Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

10-Jan-2013

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

GUIDANCE DOCUMENT ON ASSAYS FOR TESTING THE EFFICACY OF BAITS AGAINST COCKROACHES

Series on Testing and Assessment No. 183

Series on Biocides No. 5

JT03333177

ENV/JM/MONO(2013)3
Unclassified

English - Or. English

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

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FOREWORD

This *Guidance Document on Assays for Testing the Efficacy of Baits against Cockroaches* has been developed under the auspices of the Task Force on Biocides. (TFB)

A first draft document was developed by Germany and discussed at a TFB meeting in 2010. It was further reviewed and revised; there were two commenting rounds through the TFB and the Working Group of National Co-ordinators of the Test Guidelines Programme (WNT) in 2011 – 2012. The draft document was approved by the WNT in September 2012. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 21st December, 2012.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

INTRODUCTION

1. This document provides guidance on conducting laboratory tests to determine and assess the efficacy of test substances and/or the effectiveness of test products used as baits for the control of cockroach species in indoor environments. It is based upon a German guideline (Federal Environment Agency 2001) and incorporates information from the American Product Performance Guidelines 810-3500 (US EPA 1998). For the the scientific evaluation of the efficacy of a bait product and of validity of the test results, a minimum of three repetitions of the assay at different times (considering the claimed application conditions) and with different batches of test animals should be conducted.

2. The following species are considered candidates for testing as they are species of potential public health importance in Europe, North America, Australia, and Asia:

- German cockroach, *Blattella germanica* (Linné, 1767)
- Oriental cockroach, *Blatta orientalis* (Linné, 1758)
- American cockroach, *Periplaneta americana* (Linné, 1758)
- Brown-banded cockroach (or bedroom roach), *Supella longipalpa* (Fabricius, 1798)
- Australian cockroaches, *Periplaneta australasie* (this species is a pest in the South-Eastern US, Hawaii and Australia)

Of these, the German and the Oriental cockroach are the most prevalent species throughout Europe. In North America, the American cockroach replaces the latter one in relative frequency of occurrence (for more details see Product type 18 in Annex V of the EU Regulation 528/2012 concerning the placing of biocidal products on the market[1]). Information on the distribution of these pests and their public health importance is abundantly available (Rust *et al.* 1995, Cochran 1999, Pai *et al.* 2004, Gore and Schal 2007, Rust 2008).

SCOPE OF GUIDANCE DOCUMENT

3. A bait product usually contains one or more insecticidal active ingredients (a.i.) combined with an 'attractive' food/compound (which is not considered an active substance) for cockroaches. Active ingredients are: toxicants with lethal (and sometimes knock-down) effects on all mobile stages; and insect growth regulators that are active against nymphal stages. The present guidance document applies to all blatticides that are effective as baits. This includes formulations for use in bait stations as well as those applied in the open (*e.g.* in cracks and crevices). The guidance document does not deal with the physical design of bait stations, but is focused on the bait matrix.

4. A cockroach bait should be 'attractive' and effective even when alternative food sources are abundantly available (Nalyanya *et al.* 2001). In addition, it should maintain its insecticidal activity, consistency and attractiveness for the claimed period, even at damp locations such as kitchens, bathrooms and conduit pipes.

[1] Regulation (EU) No. 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. http://eur-lex.europa.eu/JOHtml.do?uri=OJ:L:2012:167:SOM:EN:HTML

DESCRIPTION OF THE TEST

Test arenas

5. A test arena is made of easy to clean materials (glass, plastic) and its design caters for the needs of cockroaches as far as size and hiding space is concerned. Examples of suitable test arenas which are essentially Ebeling boxes, are depicted in Figures 1-4 (Ebeling *et al.* 1966, Appel 1992, Le Patourel 1998, Durier and Rivault 2000a, Appel 2003). A dual-chamber system using circular boxes is shown in Figure 4. Test arenas consisting of one single large test arena (1 m^2), quadratic or circular are equally appropriate (Durier and Rivault 2000a, Durier and Rivault 2002, Wang and Bennent 2006). The latter one is quite often used, particularly in laboratories in North America.

6. To avoid contamination of arenas by insecticide residues, arena grounds are completely covered by filter paper sheets, fit accurate to size and taped tightly to the walls, thus preventing cockroaches from slipping under.

