WHO SPECIFICATIONS AND EVALUATIONS FOR PUBLIC HEALTH PESTICIDES

Bacillus thuringiensis subspecies *israelensis* strain AM65-52
+
Bacillus sphaericus strain ABTS-1743

World Health Organization

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Compliance with the specifications does not constitute an endorsement or warranty of the fitness of a particular pesticide for a particular purpose, including its suitability for the control of any given pest, or its suitability for use in a particular area. Owing to the complexity of the problems involved, the suitability of pesticides for a particular purpose and the content of the labelling instructions must be decided at the national or provincial level.

Furthermore, pesticides which are manufactured to comply with these specifications are not exempted from any safety regulation or other legal or administrative provision applicable to their manufacture, sale, transportation, storage, handling, preparation and/or use.

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INTRODUCTION

WHO establishes and publishes specifications* for technical material and related formulations of public health pesticides with the objective that these specifications may be used to provide an international point of reference against which products can be judged either for regulatory purposes or in commercial dealings.

From 2002, the development of WHO specifications follows the **New Procedure**, described in the Manual for Development and Use of FAO and WHO Specifications for Pesticides. This **New Procedure** follows a formal and transparent evaluation process. It describes the minimum data package, the procedure and evaluation applied by WHO and the experts of the "FAO/WHO Joint Meeting on Pesticide Specifications" (JMPS).

WHO specifications now only apply to products for which the technical materials have been evaluated. Consequently, from the year 2002 onwards the publication of WHO specifications under the **New Procedure** has changed. Every specification consists now of two parts, namely the specifications and the evaluation report(s):

Part One: The Specification of the technical material and the related formulations of the pesticide in accordance with chapters 4 to 9 of the above-mentioned manual.

Part Two: The Evaluation Report(s) of the pesticide, reflecting the evaluation of the data package carried out by WHO and the JMPS. The data are provided by the manufacturer(s) according to the requirements of chapter 3 of the above-mentioned manual and supported by other information sources. The Evaluation Report includes the name(s) of the manufacturer(s) whose technical material has been evaluated. Evaluation reports on specifications developed subsequently to the original set of specifications are added in a chronological order to this report.

WHO specifications under the **New Procedure** do not necessarily apply to nominally similar products of other manufacturer(s), nor to those where the active ingredient is produced by other routes of manufacture. WHO has the possibility to extend the scope of the specifications to similar products but only when the JMPS has been satisfied that the additional products are equivalent to that which formed the basis of the reference specification.

Specifications bear the date (month and year) of publication of the current version. Evaluations bear the date (year) of the meeting at which the recommendations were made by the JMPS.

* Footnote: The publications are available on the Internet under (http://www.who.int/whopes/quality/en/).

PART ONE

SPECIFICATIONS

***Bacillus thuringiensis* subsp. *israelensis* strain AM65-52 + *Bacillus sphaericus* strain ABTS-1743**

***Bacillus thuringiensis* subspecies *israelensis* strain AM65-52**

INFORMATION

Scientific name

Bacillus thuringiensis subsp. *israelensis* strain AM65-52.

Shorthand terms

Bt: all subspecies of *Bacillus thuringiensis*

Bti: all strains of *Bacillus thuringiensis* subspecies *israelensis* (flagella serotype H-14)

Bti AM65-52: the strain to which the CIPAC code 770 applies

CIPAC number

770

Identity tests

Identification is based upon the following tests.

(i) Microscopic examination: gram-positive rods; presence of spores and parasporal crystalline inclusions.
(ii) SDS-PAGE analysis of molecular weight profile of the endotoxin protein crystals.
(iii) Agarose-GE analysis of the plasmid profile (Note 1)

Definition of active ingredient

A mixture of free endotoxin protein crystals produced by Bti strain AM65-52 and the spores and cells bearing them.

Measurement of active ingredient activity

Bioassay with fourth instar larvae of *Aedes aegypti* (strain Bora Bora), results expressed as international toxic units (ITU)/mg product, relative to a reference Bti material. Note: the only reference standard currently available is Valent BioSciences Corp. strain AM65-52, lot # 093-177-W502, which has a biopotency of 6388 ITU/mg.

Note 1 : To identify to strain level, genomotyping methodology may be used.

Bacterial strains can be characterized by comparing their genomic DNA to an array of genomic DNA fragments originating from a mixture of different strains of the same species. Background information on the potential of the technology and its resolution is given in the following references (Salama et al. 2000, Leavis et al. 2007, Vlaminckx et al. 2007). This current hybridization technology has permitted the assaying of thousands of nucleic acid sequences in a single reaction on a solid substrate. Such a massively parallel system offers the opportunity of diagnostic applications for strain identification through a comparative process.

