9.1. **BACTERIAL LARVICIDE TECHNICAL CONCENTRATES (TK)**

Note for preparation of draft specifications. Do not omit clauses or insert additional clauses, nor insert limits that are more lax than those than given in the guidelines, without providing justification. From the “Notes” provided at the end of this guideline, incorporate only those which are applicable to the particular specification.

**…… [*Genus, species, subspecies* and strain of bacterium] TECHNICAL CONCENTRATE**

[CIPAC number]/TK (month & year of publication)

9.1.1 **Description** (Note 1)

The material shall consist of …… [*Genus, species, subspecies* and strain of bacterium] together with related by-products of the route of manufacture and shall be in the form of [physical description], free from visible extraneous matter and added modifying agents, except for stabilizers (preservatives) and free-flow agents (Note 2), if required.

9.1.2 **Active Ingredient** (Note 3)

9.1.2.1 **Identity**

The active ingredient shall comply with an identity test and, where the identity remains in doubt, shall comply with at least one additional test.

9.1.2.2 **Active ingredient content (biopotency)**

The …… [*Genus, species, subspecies* and strain of bacterium] content shall be declared in International Toxic Units (ITU/mg product), and when determined by the method described in Note 4, the average biopotency shall not be less than 90% of the declared minimum content.

###### 9.1.3 **Relevant impurities and contaminants**

9.1.3.1 **Microbial contaminants and impurities**

 (Note 5.)

9.1.3.2 **Chemical impurities**

The material shall be free from beta-exotoxin when tested with the fly larvae toxicity test (Notes 6 and 7) or an equivalent HPLC method.

9.1.3.3 **Water** (WHO test method M7R1)

Maximum … g/kg (Note 8).

9.1.4. **Physical properties**

9.1.4.1  **pH range** (CIPAC MT 75.3), if required

pH range … to …

# 9.1.5 Storage stability

# 9.1.5.1 Stability at elevated temperature

# (Method to be developed – Note 9.)

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Note 1 The technical concentrate is the axenic (“pure”) single organism, with all relevant biological components associated with it, e.g., toxins, cellular parts and spores. The description must include information on any genetic modifications of the strain used.

Note 2 A free-flow agent may be required to minimise static electricity and the agglomeration of particles.

Note 3 Information must be provided on the source and identification characteristics of reference material obtainable from an internationally recognised institution.

Note 4 Determination of the biopotency (toxicity) of *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* products.

 ***Principles***

Biopotency is tested by comparing mosquito larval mortality produced by the product under test with the mortality produced by the corresponding reference standard. Biopotency is measured in International Toxic Units (ITU) per mg of product.

 Presently, there are two internationally recognized reference powders that allow determination of biopotency using bioassays of bacterial preparations to mosquito larvae, when used in conjunction with the methods described below.

 The biopotency of products based on *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) is compared against a lyophilized reference powder (IPS82, strain 1884) of this bacterial species, using early fourth-instar larvae of *Aedes aegypti* (strain Bora Bora). The toxicity of IPS82 has an arbitrarily assigned toxicity of 15,000 ITU/mg powder against this insect strain.

 The biopotency of products based on *Bacillus sphaericus* (*Bsph*) is determined against a lyophilized reference powder (SPH88, strain 2362) of this bacterial species using early fourth-instar larvae of *Culex pipiens* *pipiens* (strain Montpellier). The toxicity of SPH88 has an arbitrarily assigned toxicity of 1,700 ITU/mg of powder against this insect strain.

 The toxicity of all bacterial preparations based on *Bti* or *Bsph* can be determined against the above standard powders. The toxicity (ITU/mg) of products tested is determined according to the following formula:

 titre (ITU/mg) of product tested = titre standard (ITU/mg) x LC50 (mg/l) standard LC50 (mg/l) unknown "X"

 The use of alternative bacterial larvicide reference powders and/or alternative strains of mosquitoes in this test must be approached cautiously, because it is inevitable that different results will be obtained with them. Such alternatives must be the subject of careful cross-calibration against the reference powders and/or strains identified above. Ideally, such cross-calibration should be conducted by a group of independent expert laboratories. The alternative powders/strains, and the cross-calibration data which support them, should be made available to anyone who wishes to use, or check, the test with the alternative powders/strains.

 ***Method***

 ***Apparatus and reagents***

Top-drive homogenizer or stirrer

Ice bath (container of crushed ice)

 Analytical balance (accurate to ± 0.1 mg)

 Top-pan balance (accurate to ± 10 mg), preferably with tare facility

 Deionised water

 Wetting agent (e.g. Tween 80)

 200 ml borosilicate glass or plastic beakers

 500 ml wide-necked, screw-capped, clear glass bottle

 100 ml screw-capped clear glass bottles

 Micropipette

 10 ml pipette

 12 ml plastic tubes with stoppers or caps

200 ml plastic or wax-coated paper cups

***(i) Preparation of reference standard suspensions for calibration of the bioassay***

 Before preparing the suspension, check that stirring/blending of the wetting agent/water mixture, described in the following paragraph, does not lead to foaming. If it does, dilute (e.g. 1:10) the wetting agent before use.