7. Test arenas consist of two chambers, a '*feeding'* or *activity chamber*' and a '*harbourage chamber*' connected by a translucent Plexiglas^{TM} – tube (500 mm long, diameter 50 mm). Chambers should be rectangular (245 x 395 x 300 mm; 1,12m^2, Annex: Fig 1-3) or cylindrical (1,01 m^2, Fig. 4). An impassable (physical or non-toxic) barrier should be applied to the upper inner surface to prevent cockroach escape. In cylindrical boxes, a fluoropolymer (Fluon^{TM}) can be applied, while the inner top of rectangular boxes can be coated by caterpillar glue or petrolatum. The latter ones are less suitable if early nymphal stages are tested. Surface areas in these boxes can be reduced (by a 0.1 m band of Fluon) to 0.86 m^2 and 0.78 m^2, respectively. In addition, arenas are covered by nylon screens of appropriate mesh size (*e.g.* mosquitoes-mesh netting). The *feeding chamber* contains one 50 ml water vial, placed centrally between the two baits (Fig. 3). The food is placed into the corner of a Petri dish (Fig. 3). The *harbourage chamber* contains two 'shelter (or harbourage) units' at opposite corners or walls. Each consists of three 4 mm plywood panels of 100 x 100 mm (or disposable units) separated by 10 mm spacers (surface area: 30 x 30 mm) when small test organisms are tested (*B. germanica* and *S. longipalpa*). Spacers should be 15 mm in height for tests with larger organisms, *e.g. B. orientalis* and *Periplaneta spp.* Other groups prefer harbouring shelters made from corrugated paper (in five layers) instead of plywood, this with a view to being able to easy replace them in further test.

8. If instead of the one described above, a single-chambered system is used, its area should be 1 m^2. A shelter made of corrugated cardboard is placed at one end of the arena with a cotton- plugged water flask placed into the opposite end of the arena. In between, four Petri-dishes are deployed: two with rat chow and two containing the test substance. Of these, one Petri-dish each is covered by a fine mesh to prevent test insects from entering (these serve as weight controls). Overall, other patterns should follow those described for the two chambered system (Durier and Rivault 2000a, Durier and Rivault 2002, Wang and Bennent 2006).

9. Large circular arenas (ground area 1m^2) have been in use by some companies, especially for screening bait stations. Details should be searched for in the literature.

Bait placement

10. An aliquot of bait is deployed per test arena. As a standard it is placed in the feeding unit into a position that is symmetrical to the food container relative to the opening (Fig. 3 and 4). If scattered baits or gel baits are tested, the bait is placed onto a small Petri dish cover (40 mm diameter, 7 mm height). It is recommended to change symmetrically the bait position in the replicates, so as to minimize effects potentially resulting from the variability of lighting conditions (*i.e.* patterns of light and shadow). In a

single-chamber arena the standard position of the bait is in the feeding area and for additional tests in the harbourage area, respectively.

11. Weight of the test sample is taken prior to and at the end of the test. Three additional bait specimens are placed into an open bowl outside the test arena and kept there during the entire test period; their weight is taken in the same fashion. Comparison of the two test samples will allow the determination of weight changes resulting from exogenous factors, *e.g.* moisture loss in gels. Some prefer placing the control baits (for estimating evaporative loss) into an arena identical to the test arena that is kept under identical conditions, while others recommend to put the control baits into the very same arena, well protected against access by test organisms; both methods are applicable. As humidity is a key-factor when consumption is measured it is important to secure that the weight controls and the substances given to the cockroaches are exposed to identical levels of humidity throughout the trial period.

12. For testing photosensitive gel baits, the gel can be applied into small opaque plastic tubes with a length of 40 mm and a minimum internal diameter of 9-10 mm for the German cockroach *(Blatella germnica)* and the brown-banded cockroach *(Supella longipalpa)*, 16-18 mm for the Oriental cockroach (*Blatta orientalis)*, and 18-22 mm for the American and Australian cockroaches *(Periplaneta americana* and *Periplaneta australasie)*. The tubes are positioned as described in paragraph 10 at the wall of the feeding unit in such a way that the test animals can use both sides as entrances to reach the bait. As resistance and cross-resistance is a major problem in cockroaches, it is suggested that the investigator, as far as possible, comment on the level of resistance in the strain used in relation to the active ingredient investigated. Whether a test strain is very sensitive or has elevated tolerance, this is equally important for the interpretation of the test results.

