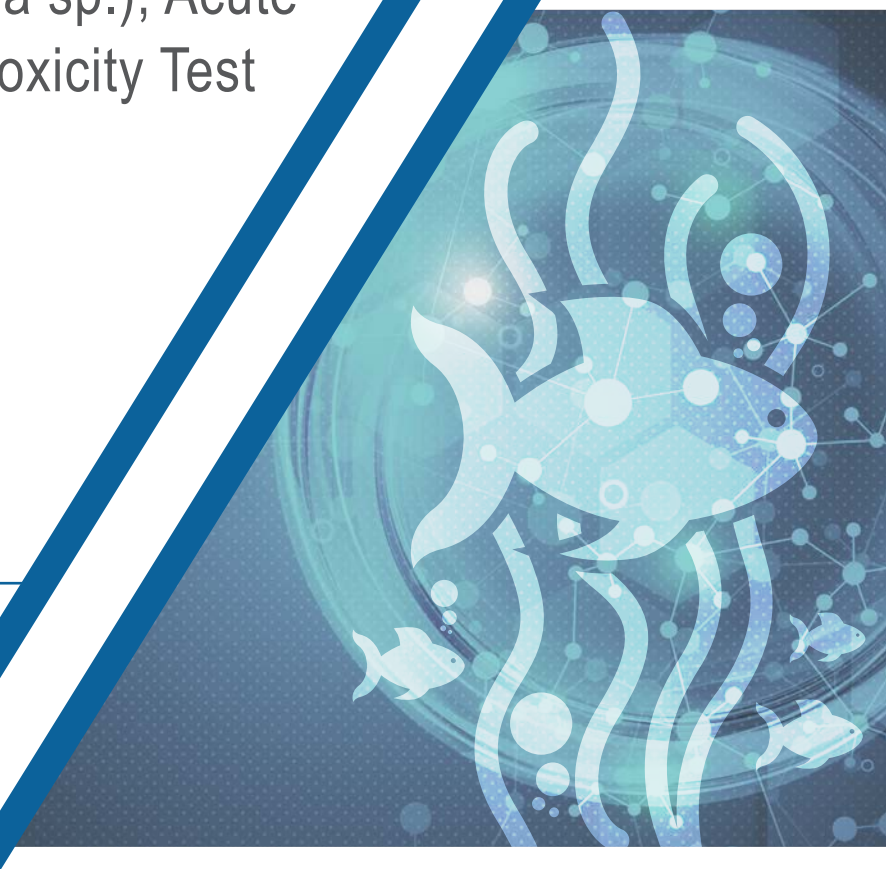
Section 2
Effects on Biotic Systems

Test Guideline No. 254

Mason bees (Osmia sp.), Acute Contact Toxicity Test



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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

MASON BEES (OSMIA sp.) ACUTE CONTACT TOXICITY TEST

INTRODUCTION

1. This test guideline describes a laboratory test method, designed to assess the acute contact toxicity of test chemicals to adult solitary bees. It is based principally on the OECD guidelines for the testing of chemicals 214 [1] and 246 [2]. The method was ring tested by the ICPPR (International Commission for Plant-Pollinator Relationships) non-Apis testing working group in 2014 to 2016. The following two species were selected and successfully used: *Osmia bicornis* and *Osmia cornuta*. The test may be applicable to other solitary bee species, although other species have not been assessed using this protocol therefore modifications may be required.

2. Pollinators, such as solitary bees, may be exposed to residues of pesticides and other chemicals either via contact (directly or via indirect transfer) or consumption of residue-containing food. To address the potential contact toxicity of a test chemical, an acute contact study can be conducted in the laboratory by exposing adult solitary bees to the respective test chemical.

INITIAL CONSIDERATIONS

3. In the assessment and evaluation of toxic characteristics of chemicals, determination of acute contact toxicity in solitary bees may be required (*e.g.*, when exposure of these bees to a given chemical is likely). The acute contact toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals. The results of this test could be used to define the need for further evaluation. In particular, this method can be used in stepwise programs for evaluating the hazards of test chemicals to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments. Test chemicals can be tested as either active ingredients (a.i.) or as formulated products.

