery method. These should be placed in locations used by ants or in areas where they are foraging. Bait application must be without risk for the user, e.g. delivered in child-resistant containers. The use of bait stations is usually required for outdoor applications (study design not included in this guidance document), and open application should be restricted to specific indoor operations, where baiting in trap devices is not feasible, e.g. for crack and crevice treatments.

(5) Due to their biology and reproduction the occurrence and development of insecticide resistance in case of sublethal doses would not be expected.

(6) For successful control of infestations with polygynous ant species all colony members including brood and queens have to be eradicated.

6. Bait efficacy testing

(1) The efficacy assessment of baits is usually made in relation to the label claim. It will take into account the species to be controlled, the method(s) of application, application rates and use patterns of the product.

(2) This guidance is aimed at testing baits that claim to demonstrate a “nest kill”, i.e. their complete elimination or “colony extermination”, since a claim such as “kills ants” is too unspecific and would also refer to the elimination of only individual ants. Furthermore, the guidance applies to nest kill of the mentioned species nesting indoors.

(3) The present guidance applies to all bait products against *M. pharaonis*, *T. melanocephalum* and *L. humile* and, although not a tropical ant species, could also be used for *Lasius niger*. This includes formulations for use in bait stations as well as those applied in cracks and crevices and comprises gel, granular and liquid baits. The applicability of the described test designs to other ant species is not confirmed yet.

(4) An ant bait should be attractive and effective even when alternative food sources are abundantly available. In addition, it should maintain its insecticidal activity, consistency and attractiveness for the claimed period.

(5) To prove the attractiveness of baits, choice tests with an alternative non-toxic food source (challenge diet, dead insects e.g. cockroaches and sugar or honey) are required.

(6) Tests should be performed for fresh bait, bait at the end of the shelf life and for residual efficacy demonstration.

Test animals

(7) For efficacy tests with baits, whole healthy (wild or laboratory) ant colonies should be used. Healthy ant colonies consist of queens, active workers and different stages of brood without fungi or parasites inside the nest. The relevant test species (*Monomorium*, *Tapinoma*, *Linepithema*) are polygynous, and a colony contains from a few up to several

thousands of queens. Therefore, 5 to 10 queens, brood and several hundred workers should be introduced into the test arenas. Exact counting of brood and workers is not requested to avoid harm of the test animals.

Test arenas

(8) For standardization the test arenas (fig. 1) should measure about 800 cm^2 ± 25% (Hooper-Bui and Rust 2000, Rust et al. 2004, Krüger et al. 2017) and a wall height of about 10 to 20 cm should be used. On the upper rim of the walls, a barrier (e.g. sticky insect glue or polytetrafluorethylene; generally non-toxic substances which do not influence ants behavior should be used) should be applied to prevent the ants from escaping.

(9) The container for the ant nest can be made of wood or plastic. Examples for design and size can be found in fig. 4 or fig. 5. Small holes in the walls are needed to enable the ants access into the interior. A layer on the floor made of plaster to provide humidity for the ants is suitable in plastic nests. The nests should provide darkness for the ants, but also enable observation of the colony inside the nest during the test procedure once a week. Therefore, a clear glass plate can serve as a lid with an additional dark plate on top can be used. To observe the nest the dark plate can be removed. Disturbance of the ants inside the nest is minimized because of the glass plate.

(10) The ant nest should be placed close to the wall (in case of rectangular arenas at the short side, see fig. 1) and adequate food (e.g. dead cockroaches provided *ad libitum* (Adams et al. 1999)), sugar or sugar solutions and water should be placed opposite to the nest as far as possible.

Test procedure

(11) Ant colonies are transferred from breeding containers into the test arenas (e.g. within the nests) and are allowed to acclimatize for at least 7 days before the bait is introduced into the test arena. The bait product should be provided according to the label claim (within a bait box or as bait point on a small petri dish when used in cracks or crevices) and placed opposite of the nest beside an alternative food source and water.