13. Fresh bait is replenished in all boxes if necessary. This is usually the case, when approximately 75% of the bait has been consumed in one test arena. However, certainly this may not be done when testing a product that claims being efficacious after deployment of a single dose. The new bait is applied with a short distance next to the old bait (*e.g.* gel bait on another Petri dish) to enable the estimation of the weight of the bait at the end of the test considering the desiccation data of the control baits."

Test organisms

14. Due to the specificity of baits, only effects against species that have been tested should be claimed (single species claims). In case a 'general' claim is to be made, such as '*for use against cockroaches*', usually the two species most prevalent to the region are the minimum to be tested.

15. In Europe, the species that are most often tested are *Blattella germanica* (German cockroach) and *Blatta orientalis* (Oriental cockroach), and on occasion *Supella longipalpa* (brown-banded cockroach), while *Periplaneta americana* (American cockroach) is sometimes used as a model species for large cockroaches. In North America, *Blatella germanica* and *Periplaneta americana* are the most often tested species while the testing of *Blatta orientalis* is less required.

16. Standard susceptible strains should be used, reared in a laboratory at standard conditions and fed on insecticide free diets (*e.g.* Harlan Teklad^{TM} rodent diet or other suitable diets. On occasions, dog/ cat chow has been used. A protein-carbohydrate ratio of approximately 25:75 has been suggested as optimum (Schrader *et al* 2007). For the special purpose of reflecting field conditions, it might be desirable to test field-caught strains from urban centres where resistance is known to be a problem or well characterized resistant strains where similar resistance is known to occur in field populations.

17. Test organisms should be in good health. However, there are some microbial pathogens that have been implicated as agents of chronic infections in laboratory roach colonies, such as bacteria (*Serratia*

spec), protozoa (*Gregarina spec*.), fungi (*Herpomyces spec*, *Metarhizium spec*), nematodes (*Blatticola blattae*), or acanthocephales (*Moniliformis moniliformis*). If existing, their possible influence should be investigated and controlled for (Lopes and Alves 2005, Libersat and Moore 2000).

18. For testing products against adults of *B. germanica*, 3-14 day old adult cockroaches of both sexes (only non-gravid females) are used, while the age range might be expanded in other species, for example *B. orientalis, P. americana* and/or others to include adults aged up to 21 days. They are released to each arena by random allocation of a representative sample drawn from the rearing facility.

19. For testing products against nymphs, early nymphs, medium nymphal stages or late nymphal stages (*e.g.* for *B. germanica:* N1/N2, N3/N4 and N5/N6 respectively) are to be investigated. Table 2 shows examples of important test stages considering the mode of action of the active substance in the bait product.

20. Size of test populations deployed varies with species and life stages tested. For arenas as proposed here, Table 1 displays possible population sizes while exact figures should be based on power calculations done prior to conducting tests. Information on examples of recommended test duration, effects investigated et cetera is displayed in Table 2. Longer durations may apply if juvenoids are to be investigated.

21. Carbon dioxide anaesthesia can be used to facilitate roach transfer from breeding containers to test arenas. However, this may be done only once, as it affects insects' behaviour (Branscome *et al* 2005). Other procedures to facilitate transfer include the use of 'chill tables', for which dry ice or a frozen package of water is put on a tray. On top, a thin piece of sheet metal or plexiglass is placed, which gets very cold from the ice below. Test insects placed on top of this cold surface are then slowed down.

22. Test organisms are allowed to habituate in the arenas for three days. Up to four hours after introduction of test animals into the arena, dead and moribund animals can be removed and replaced by fresh ones. Acclimatisation is considered successful, when – at day light conditions – most test organisms (about 80% or more) reside in the hiding towers. Before beginning the test, dead and moribund organisms are removed from the arenas without replacement. A mortality rate of four percent of the test animals during the habituation period should not be exceeded. The number of test animals at the beginning of the bait test is counted as initial number for efficacy evaluation.