4. The method aims to determine the LD50 (see Annex I for definitions) following a single exposure of adult female solitary bees to a test chemical. This test guideline on solitary bees should be seen as a lower tier test in the context of an overall risk assessment scheme for pollinators.

PRINCIPLE OF THE TEST

5. Adult female solitary bees are exposed to a range of doses of the test chemical dissolved in an appropriate carrier (an organic solvent or a water solution with a wetting agent), by direct application to the dorsal side of thorax (1µL aliquots, in case of low solubility up to 3 µL). The test duration is 48 h (or up to 96 h, if ongoing mortality makes prolongation necessary) and bees are fed *ad libitum* with sugar solution throughout the test. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the LD_{50} at 48 h, as well as at 72 h and 96 h if the duration of the study is extended. Sublethal effects, if any, should also be recorded.

VALIDITY OF THE TEST

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

- The average mortality across replicates for the untreated control and solvent control groups should be ≤ 15% at the end of the test.
- The average mortality in the toxic reference substance treated group should be ≥ 50% after 48 hours.

DESCRIPTION OF THE METHOD

Test organism

7. The contact acute toxicity test is conducted using recently hatched adult female solitary bees (see Annex II). Bees used for a test should come from the same supplier or from a defined geographic region with relatively uniform climatic conditions. The origin, approximate month of the previous year when the cocoons were produced and the wintering conditions (e.g. collection process and storage conditions) of the cocoons of the bees used in the test should be specified in the raw data. Any cocoons showing signs of parasitism, as well as hatched bees showing any abnormalities should be excluded from the test.

Preparation and selection of bees

8. Newly emerged healthy female bees are selected for the test (see Annex II). Cocoons containing females are generally of larger size than those containing males. Do not manipulate the cocoons to facilitate hatching and/or sexing of the bees (i.e., do not open or cut prior to hatching). When hatching proper sized cocoons, any males still present will emerge earlier than the females and should be removed from the cage. A food source (50% (w/v) aqueous sugar solution) should be provided in the hatching cage. Emerged females can be stored in the refrigerator at 4±3º C until enough bees have been collected to populate the test. See Annex II for recommendations.

9. To avoid great variation in susceptibility within a test, solitary bees of a similar size should be selected. The effect of test chemicals on solitary bees depends on their body size. As solitary bee females can vary significantly in size and related weight within a source population and especially between different source populations, they can have a different surface to volume ratio. This affects the susceptibility of these individuals to test chemicals as smaller bees have a greater surface to volume ratio and have less weight [3] [4]. Selection will be performed visually, and the bees required for the test will be picked manually from the emerged population, discarding the very small and very big bees. After selection, these bees will be distributed randomly over the test replicates. For practical reasons, bee weight is used as proxy for size and the surface-to-volume ratio. All selected bees are weighed individually during or immediately after application of the test chemical to determine their weight, which will result into an average weight, standard deviation and min-max weight of animals used in the test. In this way the LD_{50} can be calculated as µg test chemical per bee and µg test chemical per gram bee, which will make the evaluation of the LD_{50} for solitary bees more consistent.

10. Note that to acquire enough bees to populate a test, bees hatched on different days need to be pooled. After hatching, bees can be stored in the fridge and once a sufficient number of bees have been collected, they are sorted into visually homogenous groups that will be used for the different treatment groups. Care should be taken to avoid very small and very large bees or unhealthy bees. Since hatched bees are stored in the fridge, they are already anesthetized through chilling. One treatment group is processed outside of the fridge at a time, meaning that each bee is weighed, and the test chemical is applied. Subsequently, the treated bees are placed in the test cages in groups of 5 to 10 individuals. Note that using CO_2 for anesthesia can result in increased mortality for *Osmia* species and should therefore be avoided.

Handling and feeding

11. For all treatment groups, feeding solutions are prepared by dissolving sucrose in water with a final concentration of 50% (w/v), (*e.g.*, 500 g sucrose/L). The feeding solution should be provided *ad libitum*. Feeding solutions are offered to the solitary bees using an appropriate feeder as described in Annex II.