(12) Regular visual inspections of the activity of the foraging ants and the acceptance of the bait (by counting foraging ants feeding on the bait) are recommended especially for recognition of changes in behavior (e.g. disorientation, foraging queens) or the occurrence of disease symptoms in test organisms (e.g. abdominal swelling, changes in body colour, mobility and behavior). Furthermore, decreasing activity should be documented for data analysis and evaluation by counting the foraging workers outside the nest once a week. Forager counting can be done either by direct visual inspection (but should then preferably be performed simultaneously by two persons) or by counts of the number of foragers in photographs of the test arena floor. The latter method has the advantage of being more objective and re-evaluable at a later point in time. Furthermore, it should be recorded weekly if brood and queens in the nest are alive, regardless of workers' activity.

(13) Food and water are supplied *ad libitum*. The bait should be applied according to the use instructions. When an application dose is claimed for a specific area and the bait amount that should be introduced in the arena is not applicable (test arena is too small) the lowest amount should be applied (for example use 200 mg if the label claims 200 mg/m^2 even if the arena is smaller than 1 m^2). If the product is consumed then a reapplication is necessary.

(14) The baiting period in the test should not exceed the time span claimed for eradication of the ant colony indicated in the use instructions or on the label. If not otherwise stated on the label, the bait exposure period in the tests should be 50 d (Iglisch 1998). In case of surviving ants at the end of the baiting period, it should be followed by a

post-baiting observation period of two weeks. All bait should be removed for the post-baiting observation and food and water should be supplied *ad libitum*.

(15) At least five replicates and an equal number of negative control replicates are required. The control replicates should be conducted with the same test procedure either with a placebo bait (bait product without active substance) or if not possible with a non-toxic alternative food source.

Effectiveness criteria

(16) A successful bait product claiming "nest kill" has to achieve eradication of the ant colonies within the test period. That means that all worker ants, brood and queens are dead within the test period and max. two weeks post-baiting observation. Dead brood is dried out in contrast to live brood which is recognizable by the shiny surface of the brood.

(17) Worker activity in all replicates as well as the number of queens in (negative) controls should not decrease more than 10% on average from the beginning of the trial (before bait exposure) to the end (after post baiting period if applicable).

7. Repellent tests for surface treatment

(1) The test procedures described below are not only suitable for tropical ants (*Monomorium pharaonis, Tapinoma melanocephalum, Linepithema humile*) as mentioned in this guidance document but, although not a tropical ant species, also for Lasius niger. The applicability of the described test designs to other ant species is not confirmed yet.

(2) A successful repellent product against ants should achieve a reduction or prevention of invading ants in houses or sensitive areas (indoor use) depending on the label claim. The application of a repellent should not result in death of the target species. Therefore, the non-insecticidal effect has to be proven, since an insecticidal effect of products may result in a rejection of the authorization as repellent.

(3) Repellent products can be fluids, gels, granules or powders and can be applied on surfaces with different methods: spray, paint, scatter or pour.

(4) The efficacy assessment with repellents is usually made in relation to the label claim. It will take into account the species to be controlled, the method(s) of application, application rates and use patterns of the product.

7.1. Screening test (laboratory choice test)

(5) A choice test similar to the design described below can be used for active substance screening and product development (Krüger et al. 2017).

Test animals

(6) Screening tests should be performed with at least 50 to 70 worker ants per replicate to guarantee a sufficient number of crossing ants for statistical analysis. Testing is species-specific, and the ants should belong to the target species for which the repellent is intended.

Test arena

(7) The test arena should consist of a petri dish standing on a beaker surrounded by

water forming an artificial island for the ants. Two microscope slides should serve as bridges, connecting the petri dish island with two separate beakers (fig. 2). The upper rim of the petri dish, the beakers and the underside of the slides should be blocked by a barrier (polytetrafluorethylene or sticky insect glue; generally non-toxic substances which do not influence ant behavior should be used). The surface of the bridges may vary, and both porous and non-porous substrate fixed to the microscope slide surface can be used for testing representative surfaces. Test surfaces should reflect the intended use (e.g. use on ceramic tile, plywood, painted plywood, stainless steel, concrete). The test principally exploits the escape response of ants, which are placed on an unprotected surface (here: a petri dish). The repellent is then placed on one of two escape paths.