Test design – Test conditions

23. Tests can be designed as no-choice test (no alternative food available) or as choice test (alternative food available). No-choice testing is employed to determine if a bait matrix is palatable to cockroaches and to detect repellency to the bait matrix and/or level of active ingredient in the formulation. Other purposes of no-choice testing include the screening for the amount of active ingredient required to kill cockroaches consuming the bait and detecting behavioral avoidance of a bait type or bait station, especially in cockroaches collected from urban areas (Wang *et al* 2004). Choice testing, in contrast, will give additional insights that are important, but not necessarily evident from no-choice tests; this relates particularly to an evaluation of the attractiveness of test baits in comparison to alternative food sources. However, the issue of behavioural avoidance and palatability of the product is addressed more appropriately in choice tests than in no-choice tests.

24. Room temperature, *i.e.* testing temperature should be maintained fairly constant. It might depend upon the climatic region with the range potentially spanning from a low 19° C to a high 35 or 40° C, where the latter can be used when products are tested for which efficacy in hot climates is claimed. It is recommended that testing be carried out at temperatures that are designated as 'room' temperatures in the

region where the product be sold. This is around 22 ± 3° C in temperate regions, for example Central Europe, but may certainly differ in other regions. Relative humidity is maintained at 55 ± 10 %, if no particular requirements apply (such as the testing of baits designed for damp locations, at which occasion a company might wish to carry out tests at a higher humidity). Conditions are recorded throughout the test. Arenas can be kept at normal periods of light and dark with seasonally adjustment to the length of the photoperiod; likewise a standardized photoperiod can be chosen with a minimum of 8 hours darkness each day. While the influence of the photoperiod, or darkness, onto the test organisms has not been elaborated upon in great depth, testing in complete darkness is sometimes carried out so as to mimic conditions in sewers and/or underground environments.

25. Water should be supplied *ad libitum* and should be changed regularly.

26. A minimum of three replicate groups are dosed with each test concentration. The numbers of test individuals per replicate group and dose level (treatment group) as well as the number of replicates in the entire study need to be established prior to conducting the tests. As the improvement in power wears off substantially as the number of replicates increases beyond five, it is usually sufficient to conduct three, four or five replicate tests at each dose level**.** The precise number will depend on the size of the variances between and within the replicates. A power analysis should be carried prior to conducting tests in order to determine adequate sample sizes and number of replicates required. Examples are displayed in Table 1.

27. Refer to paragraph 16 for food used in the choice and/or control tests Food must be free of any insecticide. When investigating specific issues, food could be used that is akin to the test bait formulation.

28. Egg cases deposited during the test and/or gravid females are transferred to another container and kept there at least for seven days (*Blattella, Supella*) or ten weeks (*Blatta, Periplaneta*) for products for which relevant claims are made. Oothecae and gravid females are collected and kept separately during day one and two, and combined for all subsequent days. This allows investigating any ovicidal effects of the bait and/or its potential effects on eclosion. Note: The number of gravid females removed should be taken into account in the statistical analysis of results.

Use of reference products

29. At least two negative (not treated) controls should be included, that are of the same strain and batch as the test insects. Since negative controls are employed to confirm that the test is not observing an unrelated effect, some groups favor provision of negative controls that should equal the bait as close as possible except for not containing the active ingredient. However, others prefer using the regular feed as negative control and this is as well acceptable.

30. A reference product ('*positive control*') should be included in each trial (at least two replicates). This should preferably be a product registered for the intended use and with the same mode of action according to the IRAC scheme (IRAC 2010), since at the moment, no internationally agreed standard reference products are available for this purpose in the EU (Technical Notes for Guidance 2010).

Effects

31. Cockroaches in permanent supine position and permanent not responding by movements of antennae or extremities upon prodding are classified as dead. Insects in supine position and those on ventral position without ability to move forward and exhibiting uncoordinated or sluggish movements of legs are classified as moribund. Moribund test organisms are counted as dead, if they die within the test duration (21 days). If moribund animals did not die until day 21, a post treatment observation period of one week is advised to ensure if these animals can recover or not. During this period the animals are offered standard food and the bait is removed. See Annex for a sample reporting form.