Test cages

12. Easy to clean and well-ventilated cages should be used. Any appropriate material can be used (*e.g.,* stainless steel, wire mesh, plastic, disposable plastic cages); however, the cage material should not adsorb or affect the exposure levels of the test chemical and be free of previous contamination. The size of test cages should be appropriate to the number of bees (*i.e.*, providing adequate space and feeding opportunity such that all individuals have access to the sugar solution). Provide cage enrichment like a piece of gauze, (filter) paper or a small wire mesh, since *Osmia* bees need suitable structure and hiding places. This reduces stress for the test organisms as well as the tendency to form congregations of individuals. See Annex II for details.

Analytical verification

13. Directly after preparing the test solutions, at least one aliquot of the lowest and highest test concentrations and the control should be collected and stored in a freezer at or below −18 °C for analytical verification using a validated analytical method. If a stock solution has been used for the preparation of test chemical solutions, one additional aliquot of this stock solution should be sampled for analytical determination of the test chemical concentration.

14. If the concentrations of the test chemical are within ± 20% of the nominal, then results can be based on nominal concentrations. If the deviation from the nominal is greater than ± 20%, results should be expressed in terms of the actually measured values. It should be noted that it is often useful to report both measured and nominal effect concentrations (see Guidance Document No. 23 [5].

15. If the Test Guideline is used for the testing of a mixture, a substance of Unknown or Variable composition, Complex reaction products or biological materials (UVCB) or a multi-constituent substance, its composition should, as far as possible, be characterised, e.g. by the chemical identity of its constituents, their quantitative occurrence and their chemical-specific properties. Recommendations about the testing of difficult test chemicals like mixtures, UVCBs or multi-constituent substances are given in Guidance Document No. 23 [5]. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline,

upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

TEST PROCEDURE

Test chemical and control groups

16. The number of doses and replicates tested should meet the statistical requirements for the determination of an LD_{50} with 95% confidence limits. Normally, five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for $LD_{50,}$ are required for the test. Generally, the number of doses and the spacing factor should be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate doses.

17. A test will preferably consist of 8 treatments: Two separate control groups (one water control, in which only water is applied, and one containing the solvent or the wetting agent), at least five treatments with different doses of the test chemical, and a treatment with the reference substance.

18. Thirty (30) solitary bee females will be used per treatment. Generally, replicates consist of 5 to 10 bees housed in groups. If the recommended minimum of three replicates are used per treatment, each replicate should consist of ten bees. More replicates per treatment with fewer bees per replicate may facilitate handling. Replicates can for instance also be executed as 5 replicates of 6 bees or 6 replicates of 5 bees per treatment level.

Reference substance

19. One dose of the reference substance leading to an expected mortality of ≥ 50% after 48 hours should be used as a positive control to demonstrate the sensitivity of the solitary bees and the reliability of the test system. As solitary bees differ more in size/weight than honeybees, a larger variation in LD_{50} values can be observed. For *O. bicornis* and *O. cornuta*, to reach mortalities of >50%, a dose of 2.0 µg dimethoate/bee (corresponding to approximately 0.02 µg dimethoate/g bee) can be used. For other solitary bee species, the LD_{50} may be significantly different. The use of other toxic reference substances would be acceptable if sufficient data can be provided to demonstrate the expected sensitivity of solitary bees.

Preparation of doses

20. The test chemical is applied as a solution in a carrier (either an organic solvent or a water solution containing an appropriate wetting agent). For test chemicals of low water solubility, an organic solvent can be used. In the case of good water solubility of the test chemical, water is used as the solvent and an appropriate wetting agent is added in the same concentration to all treatments. The wetting agent reduces surface tension of the application solution for an equal distribution of the test chemical on the animal, preventing the droplet of test solution from draining off the bee's hairy back (see Annex II, section “Wetting agent”).

21. Appropriate control solutions should be prepared. Two separate control solutions should be used: one water control (handling control, applied with only water) and one solvent/wetting agent control containing either the organic solvent or the wetting agent (if water is used to dissolve the test chemical). The concentration of the solvent or wetting agent should be the same as in the test chemical application solutions.