Test procedure

(8) The formulated product (spray, liquid, gel, powder, dust, etc.) is applied in the middle of one bridge over the entire width as a stripe; the second bridge remains untreated. Typically, the stripe should reflect the intended use.

Within the investigation period the number of ants crossing more than half of the untreated or the treated bridge, by crossing the applied repellent, is recorded. Usually, the test period is about 30 min, but can be longer or shorter, depending on the ant behavior. Subsequently, ants that crossed the middle of the bridge are transferred softly with a paintbrush into the associated beaker at the end of the bridge. To exclude bias due to pheromone trails or side preferences, the locations of the treated and untreated bridges should be alternated after half of the ants crossed the bridges.

(9) After the trial, all ants should be kept with food and water supply. To exclude an insecticidal effect of the repellent possibly caused by contact and crossing, mortality is recorded 24 hours after exposure. Ants should not be used twice for trials, only naïve ants should be used in the tests. The non-insecticidal effect has to be proven, since an insecticidal effect of products may result in a rejection of the authorization as repellent.

(10) For testing residual efficacy, microscope slides should be treated with the repellent and the slides should then be stored for the desired period before they are used in the tests. The conditions of storage should reflect the real-use scenarios, according to label claim for application (temperature, humidity, light, dust etc.). The temperature should be kept at 22 ± 4 C° (unless otherwise justified), with a relative humidity of 40 - 60%, and a photoperiod ranging from 12 hours of light and 12 hours of darkness to 16 hours of light and 8 hours of darkness. The temperature during the test should be kept as constant as possible because changes can affect the performance of the product treatments. Storage should not be conducted in closed containers. When the product is claimed for outdoor use the storage should be conducted at an air temperature within the range of 19 - 29°C°, rain fall (if necessary this could be mimicked by artificial watering) at least 20% of the storage period (for specific claims e.g. ‘rainfast 1 hr after application’ or ‘water resistant’ additional testing is considered) and direct sunlight for at least 30 to 40% as most products with outdoor use will typically be applied during the summer months when sunlight is considerable for the majority of the day (6 to 12 hours per day; may depend on the claimed region to be used).

(11) To exclude side preference effects, a control treatment without the product or the active substance on any bridge should be conducted with ants from the same colony.

(12) At least 5 replications and 5 non-treated controls should be performed.

Effectiveness criteria

(13) A repellent should repel at least 90% of the total number of ants entering the treated bridge within the test period of 30 minutes (or according to the claim), directly after

substance/ product application and at the end of the claimed period (Krüger et al. 2017).

(14) After 24 hours, mortality in the treatment group should be similar to or less than that of the control group and neither group should exceed 10% mortality. In the control replicates a mortality of 10% should not be exceeded.

7.2. Simulated-use test

(15) A choice test similar to the design described below should be performed for product authorization (Krüger et al. 2017).

(16) The simulated-use tests are designed to mimic the practical use situation and are suitable for testing the performance of products. The tests should be relevant to the intended use and label claims.

Test animals

(17) For simulated-use tests with repellents, whole healthy (wild or laboratory) ant colonies should be used. Healthy ant colonies consist of queens, active workers and different stages of brood without fungi or parasites inside the nest. The mentioned species are polygynous, i.e. a colony contains variable number of queens. Therefore, 5 to 10 queens, brood and several hundred workers should be introduced into the test arenas.

Test arena

(18) For standardization arenas of at least 800 cm^2 ± 25% (Hooper-Bui and Rust 2000, Rust et al. 2004, Krüger et al. 2017) with a wall height of 10 to 20 cm divided into two similar compartments by an at least 1 cm wide line of slick insect barrier (sticky insect glue or polytetrafluorethylene; generally non-toxic substances which do not influence ants behavior should be used) should be used (fig. 3). Alternatively, a double chamber can be used. In both cases, the compartments are connected via bridges. One compartment contains the nest, the other food and water. The repellent is tested on small bridges, which connect the compartments. During acclimatization, ants can reach the food via one bridge and build a pheromone trail. Different bridges should be used for both porous and non-porous surfaces (e.g. ceramic tile, plywood, painted plywood, stainless steel, concrete), according to the label claim.