32. Mortality is counted at day 1, 2 and at 24 h intervals up to 21 days and is recorded separately for gender, developmental stage and location where dead and moribund bodies were found. In case of knock-down, the potential for recovery should be assessed. Dead cockroaches are removed from the arenas unless a reason exists not to do so, for example if secondary transfer of toxicity to the colony mates is under investigation (Flynn 1966, Buczkowski 2001, Buzkowski 2008). To cater for week-ends, it seems useful to recommend starting on a Friday with the baits placed the following Monday. This would allow reading results easily on day 1,2,3,4, and 7, etc.

33. If control mortality exceeds five percent in any specific developmental stage or gender, mortalities are corrected by control mortality using Abbott's formula (Abbott 1925):

$$\text{Efficacy} = 100 * (t-c) / (100\ c)$$

where: t = % treated mortality
c = % control mortality

34. Time-mortality responses can be analysed by adequate statistical analyses. Historically these had been probit analyses that yield lethal time values, LT_{50} and LT_{95} (Finney 1971). However, non-parametric analysis will allow the characterization of data without assuming an underlying normal distribution. Non-parametric analysis may also be useful in determining how long an application of a fixed amount of bait will last; this analysis supports the collection of bait consumption data as described in paragraph 35. Therefore, it has been recently switched to performing non-parametric tests, such as failure analysis, *e.g.* Weibull analysis or event analysis, *e.g.* Kaplan-Meier survival analysis. A significance level of $p = 0.05$ (two-sided testing) is usually considered sufficient. Further advices can be obtained from an OECD Guidance Document (OECD 2006). While 95% performance standards are usually considered in Europe (European Commission 2012), the US EPA proposes to use a 90% performance standard.

35. Bait consumption is calculated as follows:

$$BC = (MTSB - MTSE) * MCSE / MCSB$$

where: BC = bait consumption (in mg)

MTSB = mass of test substance at baseline (in mg)
MTSE = mass of test substance at test end (in mg)
MCSE = control substance mass at test end (in mg)
MCSB = mass of control substance at baseline (in mg)

Efficacy evaluation

36. Efficacy is usually considered sufficient if – at the end of the test (see Table 2 in Annex 2) – 95% mortality rate, corrected according to Abbott in a 'no-choice-test', and 90% in a laboratory 'choice-test' is achieved.

Validity of the test

37. For a test to be valid, the following conditions apply:

- Average mortality in (negative) controls must not exceed 10% at the end of the test.
- Mortality rate in one (negative) control must not exceed 15% at the end of the test.

Test report

38. The test report should contain the following information:

Test Substance/Product**:** chemical identification data (CAS number of active ingredients and all formulants and their concentrations if relevant), batch number, physical nature and physicochemical properties

Test Conditions**:** Test design: number and description of test arenas, number of test insects per replicate, number of replicates, temperature and humidity recordings.

Test Organisms: Scientific name, strain, stage, age, health status, source, method of rearing and handling including specification of feed used day of preparation.

Disease Symptoms in Test Organisms**:** abdominal swelling, changes in body colour, odour, mobility and/or behaviour.

Data Management**:** Results should be summarised in tabular form, showing for each treatment and control group, the number of cockroaches used, mortality observed at each observation time and number of cockroaches with adverse behaviour. Data management procedures and statistical analysis are recorded.

Deviations: Any deviation is recorded and mentioned in the report. Deviations are possible, but should be justified.

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

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ANNEX 1

Abbreviations

a. i.	active ingredient
BC	bait consumption
d	day/s
FP	food placement area
h	hour/s
HU	hiding unit
IGR	Insect Growth Regulator
Imp	moribund test organisms
LT_{50}, $LT_{95,}$	lethal times: the time at which 50% and 95% of test organisms are dead respectively
MCSB	mass of control substance at baseline
MCSE	mass of control substance at the end of the test
MTSB	mass of test substance at baseline
MTSE	mass of test substance at the end of the test

Glossary

Active ingredient	The insecticide component of a formulated product
Eclosion	The emergence of a nymph or adult from a previous developmental stage, for example of an adult from the pupa case
Health Status	Populations will be checked for clinical symptoms of diseases and/or parasites like *Gregarina spec*., *Serratia spec*, *Moniliformis spec*., *Metarhizium spec.,* etc.
Moribund	approaching death
Mortality	the state of a test organism in which all vital functions have ceased
Ootheca	Egg case, the structure in which female cockroaches place their eggs and encase them with a hard covering.