***Bacillus sphaericus* strain ABTS-1743**

INFORMATION

Scientific name

Bacillus sphaericus strain ABTS-1743.

Shorthand terms

Bs: All subspecies of *Bacillus sphaericus* (serotype H5a5b)

Bs, strain ABTS-1743: The strain to which CIPAC code 978 applies and the subject of the current evaluation

CIPAC number

978

Identity tests

Identification is based upon the following tests.

(i) Microscopic examination: gram-positive rods, presence of a spherical spore within a visible swelling at one end of the cell, prior to lysis.
(ii) SDS-PAGE analysis provides a profile of the two Bs crystalline endotoxin proteins of sizes 51kDa and 42kDa.
(iii) Genomotyping analysis determines the overall genetic relatedness to highly similar strains within the same species (Note 1).

Definition of active ingredient

The active ingredient of Bs strain ABTS-1743 is defined as a mixture of the cells, spores, and the crystalline inclusion associated with the spores.

Measurement of active ingredient activity

The content of active ingredient activity (biopotency) is measured and expressed as Bs International Toxic Units (BsITU) per mg of product. Bioassay with early L3 third instar larvae of *Culex quinquefasciatus. R*esults expressed as international toxic units (ITU)/mg product, relative to a reference ABTS-1743 material. Note: The only reference standard currently available is Valent BioSciences Corporation Bs strain ABTS-1743, Lot # 089-273-W501, which has a biopotency of 1639 Bs ITU/mg.

Note 1 : To identify to strain level, genomotyping methodology may be used.

Bacterial strains can be characterized by comparing their genomic DNA to an array of genomic DNA fragments originating from a mixture of different strains of the same species. Background information on the potential of the technology and its resolution is given in the following references (Salama et al. 2000, Leavis et al. 2007, Vlaminckx et al. 2007). This current hybridization technology has permitted the assaying of thousands of nucleic acid sequences in a single reaction on a solid substrate. Such a massively parallel system offers the opportunity of diagnostic applications for strain identification through a comparative process.

Bacillus thuringiensis subspecies *israelensis* strain AM65-52 + *Bacillus sphaericus* strain ABTS-1743

GRANULES

WHO specification 770+978/GR (April 2016)

This specification, which is PART ONE of this publication, is based on an evaluation of data submitted by the manufacturer whose name is listed in the evaluation report (770+978/2015). It should be applicable to relevant products of that manufacturer but it is not an endorsement of those products, nor a guarantee that they comply with the specification. The specification may not be appropriate for the products of other manufacturers. The evaluation reports (770+978/2015), as PART TWO, form an integral part of this publication.

1 **Description** (Note 1)

The material shall consist of granules containing *Bacillus thuringiensis* ssp. *israelensis* strain AM65-52 + *Bacillus sphaericus* strain ABTS-1743 (Note 2), together with suitable carriers and any other necessary formulants. It shall be in the form of free flowing small granules with a narrow particle size distribution (see clause 5.3), intended for direct application to mosquito larval habitats. The formulation shall be dry, free from visible extraneous matter and hard lumps, free-flowing, essentially non-dusty and intended for application by machine or hand.

2 **Active ingredient** (Note 1)

2.1 **Identity**

The active ingredients shall comply with the identity tests described in Note 3.

2.2 ***Bacillus thuringiensis* ssp. *israelensis* strain AM65-52 + *Bacillus sphaericus* strain ABTS-1743 content** (Note 4)

The biological activity (biopotency) for *Bacillus thuringiensis* ssp. *israelensis* strain AM65-52 and *Bacillus sphaericus* strain ABTS-1743 shall not be less than 50 Bs International Toxic Units/mg, when determined by the method described in Note 4.

3 **Relevant impurities** (Note 1)

3.1 **Water** (MT 30.5, CIPAC Handbook J, p.120, 2000)

Maximum: 60 g/kg.

4 **Bacterial contaminants** (Note 1)

4.1 ***Staphylococcus aureus*** (Note 5)

Staphylococcus aureus shall not be detected when tested by the method described in Note 5.

4.2 ***Salmonella* species** (Note 6)

Salmonella species shall not be detected when tested by the method described in Note 6.

4.3 ***Pseudomonas aeruginosa*** (Note 7)

Pseudomonas aeruginosa shall not be detected when tested by the method described in Note 7.

4.4 ***Escherichia coli*** (Note 8)

Escherichia coli shall not exceed 100 colony-forming units (CFU)/g of GR when tested by the method described in Note 8.

5 **Physical properties** (Note 1)

5.1 **pH range** (MT 75.3, CIPAC Handbook J, p.131, 2000)

pH range: 4.0 to 7.0.