Administration of doses

22. Anaesthetized (cooled down) bees are individually treated by topical application. The bees are randomly assigned to the different treatment and control groups. A volume of 1 µL (depending on solubility, 2 or 3 µL can also be used) of control solution, reference solution, or application solution containing the test chemical at the suitable dose should be applied with a micro-applicator or pipette to the dorsal side of the thorax of each bee between the two wing bases. After application, the bees are allocated to test cages. Bees should be placed upright to allow maximum time for the test chemical to dry.

Test conditions

23. The bees are kept under 16:8 h light:dark conditions in a climate-controlled room at a temperature of 22±2° C and a relative humidity of 60±20%. During the test, the bees have access to 50% (w/v) aqueous sucrose solution *ad libitum*. If artificial light is chosen, ensure that daylight is mimicked as closely as possible, as the type of light used can influence the likelihood of bees finding feeders.

24. Test conditions comprising light intensity, relative humidity, and temperature should be recorded throughout the test. Light intensity should be recorded at least once at the start of the test, relative humidity can be documented once per day, while temperature should either be recorded continuously using a logger or by using a min-max thermometer that will be documented daily. Note that short-term deviations (≤ 2 h) from the recommended ranges are often unavoidable (e.g., due to handling of the set-ups) and will normally not result in major disturbances of the test performance.

Test duration

25. The test duration is at least 48 h. If the mortality rate increases by >10% between 24 h and 48 h in at least one test chemical treatment whilst control mortality remains at an acceptable level (i.e., <15%), it is appropriate to extend the duration of the test to 72 h, or up to a maximum of 96 h in case mortality increases by >10% between 48 h and 72 h.

Observations

26. Mortality is first recorded at 4 h after test chemical application, as well as after 24 h and 48 h. If prolonged observation is required, further assessments should be made after 72 h and if applicable after 96 h. The results are analysed to calculate the LD_{50}, if possible, at 48 h, as well as at 72 h and 96 h if the test is prolonged.

27. Additionally, the number of bees displaying sublethal effects and any other observations should be recorded daily at the same time as mortality assessments. Sublethal effects should be recorded as follows:

- **unaffected** = bees do not exhibit abnormal behavior (including natural occurring phases of inactivity).
- **affected** = bees displaying signs of reduced coordination, cramping, increased grooming or other signs of intoxication (symptoms should be specified in the report).
- **moribund** = bees are unable to walk and show only very feeble movements of legs and antennae; only weak response to stimulation (*e.g.*, light or blowing); may recover but usually die.

LIMIT TEST

28. In some cases (e.g., when a test chemical is expected to be of low toxicity) it may be appropriate to conduct a limit test, using a single test dose (*e.g.*, 100 µg a.i. or test chemical per bee or at the limit of solubility) to demonstrate that the LD_{50} is greater than this value. The above-described test procedure should be used (including relevant controls, and the use of the toxic reference substance), but instead of using 30 bees per treatment group, 50 bees (caged in replicate groups of 5, 6 or 10) are used for the test chemical treatment and control groups. For the toxic reference substance at least 30 bees are used. If a statistically significant mortality occurs in the test chemical group compared to the control group, a full dose-response study should be conducted. If sublethal effects are observed, these should be recorded as described above.

DATA AND REPORTING

Data

29. Data should be summarized in tabular form, showing the number of bees used, mortality and sublethal effects (*e.g.*, adverse behavior) at each observation time in the treatment, control and toxic standard groups.

30. The water control and solvent/wetting agent control are tested for statistically significant differences. If there is no statistically significant difference, both controls may be pooled for further statistical evaluations. In the case of a statistical difference, the solvent/wetting agent control is used for LD_{50} calculation and for possible mortality corrections of the test chemical treatments. Corrections for control mortality using the Abbott formula [6] should be used with caution [7] and only when scientifically justified.