ANNEX 2

Table 1:

Recommended size of test populations per arena (dual chamber box) for different species

Species	Adults*		Adults and late nymphs**			Early nymphs***
	♂	♀	nymphs	♂	♀	nymphs
Blattella spp., Supella spp.	25	25	40	20	20	100
Blatta spp.	25	25	30	20	20	80
Periplaneta spp.	25	25	30	15	15	50

* Tests with adults only (first two columns)

** Test with adults and late nymphs

*** Tests with early nymphs only

Table 2:

Recommended duration of tests, developmental stages and variables investigated when testing efficacy of cockroach bait formulations

Test Substance	Life Stages	Minimum Test Duration (weeks)	Variables/Endpoints
Classical insecticides	Adults, late nymphs	3	Knock down, recovery, mortality
Juvenoids	Early nymphs, gravid females (*e.g.* when testing *B. germanica*)	8	Duration of development; deformities, sterility, mortality
Chitin synthesis inhibitors	medium nymphal stages	6-8	Deformities, behavioural anomalies, mortality

Figure 1. Arenas for testing cockroach baits: dual chamber box with rectangular units, side view

Figure 2 Arenas for testing cockroach baits: harbourage unit of dual chamber box, top view

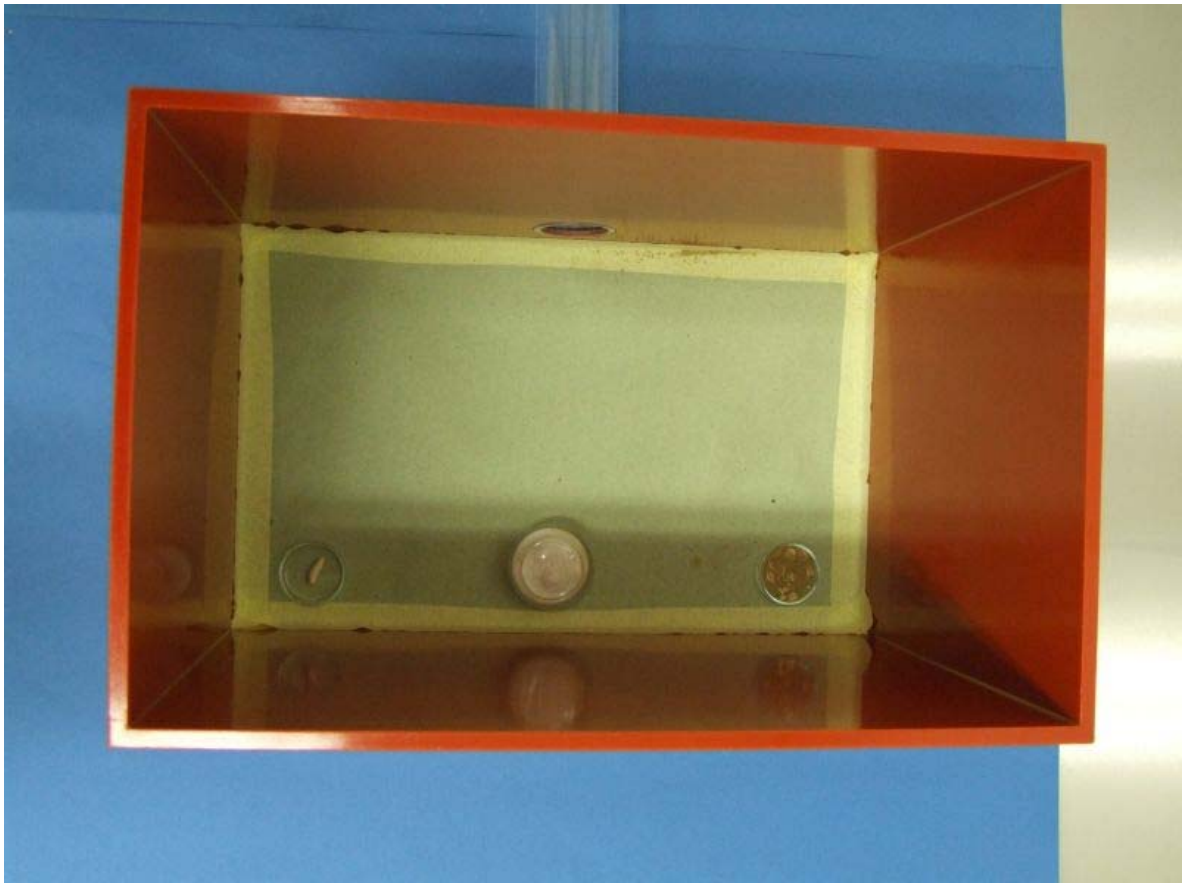

Figure 3 Arenas for testing cockroach baits: feeding unit of dual chamber box, top view.