5.2 **Pour and tap density** (MT 186, CIPAC Handbook K, p.151, 2003)

Pour density: 0.5 to 0.7 g/ml.

Tap density: 0.6 to 0.8 g/ml.

5.3 **Nominal size range** (MT 170, CIPAC Handbook F, p.420, 1995)

Not less than 900 g/kg of the formulation shall be within the size range of 500 to 2000 µm.

5.4 **Dustiness** (MT 171, CIPAC Handbook F, p.425, 1995) (Note 9)

The formulation shall have a maximum collected dust of 30 mg by the gravimetric method or a maximum dust factor of 25 by the optical method.

5.5 **Attrition resistance** (MT 178, CIPAC Handbook H, p.304, 1998)

Minimum 97% attrition resistance.

6 **Storage stability**

6.1 **Stability at elevated temperature** (MT 46.3, CIPAC Handbook J, p.128, 2000) (Note 10)

After storage for 14 days at 54 ± 2ºC, the determined average biopotency relative to the 5℃ control shall not be lower than 70%, relative to the determined average found before storage (Note 11), and the formulation shall continue to comply with the clauses for:

- nominal size range (5.3);
- dustiness (5.4);
- attrition resistance (5.6).

Note 1 A sample consisting of at least two sealed bags (or the smallest packaging units) should be taken from each batch for testing. Prior to testing, sealed bags must not be opened and must be kept away from direct sunlight and other heat sources. The material to be tested for bacterial contaminants (clauses 4.1 to 4.4) must be taken from a bag freshly opened under aseptic conditions.

Note 2 The active ingredients, Bti strain AM65-52 and Bs strain ABTS-1743, is defined as a combination of endotoxin protein crystals and the cells and spores bearing these endotoxin crystals.

Note 3 **Identification of Bti strain AM65-52**

Identification is based on the following tests.

(i) Microscopic examination of the bacterial cells after gram staining (gram positive rods), and of spores and adherent crystalline proteins without gram staining.
(ii) SDS-PAGE analysis of molecular weight profile of the Bti crystalline endotoxin proteins.
(iii) Agarose gel electrophoresis of the plasmid DNA coding for the endotoxins.

In test (i), gram staining is a universally-used bacteriological test and is not described below. *Bacillus thuringiensis* is observed as gram-positive rods in test (i) but this result identifies only the broad group of bacteria which includes Bt. Microscopic observation of spores and adherent (irregularly round) crystals supports identification as Bt but is not definitive. Tests (ii) and (iii) identify the product as Bti. Identity as a Bti may be established using either test (ii) or (iii) in combination with test (i) but, in cases of doubt, all tests should be conducted. Note that only genomotyping can determine strain of Bti.

Flagellar antigens (H-14) may also be used to identify the presence of Bti, if suitable well characterized antisera become available but it is important to note that such antisera would not identify the strain.

Identity test (ii), molecular weight profile of the Bti strain AM65-52 endotoxin protein crystals

Principle

Endotoxins of Bti strain AM65-52 occur as irregularly round inclusions, developed during sporulation. The crystals contains 4 major proteins[1,2], designated Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa. Crystals are extracted from the formulation by agitation, centrifugation and washing. The crystal proteins are dissolved and denatured (losing their secondary and tertiary structure) and molecular weights are determined by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, based on that of Laemmli *et al.*[3], as modified for Bt toxins by Brussock & Carrier[4]. Similarly-treated standard proteins are also separated on the gel, to provide molecular weight calibrants. After staining the gel and de-staining it to remove the background, 3 major protein bands should be apparent, of 135 kDa (Cry4Aa, Cry4Ba), 70 kDa (Cry11Aa), and 28 kDa (Cyt1Aa).

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1 Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of Bacillus thuringiensis. Microbiological Reviews 53: 242-255.

2 Crickmore *et al*. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev*. 62: 807-813.

3 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 (5259): 680-685.

4 Brussock, S.M. and T.C. Carrier (1990) Use of sodium dodecyl sulfate – polyacrylamide gel electrophoresis to quantify *Bacillus thuringiensis* δ-endotoxins. Chapter in: Analytical chemistry of *Bacillus thuringiensis. ACS Symposium Series.* (Hickle, L.A. and W.L. Fitch. eds.) 78-87.

Equipment and materials

Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system; resolving gels of 10% acrylamide or a linear gradient of approximately 5-20% are appropriate.

Boiling water bath (100ºC).

Micro-centrifuge (Eppendorf Microfuge, or equivalent), producing 8000 g.