Accurately weigh about 50 mg (to the nearest 0.1 mg) of the reference standard powder and transfer it to a 200 ml beaker with 100 ml deionised water (it can be transferred directly to the 500 ml bottle if the neck is wide enough to accept the stirrer/blender head). Allow the mixture to stand for 30 min and add a small drop (about 0.2 mg) of wetting agent. Place the beaker in the ice bath and either stir or blend the mixture for 2 min. Check visually for any large particulates remaining and repeat the stirring/blending if there are any. Weigh or tare the 500 ml bottle and transfer the suspension/solution to it, rinsing carefully and thoroughly the beaker and stirrer/blender. Add further deionised water to make the weight of contents to 500 g (500 ml), cap the bottle and shake vigorously to mix the contents. Confirm, by microscopic examination of a small aliquot, that no aggregates of spores and crystals persist. If any are present, the contents must be subjected to further stirring/blending in the ice bath. This primary suspension/solution contains 1 mg/10 ml and must be shaken vigorously immediately before removing aliquots.

 Transfer 10 ml aliquots of the primary solution/suspension to clean 12 ml tubes that are stoppered/capped immediately. If transferring a number of aliquots, cap and shake the primary suspension/solution at intervals not exceeding 3 min, because the spores and crystals sediment quickly in water. The aliquots can be stored for a month at 4 °C and for 2 years in a freezer at ‑18 °C. Each contains 1 mg standard powder.

To prepare a “stock solution”, weigh or tare a 100 ml bottle. Transfer one of the 10 ml aliquots into the 100 ml bottle, rinsing carefully at least twice with deionised water, and fill to a total of 100 g. Shake the mixture vigorously (or use the blender) to produce a homogeneous suspension. Frozen aliquots must be homogenised thoroughly before use, because particles agglomerate during freezing. The “stock solution” contains 10 mg/l.

 From the “stock solution”, subsequent dilutions are prepared directly in plastic cups filled (by weighing) with 150 ml de-ionized water. To each cup, 25 early L4 larvae of *Aedes aegypti* or *Culex pipiens* (depending on the bacterial species to be tested: *Aedes* for *Bti* and *Culex* larvae for *B. sphaericus*) are added first by means of a Pasteur pipette, prior to addition of bacterial suspensions. The volume of water added with the larvae is removed from the cup (by weighing) and discarded, to avoid changing of the volume in the cup. Using micropipettes, 600 µl, 450 µl, 300 µl, 150 µl, 120 µl and 75 µl of “stock solution” are added to separate cups and the solutions mixed to produce final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/l, respectively, of the reference standard powder. Four replicate cups are used for each concentration and one for the control, which contains only 150 ml de-ionized water.

 ***(ii) Preparation of suspensions of the product to be tested***

 For bioassay of preparations of dry products (TK, WP, WG, WT) of unknown toxicity, an initial homogenate is made in the same manner as described for the reference standard powder, above, except that the replicate determinations must be made on dilutions prepared by weighing separate test portions of the product. That is four replicate primary suspension/solutions must be prepared. For assay of a liquid formulation (SC), after suitable agitation, 100 mg is weighed instead of 50 mg (the “stock solution” then corresponding to 20 mg/l). Cups and larvae are prepared as described above and comparable dilutions are prepared as for the reference standard.

 For products of unknown toxicity, perform range-finding bioassays, using a wide range of concentrations of the product under test, to determine its approximate toxicity. The results are then used to determine a narrower range of concentrations for a more precise bioassay.

 ***(iii) Determination of toxicity***

 No food is added for *Aedes* larvae. For the *Culex* bioassay, finely ground yeast extract (1.5 mg) is added to the water and mixed to produce a concentration of 10 mg/l. All tests should be conducted at 28 + 2 °C, with a 12-h light/12-h dark cycle. To avoid the adverse effects of evaporation of water in low humidity, the relative humidity should be maintained at 50 ± 15%, if possible.

 Each bioassay series should preferably involve 6 concentrations x 4 replicates x 25 larvae for the reference standard and the unknown and 100 larvae for the control. The aim is to identify a range of concentrations that give mortality between 5 to 95% (because 100 larvae are used). Data giving 0 or 100% mortality are ignored for the calculation of the LC50. To prepare a valid dose-response curve, only concentrations giving values between 95% and 5% mortality should be used. A minimum of two dilution points must be above the LC50 and two below, to ensure the validity of the value. The sensitivity of the insect colony may require a slightly different 6 dilution series to be used.