(19) The container for the ant nest can be made of wood or plastic. Examples for design and size can be found in fig. 4 or fig. 5. Small holes in the walls are needed to enable the ants access into the interior. A layer on the floor made of plaster to provide humidity for the ants is suitable in plastic nests. The nests should provide darkness for the ants, but also enable observation of the colony inside the nest during the test procedure once a week. Therefore, a clear glass plate can serve as a lid with an additional dark plate on top can be used. To observe the nest the dark plate can be removed. Disturbance of the ants inside the nest is minimized because of the glass plate.

Test procedure

(20) Ant colonies (containing workers, brood and queens) are introduced in the test arena (e.g. within the nest). After an acclimatization period of at least 7 days, the formulated product (spray, liquid, gel, powder, dust etc.) is applied in the middle of the bridge, covering the entire width in a stripe. Typically, the stripe should be 1 cm wide or according to the label claim. Additionally, a second untreated bridge of the same material is provided. Therefore, ants have a choice of two paths into the food and water compartment: one, which they are accustomed to but treated with the repellent and a second unaccustomed but untreated path.

(21) Depending on the size of the ant colony and the ant activity for 1 to 5 min (longer observation periods are possible if ant activity is low), the number of ants crossing the treated bridge (over the treated surface) and the untreated bridge are counted and recorded separately.

(22) For testing residual efficacy, the test system can be let in place for the intended time period the product is claimed for. Observations at regular time intervals should be conducted. Residual efficacy testing can also be conducted with bridges treated with the repellent and the bridges are then to be stored for the desired period before they are used in the tests. The conditions of storage should reflect the real-use scenarios (temperature, humidity, light, dust etc.).

(23) Tests should be performed with 5 replicates and 5 non-treated controls should be used.

(24) A control treatment of the tested formulation(s) without active substance (= placebo product) should be included in all tests. Control trials should mimic, as far as possible, the test itself in terms of number of replications and individuals, for statistical comparison and to get a fair impression of the levels of repellence.

(25) Environmental conditions must be specified for the test itself, and during the storage of the treated surfaces (temperature, humidity, photoperiod and ventilation).

Effectiveness criteria

(26) At least 90% repellency should be achieved within the test period (or according to the claim), directly after substance/product application and at the end of the claimed period.

(27) Proof of non-insecticidal efficacy: If a mortality of 10% in the treated replicates during the test and 24 hours after the end of the test is recorded, this would lead to the rejection of the authorization of the repellent product in the European Union. Dead ants should be recorded and at the end of the test period; therefore all surviving individuals of the colony should be frozen and counted.

8. References

1. **Adams, A., Kunkel, S., Dodd, G. and Höbel, S.** (1999). "Method and Procedure for Evaluating Biological Performance of Pharaoh Ants, *Monomorium pharaonis* (Hymenoptera: Formicidae), Baits." Proceedings of the 3rd International Conference on Urban Pests: 203-209.
2. **Beatson, S.** (1972). "Pharaoh´s Ants as Pathogen Vectors in Hospitals." The Lancet 1: 425-427.
3. **Fowler, H. G.** (1993). "Ants as Potential Vector." Insect Science and its Application 14: 367-370.
4. **Hooper-Bui, L. M. and Rust, M. K.** (2000). "Oral Toxicity of Abamectin, Boric Acid, Fipronil, and Hydramethylnon to Laboratory Colonies of Argentine Ants (Hymenoptera: Formicidae)." Journal of Economic Entomology 93: 858-864.

5. **Iglisch, I.** (1998). "Richtlinien für die amtliche Prüfung von Mitteln und Verfahren auf Wirksamkeit zur Bekämpfung tierischer Schädlinge gemäß §10 c Bundes-Seuchengesetz." [In German; Guidelines for official testing of pesticides and techniques for control of health pests according to § 10c of the Federal Act on Epidemics]. Bundesgesundheitsblatt 4: 184- 189.

6. **Kim, C. W., Kim, D. I., Choi, S. Y., Park, J. W. and Hong, C. S.** (2005). "Pharaoh Ant (*Monomorium pharaonis*): Newly Identified Important Inhalant Allergens in Bronchial Asthma." Journal of Korean Medical Science 20: 390-396.