31. The mortality data should be analyzed by appropriate statistical methods (*e.g.*, Probit analysis [8], moving-average, binomial probability). A dose-response curve should be plotted and the slopes of the curves and the median lethal doses (LD_{50}) with 95% confidence limits calculated. LD_{50} should be expressed in µg of test chemical per bee, and additionally as µg test chemical per gram bee.

Test report

32. The test report should include the following information:

Test chemical and reference substance:

- source, batch and/or lot number, if available
- solubility of the test chemical in water or solvent, if available
- physical appearance and additional relevant physicochemical properties
- chemical identification, such as chemical substance name, IUPAC or CAS number and structural formula
- For products containing a single active ingredient: Purity and chemical identity of impurities as appropriate and practically feasible (including the organic carbon content, if appropriate)
- For products containing UVCBs (substances of Unknown or Variable composition, Complex reaction products or biological materials) and mixtures: characterized as far as possible by chemical identity, quantitative occurrence and relevant physico-chemical properties of the constituents

Test system:

- scientific name of the species tested, information on hatching process and conditions and holding conditions until the start of the test
- all relevant information on origin of cocoons and diseases noticed during hatching
- weight of each bee used in the test

Test conditions:

- description of the test design: number of treatment groups (including controls and reference substances); number of replicate cages and number of bees per cage; tested doses of the test chemical
- housing conditions including type, size, material of cages, feeding devices
- temperature and relative humidity of experimental room, light source, light regime/conditions
- methods of administration of test chemical used (*e.g.*, solvent, wetting agent, volume of test solution applied)
- method of anesthesia
- place and dates of the test

Results:

- results of preliminary range-finding study if performed
- raw data: mortality and sublethal effects in a quantitative way for each bee at each dose tested at each observation time (i.e., number of bees with each effect per timepoint per replicate/cage).
- graph of the dose-response curves at the end of the test, if available
- LD_{50} values, with 95% confidence limits
- statistical procedures used for determining LD_{50}
- any other biological effects observed and any abnormal responses of the bees
- nominal test concentrations used and measured concentrations of the test chemical in the treatment solutions, and details on analytical method used
- any deviation from the test guideline procedures and any other relevant information.

LITERATURE

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ANNEX I

DEFINITIONS

Acute contact toxicity: The adverse effects occurring within a maximum observation period of 96 h after a topical application of a single dose of a test chemical.

Dose: The amount of test chemical applied. Dose is expressed as mass (µg) of test chemical per test animal (µg/bee).

LD_x (lethal dose) contact: Statistically derived single dose of a test chemical that can cause death in X% of animals when administered by contact. The LD_x value is given in µg of test chemical per bee (and per gram bee). The test chemical may be either an active ingredient (a.i.) or a formulated product containing one or more than one active ingredient(s). The test duration time for which the LD_x is calculated (48 h, 72 h or 96h) is noted (i.e., 48h-LD_{50} would be the dose that causes death to 50% of animals 48 hours after exposure to the test chemical).

ANNEX II

GENERAL RECOMMENDATIONS OF THE RING TEST GROUP:

Hatching

Bees should be obtained from cocoons harvested the previous year. Hatching is temperature dependent. Cocoons should be overwintered in relatively stable, cool conditions (well-ventilated boxes with cocoons not piled too high, placed next to an open container of water in a refrigerator set at approximately 0-4°C, for instance, is appropriate).

Hatching should be synchronized within the actual flight period of the population being tested. Studies conducted outside of this timeframe result in higher mortality rates and, consequently, inaccurate toxicity outcomes. In the northern hemisphere hatching is normally possible from late February to August. However, due to the short natural flight period of *Osmia* bees, best results are achieved with bees that have hatched between April and early June. Very early in the season, the hatching period is prolonged, and it will take a few days to hatch the number of bees required for one trial. It is recommended to take the "middle hatch" fraction of the bees (hatched over as few days as possible) and it is not recommended to use bees that are older than 4-5 days at test start. Using bees that have been in the refrigerator for longer than 5 days or doing the test after July may result in high control mortalities.