Molecular weight calibration standard. Containing proteins in the range 14 kDa (lysozyme) to 200 kDa (myosin). Intermediate molecular weight proteins that may be included are ß-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and myoglobin (17 kDa). Examples of commercially available calibration kits are Mark 12 unstained standard (Invitrogen Cat.# LC5677) or Broad range SDS-PAGE standard (BioRad Cat.# 161-0317) but any suitable equivalent may be used. The calibration standard should be prepared in 2X Laemmli sample buffer.

Ethylenediamine tetra-acetic acid solution (EDTA), 5 mM in water, pH8.

Sodium chloride/EDTA solution, NaCl/EDTA, 1 M/5 mM in water, pH 8.

Sodium hydroxide solution, 0.1 M in water.

2X Laemmli buffer, 125 mM tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.2% ß-mercaptoethanol, 50% glycerol, 0.02% bromophenol blue (tracking marker) in water. Dithiothreitol (0.2 M) may be used instead of ß-mercaptoethanol.

Coomassie blue solution, 0.2% Coomassie Brilliant Blue R in water containing 50% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).

Methanol/acetic acid, water containing 25% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).

Deionised water.

Micropipette.

Method

i. Weigh approximately 1 g of granules into a 50 ml screw cap tube. Add 3 ml of 0.2% Tween 80 and agitate granules in the solution by shaking for 30 minutes.
ii. Aliquot 200 µl of suspension, avoiding granules, into a clean microfuge tube.
iii. Add 1 ml of NaCl/EDTA solution and disperse the product. Centrifuge at > 8000 g until the suspended solids form a pellet (typically 5 min at 14000 g). Discard the supernatant.
iv. Wash the pellet twice in 5 mM EDTA pH 8.0, centrifuge as above and discard the supernatant each time.
v. Solubilize the endotoxin crystals in the pellet by re-suspending them in 100 µl NaOH solution for 30 min at 37°C.
vi. Centrifuge the suspension, as above, to remove insoluble materials. Collect the supernatant and discard the pellet.
vii. Add 100 µl 2X Laemmli buffer to the supernatant, mix and immediately heat the mixture at 100°C for 5 min.
viii. Cool, then centrifuge the mixture for 5 min at ≥ 8000 g to remove insoluble materials. Collect the supernatant and discard the pellet.
ix. Load a small volume (approximately 10-20 µl) of supernatant onto an SDS-PAGE gel. Also load the gel with an appropriate quantity of molecular weight calibration standard in 2X Laemmli buffer. Perform the electrophoresis according to the gel rig manufacturer's instructions.
x. Stain the gel with Coomassie blue solution, to visualize the proteins, then de-stain it with methanol/acetic acid until the background is clear.
xi. Observe the positions of the main distinct bands in the sample relative to the molecular weight calibration standard. Bti strain AM65-52 endotoxins are expected to produce bands at positions corresponding to approximately 135, 70 and 28 kDa.

Identity test (iii), agarose gel electrophoresis of the plasmid DNA

Principle

Bti spores are separated from the formulation, cultured in Luria broth, then lysed and centrifuged to remove insolubles. Following plasmid precipitation by low temperature ethanol and centrifugation, residual proteins and RNA are removed with proteinase and RNases, respectively. The plasmid DNA is separated by agarose gel electrophoresis and visualized using ethidium bromide fluorescence under UV light. Under these conditions, the Bti strain AM65-52 plasmid produces visible DNA bands corresponding to approximately 3.3, 4.2, 4.9, 10.6, 68, and 75 MDa, the last of which contains the Bti toxin genes. The 68 and 75 MDa components will generally appear as one band above the chromosomal smear. Additional plasmids known to be present are 105 and 135 MDa bands, which are too large to isolate easily.