7. **Krüger, A., Knobelspieß, S. and Schmolz, E.** (2017). "Development and Evaluation of Testing Methods for Ant Repellents." Proceedings of the 9th Conference on Urban Pests: 277-280.

8. **Lima, W. R., Marques, S. G., Rodrigues, F. S. and Rebelo, J. M.** (2013). "Ants in a Hospital Environment and their Potential as Mechanical Bacterial Vectors." Revista da Sociedade Brasileira de Medicina Tropical 46: 637-640.

9. **Markin, G. P.** (1970). "Food Distribution within Laboratory Colonies of the Argentine Ant, *Iridomyrmex humilis* (Mayr)." Insectes Sociaux 17: 127-158.

10. **Moreira, D. D. O., Moreis, V., Vieira-Da-Motta, O., Campos-Farinha, A. E. d. C. and Tonhasca, A.** (2005). "Ants as Carriers of Antibiotic-Resistant Bacteria in Hospitals." Neotropical Entomology 34: 999-1006.

11. **Rust, M. K., Reierson, D. A. and Klotz, J. H.** (2004). "Delayed Toxicity as a Critical Factor in the Efficacy of Aqueous Baits for Controlling Argentine Ants (Hymenoptera: Formicidae)." Journal of Economic Entomology 97: 1017-1024.

12. **Wetterer, J. K.** (2009). "Worldwide Spread of the Ghost Ant, *Tapinoma melanocephalum* (Hymenoptera: Formicidae) " Myrmecological News 12: 23-33.

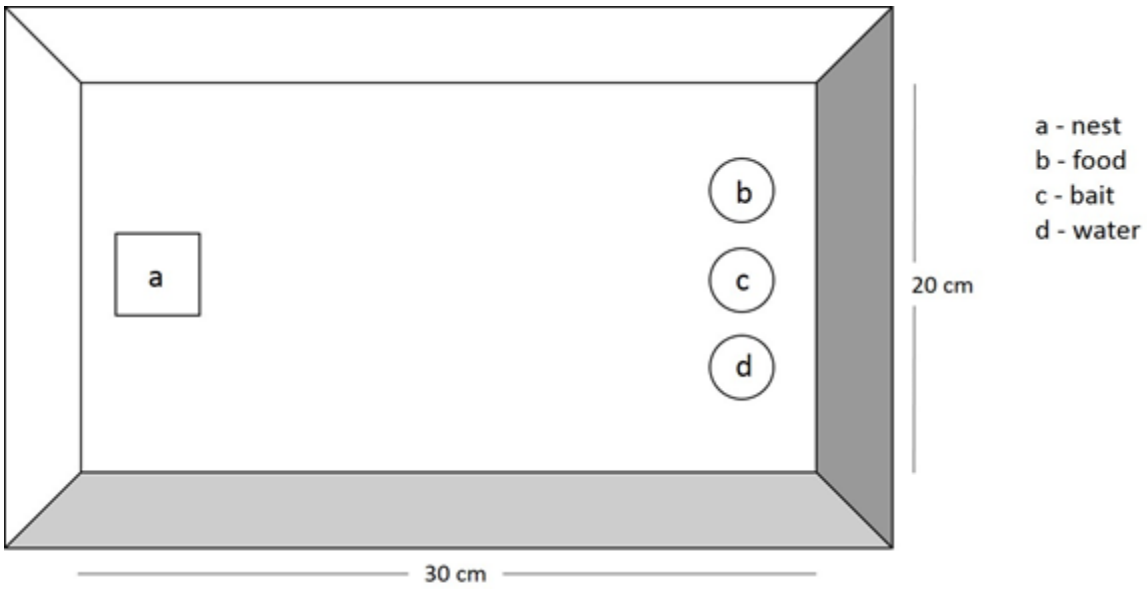
13. **Wetterer, J. K.** (2010). "Worldwide Spread of the Pharaoh Ant, *Monomorium pharaonis* (Hymenoptera: Formicidae)." Myrmecological News 13: 115-129.