 Mortality is determined at 24 and 48 h by counting the live larvae remaining. If pupation occurs, the pupae should be removed and their numbers excluded from the calculations. If more than 5% of larvae pupate, the test is invalidated because larvae do not ingest 24 h before pupation and too many larvae may have survived simply because they were too old. Because of the very rapid killing action of *Bti,* usually there is no difference between the 24 and 48 h mortality. In this case, the 48-h count confirms the 24-h reading and provides a check on the possible influence of factors other than *Bti* components. Mortality is recorded at 48 h for *Bsph* preparations, due to its slower rate of action.

 If the control mortality exceeds 5%, the mortalities of treated groups should be corrected according to Abbott’s formula [Abbott, W. S. (1925). A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology*, **18**, 265-267]:

 X – Y

percentage (%) control = ————

 X x 100

where X = % survival in untreated control,

 Y = % survival in treated sample.

Tests with a control mortality greater than 10%, or any pupation greater than 5%, should be discarded. Mortality-concentration regression lines may be drawn on gausso-logarithmic paper but this is rather subjective. It is preferable to use a statistical program, such as SAS, which incorporates a Log Probit Analysis. With such a statistical program, Abbott’s formula is not required because the correction is automatically carried out by the program. The toxicity of an unknown preparation is determined by estimation and comparison of the LC50s of the tested product and reference standard preparations, using the formula described above. The toxicity of *Bti* preparations is defined by the count at 24 h after initiation of the test, whereas the toxicity of *Bsph* is defined by the count after 48 h of larval exposure.

 For increased accuracy, bioassays should be repeated on at least three different days, concurrently with the assay of the reference standard, and the standard deviation of the means calculated. A test series is valid if the relative standard deviation (RSD or coefficient of variation, CV) is less than 25%.

 ***(iv) Production of test larvae***

 L4 larvae are representative of the total sensitivity of the target population and convenient to handle. It is very important to use a homogenous population of early fourth instars, which are obtained within five days of hatching using standardized rearing methods.

 For *Aedes* *aegypti, e*ggs are laid in a cup lined with filter paper and filled one third with deionised water. The paper is dried at room temperature and kept for several months by storing in a sealed plastic bag at room temperature. When larvae are needed, the paper is immersed in de-chlorinated water. To synchronise hatching, add larval feed to the water 24 h prior to adding the eggs. The bacterial growth will deoxygenate the water and this triggers egg hatching. This usually induces the first instars to hatch within 12 h. These larvae are then transferred to a container (25 x 25 x depth cm) containing 2 litres of de-chlorinated water, to obtain a population of 500 to 700 larvae per container. Larval feed may be flakes of protein as used for aquarium fish, or powdered cat biscuit, and the containers are held at 25 + 2 °C. It is important that the amount of food is kept low to avoid strong bacterial growth that kills the larvae. Several feedings with one or two days interval and daily observation of the larvae is optimal. If the water becomes turbid, replace all water by filtering out the larvae and transfer to a clean container with clean water and feed. Five to seven days later a homogenous population of early fourth instars (5 days old and 4 to 5 mm in length) should be obtained.

 For *Culex pipiens pipiens* larvae, it is more difficult to obtain a homogenous population of fourth instars. Firstly, a large number of egg rafts must be laid and collected on the same day. These can be stored at 15-18 °C in order to accumulate more eggs for hatching. The first instars are fragile and thus should not be handled. Development to the second instar usually takes 3-4 days at 25 + 2 °C after the eggs are laid. When ready, second instars are grouped in a tray with 3 L dechlorinated water of 4-6 cm depth, 800 – 1000 larvae per tray. Food (yeast extract and dog or cat biscuits) is provided as needed. Early fourth instars suitable for testing are usually obtained within 7 days, though sometimes 8 or 9 days are required.

Note 5 The maximum acceptable levels of microbial contaminants have not yet been determined.

Note 6 Fly larvae toxicity test: Bond R. P. M., *et al*. The thermostable exotoxin of *Bacillus thuringiensis*. In: Burges H. D. and Hussey N. W., eds. Microbial control of insects and mites. Academic Press, London, 1971.

Note 7 No test is required if the manufacturer has shown that the *Bacillus thuringiensis* strain is not capable of producing beta exotoxin. No test is required for *Bacillus sphaericus*, because this species is not known to produce exotoxins.

Note 8 Generally, the water content should not exceed 5%, to preclude premature degradation of the product.

Note 9 Microbial larvicides should be stored at cool temperatures but accelerated storage stability tests would be most useful for rapid checks on the storage stability of products. At present, no standardised method is available. In the absence of an accelerated storage stability test, it is recommended that the following minimum standards be met:

a) no more than 10% loss in biopotency below the labelled potency value when stored at 5 ºC for 2 years; and

b) no more than 10% loss in biopotency below the labelled potency value when stored at 20 to 25 ºC for 1 year.

 These storage stability tests shall be performed using representative product samples and the biopotency shall be assessed using the test method described in Note 4.

 Results from the biopotency test may vary by up to ± 25% from the average and this must be taken into account in determining the potency loss. If one- and two-year test data are not available at the time of drafting a specification, an estimate of the storage stability may be acceptable, pending completion of the tests.