Equipment and materials

Incubator, 37ºC.
Water bath, 68ºC.
Shaker water bath, 28ºC.
Refrigerator, 4 ± 2ºC.
Freezer, -18 ± 2ºC.
Ice, crushed.
Bench-top centrifuge, taking 50 ml tubes, to operate at 4000 g.
Micro-centrifuge, refrigerated (Eppendorf Microfuge, or equivalent), to operate at 14000 g.
Vortex mixer.
Vacuum dryer, Savant Speedvac or equivalent.
Luria broth, Sigma-Aldrich L3522 or equivalent, reconstituted according to the manufacturer's instructions and sterilized in an autoclave.
Water, double-distilled.
Hydrochloric acid, concentrated.
Acetic acid, glacial.
Tris buffer solution, 1 M. Dissolve 121.1 g tris base in about 800 ml water, adjust to pH 7.6 with concentrated HCl (about 60 ml) and make to 1 l with water.
Sodium chloride solution, 5 M. Dissolve 292.2 g NaCl in water and make to 1 l.
EDTA solution, 0.5 M in water, adjusted to pH 8.0.
Sodium dodecyl sulfate (SDS) solution, electrophoresis grade, 10% in water. Dissolve 100 g SDS in about 900 ml (heating to 68ºC to assist dissolution), adjust to pH 7.2 with a few drops of concentrated HCl and make to 1 l with water.
TES buffer solution. Dilute a mixture of 3 ml tris buffer; 1 ml EDTA and 1 ml NaCl solutions (as above) to 100 ml with water.
Sucrose medium. Dilute 12.50 g sucrose together with 1 ml NaCl and 2.5 ml tris solutions to 50 ml with water.
SDS-NaCl solution. Dilute a mixture of 2 ml SDS and 1.4 ml NaCl solutions to 10 ml with water.
Sodium acetate solution. Dissolve 40.81 g sodium acetate·$3H_2O$ in about 80 ml water, adjust to pH 5.6 with glacial acetic acid and make to 100 ml with water.
Tris-borate buffer solution. Dissolve 108 g tris base, 55 g boric acid and 5 ml EDTA solution in about 800 ml water, adjust to pH 8.3 and dilute to 1 l (10X tris-borate buffer). Dilute 1+9 with water to produce 1X tris-borate buffer.
Lysozyme solution. 50 mg/ml in sucrose medium.
Ethanol, 100% and 70% aqueous solution, cooled to 4ºC.
T1 RNase solution, 100 U/ml.
RNase A solution, 10 mg/ml.
Proteinase solution, 10 mg/ml.
Agarose gels. Prepare 0.8% gels in 1X tris-borate buffer. A gel 20cm long, 10cm wide and 3-4mm deep requires about 100 ml agarose solution. Use 1.5% agar for end plugs, if required. When the gel has solidified, cover its surface minimally with tris-borate buffer (approximately 40 ml).
PVDC film wrap ("cling-film"), Saran™ or equivalent.
Electrophoresis apparatus, suitable for running agarose gels. BioRad; GE (formerly Pharmacia) or equivalent.

DNA molecular weight marker solution, 1kB ladder (Invitrogen), Pulse marker (Sigma) or equivalent.

Tracking dye for electrophoresis, containing 0.25% bromophenol blue and 15% Ficoll 400.

Ethidium bromide solution, 5 µg/ml in water (note: wear nitrile gloves for handling solution and treated gels).

UV lamp, for visualization of DNA bands.

Method

i. Aseptically transfer about 100 mg of granules into 2 ml medium/water in a sterile bottle and mix thoroughly.
ii. Maintaining aseptic conditions, inoculate 100 µl of the suspended Bti cell mixture into 20 ml Luria broth and incubate, with shaking, at 28ºC for about 16 hours.
iii. Sediment the cells in a bench-top centrifuge at maximum speed for 15 minutes and discard the supernatant.
iv. Add 1 ml TES buffer, vortex to re-suspend the pellet, transfer the suspension to a microcentrifuge tube and sediment the cells for 2 minutes at 5ºC. Discard the supernatant.
v. Add 180 µl sucrose medium and vortex to re-suspend the pellet. Add 20 µl lysozyme solution, mix gently by hand (do not use a vortex mixer) and incubate at 37ºC for 60 minutes.
vi. Add 48 µl NaCl solution, 12 µl EDTA solution and 260 µl SDS-NaCl solution and slowly invert the tube, twice. Incubate the mixture for 10 minutes at 68ºC, then stand the tube in ice for 60 minutes. Centrifuge at 4ºC for 15 minutes to sediment the cell wall debris and transfer 300 µl of supernatant to another micro-centrifuge tube.
vii. Add 33 µl sodium acetate solution and 670 µl cold 100% ethanol, vortex to mix and place in the freezer for ≥1 hour. Centrifuge at 5ºC for 15 minutes and discard the supernatant.
viii. Add approximately 200 µl cold 70% ethanol, vortex to mix, then centrifuge at 5ºC for 10 minutes and discard the supernatant. Dry the pellet in a vacuum dryer for about 30 minutes. Add 200 µl TES buffer to the dried pellet, vortex to re-suspend it, allow the mixture to stand at room temperature for 15 minutes and then vortex again to mix.
ix. Add 2 µl T1 RNase solution and 2 µl RNase A solution, mix and incubate the mixture at 37ºC for 30 minutes. Add 20 µl proteinase solution, mix and incubate the mixture at 37ºC for 1 hour.
x. Mix 15 µl sample solution with 3 µl tracking dye and transfer the whole to a well in the agarose gel. Include an appropriate amount of DNA molecular weight marker solution, according to the manufacturer's directions, in an adjacent well.
xi. Run the gel at 50 V for about 15 minutes. Turn off the voltage before removing excess buffer from the surface of the gel and then cover it with PVDC film. Adjust the voltage to give 20 mA current and run the gel overnight (16-17 hours). Reverse the voltage polarity for 30 seconds immediately before switching off and removing the gel.
xii. Stain the gel in ethidium bromide solution for 20 minutes, with gentle rocking. Destain the gel in 1X tris-borate buffer for 20 minutes, changing the buffer 3 times during this time. Place the gel under a UV lamp and photograph it. Bti strain AM65-52 plasmid should produce 5 fluorescent bands below the chromosomal smear and, depending upon the separation quality of the gel, 1 band may appear above the chromosomal smear. Due to possible conformational changes from super-coiled to relaxed forms of the plasmid during preparation, the actual sizes of the plasmids are best determined by comparison on the same gel with a Bt strain having known plasmid sizes, such as Bti reference standards HD-1 or HD-2.