14. **Wetterer, J. K., Wild, A. L., Suarez, V., Roura-Pascual, N. and Espadaler, X.** (2009). "Worldwide Spread of the Argentine Ant, *Linepithema humile* (Hymenoptera: Formicidae)." Myrmecological News 12: 187-194.

15. **Zarzuela, M. F. M., Campos-Farinha, A. E. d. C. and Pecanha, M.** (2004). "Evaluation of Urban Ants (Hymenoptera: Formicidae) as Carriers of Pathogens in Residential and Industrial Environments: I. Bacteria." Sociobiology 44: 9-14.

16. **Zarzuela, M. F. M., Campos-Farinha, A. E. d. C., Russomanno, O. M. R., Kruppa, P. C. and Goncalez, E.** (2007). "Evaluation of Urban Ants (Hymenoptera: Formicidae) as Vectors of Microorganisms in Residential and Industrial Environments: II. Fungi." Sociobiology 50: 653-658.

546 **Figure 1. Example for the experimental set-up for efficacy testing of baits against tropical ants.**



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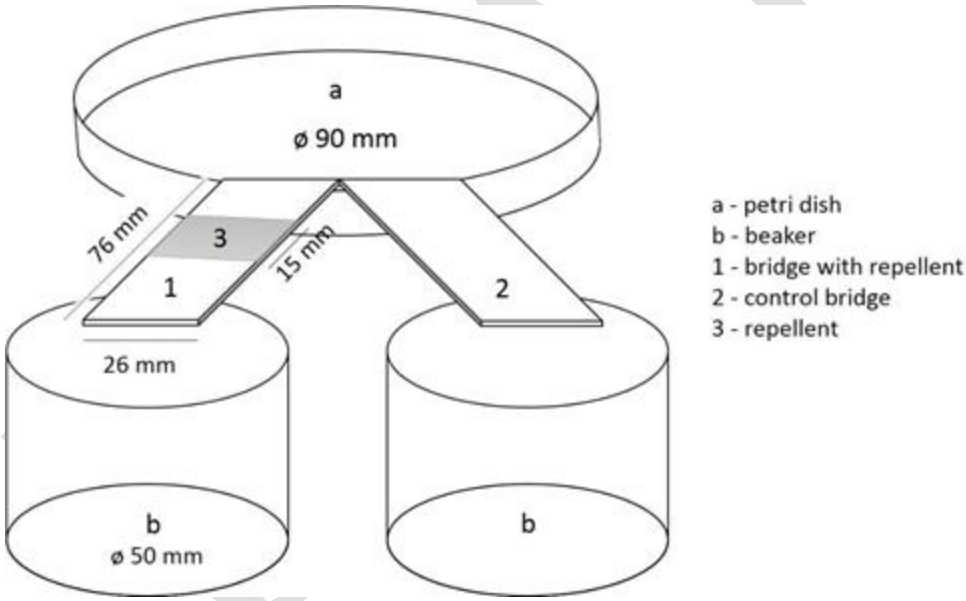
548 **Figure 2. Example for the experimental set-up of the laboratory test for repellents against ants.**