To find the optimal hatching period for the respective batch of bees, a preliminary test can be done by hatching a few cocoons from the population that is to be used for the test. Males (from small cocoons) should start emerging within 1-2 days following exposure to 22-23°C and the peak of emergence in females should not exceed 10 days. Conversely, if many males have already emerged within the wintering chamber or females emerge just a few hours after incubation, the population has already exceeded its optimal emergence period.

Males/Females/Mating

Although female bees are recommended for use in the test, the protocol seems to be just as applicable to male bees. However, only tests performed on females were considered for the test development as females are bigger and live longer than males and as females are ecologically more relevant (reproduction, collection of pollen).

Originally, strictly unmated female bees were used for the test. However, as some males hatch late (when females are present already), it is hard to ensure that the bees are unmated. Additionally, during the course of the ring tests, it was observed that mating seems to have no influence on the test results.

Photo: Bettina Wenzel

Figure 1. Male and female Osmia bicornis.

Sexual dimorphism is clear for many solitary bee species. Here is an example of a male (left) and a female (right) *Osmia bicornis* bee. Besides being smaller, male bees have white markings on their face while the females face is completely black.

Wetting agent

A wetting agent is any surfactant, solubiliser, dispersant, or similar and is needed to ensure an equal distribution of the droplet applied to the thorax, as a droplet of water without a wetting agent will just drain off the hairy thorax. In the international ICPPR ring test, Triton X-100 (at a concentration of 0.1%) was the wetting agent of choice. If shown to be non-toxic to the bees (as shown by the solvent/wetting agent control group), other wetting agents reducing surface tension can be used as well (e.g. Etalfix, Agral, Citowett, Lubrol), or any other appropriate wetting agent, normally in concentrations of no more than 1.0%. Note that Tween 80 at a concentration of 0.5% is not recommended as it did not sufficiently reduce the surface tension and therefore caused an improper exposure of the bees during the ring test. In any case, it is important that the drop actually disperses on the thorax immediately after application, so that exposure can be guaranteed.

Organic solvents

Organic solvents of low toxicity to bees (as shown by the solvent control group) may be used for test chemicals that are not soluble in water. When the test chemical is dissolved in an organic solvent, normally no wetting agent is required. The concentration of solvent used depends on the solubility of the test chemical (can be up to 100%) and should be the same for all test chemical treatment levels and the solvent control. Any solvent can be used as long as the validity criterion of the solvent control group is met. However, even with low solubility, more than 3 µL solvent/bee should not be used. Note that the frequently used organic solvent acetone may result in tremors when applied at high volumes (i.e., 3 µL/bee).

Test cages and feeding devices

Some examples of test cages are shown in Figure 2. Different test cages and feeders were used during the ring test. Feeding the bees was a problem in some cases, as the bees did not readily feed on the provided food, causing unacceptable mortality. Some points that seemed to help with feeding were:

- Feeders positioned on the ground appear to work better than suspended feeders
- Feeders containing a reservoir with some kind of wick or cotton from which the solitary bees feed give good results. Similarly, a feeder with an opening large enough so that the food is not “hidden” gave better results. However, if the food is provided in a large open cup, the bees might just bathe in the food instead of feeding on it.
- Using yellow-, blue- or purple-coloured feeders
- Using bigger cages with multiple feeders or using less bees per cage but increasing the number of cages per treatment level. In principle, however, groups of either 5, 6 or 10 bees per cage have been tested and proven to work.
- Ideally, the same type of feeder used in the test is included in the hatching unit so that the bees can start getting used to it.

a)

b)

c)

d)

Figure 2. Some examples of test cages with feeders positioned on the ground. a) 'Open access' feeder cup in the corner. b) 'Open access' feeder cup (Nicot system) with refill construction (stopper) in test cage lid. c) Feeder tube with wick, note that refilling requires full replacement as wick will solidify due to sugar. d) Example of large feeder in corner allowing multiple bees feeding simultaneously.

Recent publications suggesting different feeding methods may also help to solve problems due to difficulties feeding the bees. For example, it is suggested to use feeders equipped with a flower petal (in which case the flower petal needs to be either artificial or completely free of any residues; [9]), or providing food via syringes with the syringe tip removed [10].