Identification of Bs strain ABTS-1743

Identification is based on the following tests.

(i) Microscopic examination of the bacterial cells after gram staining (gram positive rods), and presence of a terminal round spore causing a swollen appearance at that end of the mother cell.

(ii) SDS-PAGE analysis of molecular weight profile of the Bs crystalline endotoxin proteins.

(iii) Genomotyping hybridization analysis determines degree of relatedness within the same species, thus providing strain specific identity.

In test (i), gram staining is a universally-used bacteriological test and is not described below. *Bacillus sphaericus* is observed as gram-positive rods in test but this result identifies only the broad group of bacteria which includes Bs. Microscopic observation of terminally located round spores, causing the cell terminus to swell, and observation of spore associated crystalline structures, supports the identity but is not definitive. Test (ii) endotoxin protein analysis identifies the active ingredient as Bs by observation of both the 51kDa and 42kDa proteins, but this method is not strain specific. Only test (iii) genomotyping can establish strain specificity.

Identity test (ii), molecular weight profile of Bs, strain ABTS-1743 endotoxin protein crystals using SDS-PAGE analysis

Principle

Endotoxins of Bs, strain ABTS-1743 occur as irregular crystalline inclusions closely associated with the spore. The crystal inclusions contain 2 major proteins, designated BinA (42kDa) and BinB (51kDa) which are encoded on the chromosome.[2] Crystals are extracted from the formulation by agitation, centrifugation, and washing. The crystal proteins are dissolved and denatured (losing their secondary and tertiary structure) and the molecular weights determined by separation by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, based on that of Laemmli *et al.*[3] Similarly, treated standard proteins are also separated on the gel, to provide molecular weight calibrants. After staining the gel and de-staining it to remove the background, two major protein bands should be observed.

Equipment and materials

Laemni sodium.
Boiling water bath (100ºC).
Micro-centrifuge (Eppendorf Microfuge, or equivalent), producing 8000 g.
Molecular weight calibration standard. Containing proteins in the range 14 kDa (lysozyme) to 200 kDa (myosin). Intermediate molecular weight proteins that may be included are ß-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and myoglobin (17 kDa). Examples of commercially available calibration kits are Mark 12 unstained standard (Invitrogen Cat.# LC5677) or Broad range SDS-PAGE standard (BioRad Cat.# 161-0317) but any suitable equivalent may be used. The calibration standard should be prepared in 2X Laemmli sample buffer.
Ethylenediamine tetra-acetic acid solution (EDTA), 5 mM in water, pH8.

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[1] Baumann, P. et al. (1985) Purification of the larvicidal toxin of *Bacillus sphaericus* and evidence for high molecular weight precursors. J. Bacteriol. 163:738-747.

[2] Liu, J.-W., J. Hindley, A. G. Porter, and F. G. Priest. (1993). New high-toxicity mosquitocidal strains of *Bacillus sphaericus* lacking a 100-kilodalton-toxin gene. Appl. Environ. Microbiol. 59:3470-3473.

[3] Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage. Nature. 227:680-685.

Sodium chloride/EDTA solution, NaCl/EDTA, 1 M/5 mM in water, pH 8.
Sodium hydroxide solution, 0.1 M in water.
2X Laemmli buffer, 125 mM tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.2% ß-mercaptoethanol, 50% glycerol, 0.02% bromophenol blue (tracking marker) in water. Dithiothreitol (0.02 M) may be used instead of ß-mercaptoethanol.
Coomassie blue solution, 0.2% Coomassie Brilliant Blue R in water containing 50% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).
Methanol/acetic acid, water containing 25% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).
Deionised water.
Micropipette.