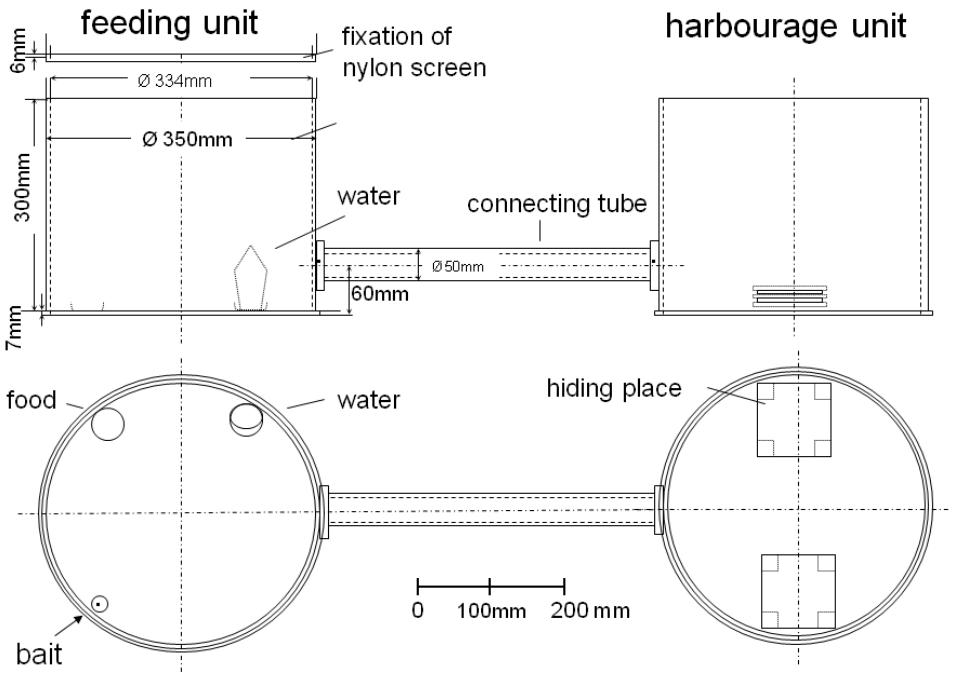

Fig.4. Arenas for testing cockroach baits: schematic representation of circular arenas, side and top views

ANNEX 3

Example of a reporting form for the testing of bait substance/product
(for use in dual chamber boxes)

Test Design: ……..…. (Choice/No-Choice Test)

Test Item: …….. Date / Time: ……..…..……

Roach Species: …… Start of Habituation Date/Time: ....….……..

Number of Test Organisms: ……...…m; ……..… fem

Test Arena No.													
Name/Date /Time	Control Time	**Harbourage Chamber**				**Feeding Chamber**				∑ **m + dead**	Deads rremoved removed	Oothecas removed	Remarks
		Outside HU*		Inside HU		Outside FP*		Inside FP					
		mb	**vital**	**mb**	**vital**	**mb**	**vital**	**mb**	**vital**				
	H 0 d												
	H 3 d												
Placement of Test Organisms into Arenas													
	1 h												
	3 h												
	6 h												
	24 h												
	48 h												
	72 h												
	96 h												
	7 d												
	8 d												
	9 d												
	10 d												
	11 d												
	14 d												
	15 d												
	16 d												
	17 d												
	18 d												
	21 d												
	End												

***** Legend: HU = Hiding Unit; FP Food Placement Area; mb = number moribund test organisms, vital: number of organisms that are not moribund