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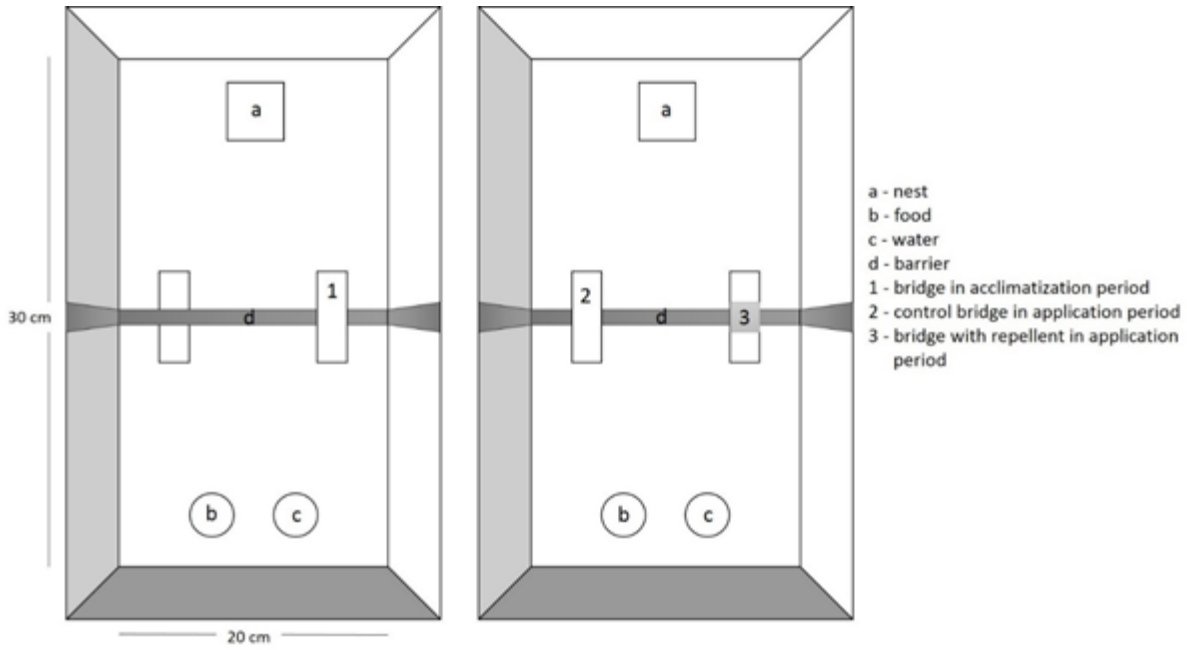
551 **Figure 3. Example for the experimental set-up of the simulated use-test for repellents against ants.**



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554 **Figure 4. Example for ant nests (3 cm by 3 cm by 1.6 cm) in simulated use tests. An additional plate**
555 **for providing darkness in the nest should be placed on top.**

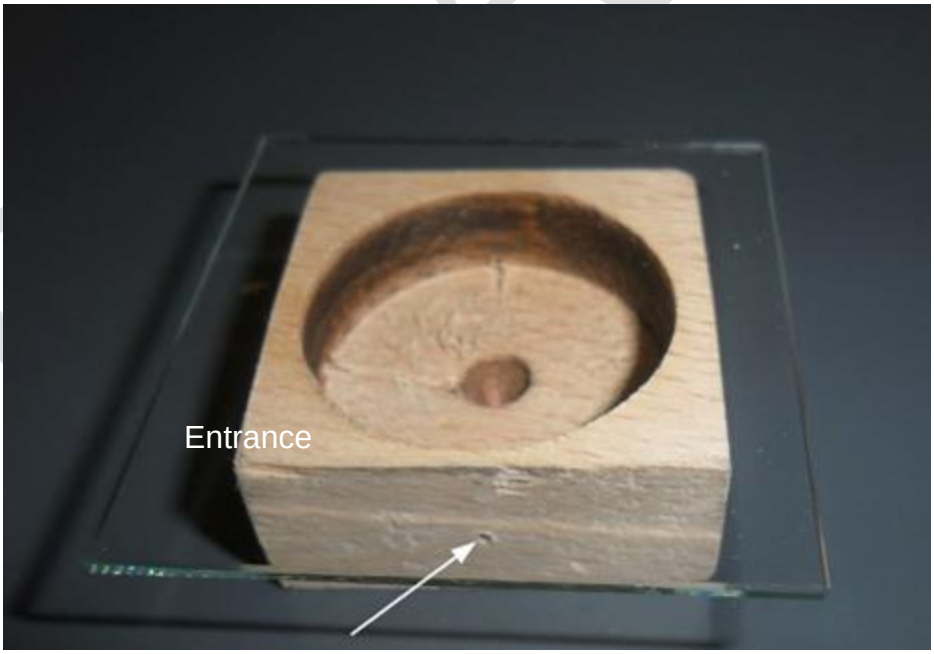

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Figure 5. Example for ant nests (5.8 cm by 3.7 cm by 2.6 cm) with a layer of plaster (about 0.5 cm) on the floor for use in simulated use tests. For providing darkness in the nest the nest box should be made of black plastic or an additional plate should be placed on the top.