Method

i. Weigh approximately 1 g of granules into a 50 ml screw cap tube. Add 3 ml of 0.2% Tween 80 and agitate granules in the solution by shaking for 30 minutes.
ii. Aliquot 200 µl of suspension, avoiding granules, into a clean microfuge tube.
iii. Add 1 ml of NaCl/EDTA solution and disperse the product. Centrifuge at > 8000 g until the suspended solids form a pellet (typically 5 min at 14000 g). Discard the supernatant.
iv. Wash the pellet twice in 5 mM EDTA pH 8.0, centrifuge as above and discard the supernatant each time.
v. Solubilize the endotoxin crystals in the pellet by re-suspending them in 100 µl NaOH solution for 30 min at 37°C.
vi. Centrifuge the suspension, as above, to remove insoluble materials. Collect the supernatant and discard the pellet.
vii. Add 100 µl 2X Laemmli buffer to the supernatant, mix and immediately heat the mixture at 100°C for 5 min.
viii. Cool then centrifuge the mixture for 5 min at ≥8000 g to remove insoluble materials. Collect the supernatant and discard the pellet.
ix. Load a small volume (approximately 10-20 µl) of supernatant onto an SDS-PAGE gel. Also load the gel with an appropriate quantity of molecular weight calibration standard in 2X Laemmli buffer. Perform the electrophoresis according to the gel rig manufacturer's instructions.
x. Stain the gel with Coomassie blue solution, to visualize the proteins, then de-stain it with methanol/acetic acid until the background is clear.
xi. Observe the positions of the main distinct bands in the sample relative to the molecular weight calibration standard. Bs strain ABTS-1743 endotoxins are expected to produce bands at positions corresponding to approximately 42 and 51 kDa.

Identity test (iii) Genomotyping Analysis

Principle

Genomotyping describes the analysis of bacteria by comparison of their genomes using microarrays. DNA microarray analysis allows for gene by gene comparison of strain genomes, and also provides data determining the presence or absence of genes. The technology allows for the assaying of up to thousands of nucleic acid sequences in a single reaction on a solid substrate, allowing for determination of strain relatedness and strain specific identity within a group of closely related strains.

Note 4 **Determination of biopotency for Bs**

Principle

Biopotency for Bs based products is measured in Bs International Toxic Units (BsITU) per mg of product. Biopotency is tested by comparing mosquito larval mortality produced by the product under test (test substance) with the mortality produced by a spray-dried reference powder of *Bacillus sphaericus,* using two day old, third instar larvae of *Culex quinquefasciatus*. The toxicity (BsITU/mg) of products tested is determined according to the following formula:

$$\text{BsITU/mg of product tested} = \frac{\text{reference standard BsITU/mg} \times LC_{50}\ \text{(mg/l) standard}}{LC_{50}\ \text{(mg/l) product tested}}$$

Equipment and materials

Metered dispensing pump.
Refrigerator.
Analytical balance.
Mosquito cages.
Shaker.
Water bath sonicator.
Plastic stackable pans.
Repeating pipette.
Graduated cylinder (10 ml, 100 ml, 1000 ml).
Dessicator jar.
Analytical balance.
Tween 80 (polyoxyethylene-sorbitan mono-oleate) as wetting agent.
Yeast extract.
Paper, wax-coated cups (4 oz.).
Paper dilution cups, wax-coated (8oz.).
Wax paper.
Parafilm®.
Screw-capped, clear glass bottles.
Test trays.
Disinfectant (sodium hypochlorite, or equivalent).
Dishwashing liquid.
Deionized water.
Pelletized guinea pig chow.
Blender.
Sucrose solution, 10% concentration.
Beef blood, defibrillated.
Mosquito larvae *Culex quinquefasciatus.*
Reference Standard (*Bacillus sphaericus* strain ABTS-1743 for the purpose of testing product compliance with the specification).

Method

(i) Production of test larvae

All life cycles stages of this species are reared at 28 ± 2°C (12L:12D photophase) with an exception of test larvae which are reared at 29.5 ± 2°C.

Plastic cups (100 x 50 mm diameter) are used as oviposition containers where the cups are filled half-full with deionized water. Approximately 72 hours after blood feeding, the females should begin to lay eggs and continue for 2 consecutive days. Therefore, 2 days after blood feeding, oviposition containers are placed in the cage, left overnight, and egg-laden containers are removed every morning. The eggs are deposited in rafts, variable in size, on the water surface. To hatch eggs, the egg-laden oviposition containers are placed in 28 ± 2°C incubator overnight. Larvae hatch from the egg rafts overnight are ready to use the

next morning. It is possible to store the eggs for a few days in the refrigerator between 2-11°C before hatching. For best results, incubate the first day eggs laid at 30°C to hatch. For second day eggs laid, incubate the eggs at ambient temperature to hatch.

To maintain larvae, they are fed with pelletized guinea pig chow that has been blended to a fine powder. Larvae for testing are grown at an approximate density of 1 larval/mL of water in white plastic stackable pans, which are nested to provide a cover for each pan. These covers help reducing contamination and evaporation. Pans are filled to an approximate depth of 1" (approximately 2 L) with dechlorinated water. Dechlorination of tap water occurs by allowing each pan to stand for a minimum of 16 hours. Using sieves, newly hatched larvae are poured off. The sieves are then rinsed with deionized water and the mosquito larvae are placed into shallow container. Using a repeating pipette, *Culex* mosquito larvae are pipetted into each plastic pan. Each pan is given guinea pig chow for larval feeding. Pans are placed on movable racks in 29.5 ± 2°C incubator and labeled with proper test date. Larvae are reared to third instar in approximately 2 days.

(ii) Preparation of reference standard (RS), check sample (CS), and product tested (TS = test substance) suspensions

To achieve valid mortality dose response, the appropriate quantity of RS, CS, and TS are weighed. The actual weight should be ± 2% of target weight. For the RS and CS, historical data is used to determine the amount to weigh (in mg) and the dilution method to use.

Initial stock suspension (ISS) is prepared by adding 100 ml of 0.2% Tween 80 into each RS bottle. Place each ISS bottle on wrist action shaker and shake each bottle for 20 minutes. Bottles are then placed in water bath sonicator for 2 minutes.

Final stock suspension (FSS) is the suspension used to prepare the six test concentrations (C). The six Cs are the concentrations test larvae are exposed to.

(iii) Bioassay procedure

The 120 ml paper cups are placed on the appropriate test trays by following the replications described below:

RS has 3 replicates per test day.

CS has 1 replicate per test day.

TS requires a minimum of 2 replicates per day (a total of 4 replicates for 2 test days).

Each concentration is comprised of 3 cups.

UTC contains of 3 cups per test day.

Dispense 90mL of DI water into each test cup using a metered dispensing pump. Using pipette, 1-10 ml DI water is dispensed into each cup as appropriate (Table 1). Add the FSS for the RS, CS, or TS to each cup. See Table 1 for the amounts of FSS added to achieve the test concentration (C) for each cup and preparation of untreated controls.

Table 1. Preparation of Test Concentration (C)

C	*Volume (ml)*		
	FSS	*DI water*	*Initial DI water from pump*
1	10.0	0.0	90
2	8.5	1.5	90
4	7.0	3.0	90
5	4.0	6.0	90
6	2.5	7.5	90
UTC	0.0	10.0	90

Infest each cup with 20 *Culex* larvae. Larvae are 2 days old (third instar), incubated at 29.5 ± 2 ℃ and visually uniform in size. Into each cup, add 0.5 ml yeast solution. See Table 2 below for yeast solution preparation.

Table 2. Preparation of Yeast Solution

Yeast extract (mg)	*DI H_2O (ml)*
600	100
1200	200
1800	300
2400	400
3000	500

Each test tray is covered with an appropriate-sized piece of wax paper. Make sure the wax paper covers all test cups. This tray is then with another tray, and place on an appropriate cart. Once all trays have been covered and placed on cart, place the cart directly in the incubator (environmental chamber). The cups are incubated for 42-45 hours at 29.5 ± 2℃.

Following incubation period, the numbers of all live and dead larvae are recorded into bioassay data sheets. A larva is considered alive if it responds, even weakly, to probing.

(iii) Determination of toxicity

All test larvae are 2 days old, third instar and of visually uniform in size. The test cups are incubated at 29.5 ± 2℃.

Acceptance criteria for replicates:

- Estimated value is too close to either the highest or the lowest tested concentration.
- For LC_{50} to be valid, a minimum of two concentrations must be above and two below the LC_{50}, to ensure the validity of the LC_{50} value (the sensitivity of the insect colony may require a slightly different 6-dilution series to be used).
- LC_{50} value of the RS or TS does not fall within the tested concentration range.
- If 1-2 replicates of the RS are acceptable then the data is still valid.

Acceptance criteria for entire day test:
The results of all tests will be considered unacceptable if:

- Larval mortality of the UTC is greater than 15%.
- The entire day test is rejected if all 3 replicates of RS are rejected.

Any data from replications with unacceptable results will be retained as part of the study raw data package.

To estimate the biopotency of the TS and CS for each replicates, the following formula is used. The mean of biopotency of all replicates over the necessary days will be calculated.

$$\text{Potency of TS or CS (ITU/mg)} = \frac{\text{Estimated LC50 of RS}}{\text{Estimated LC50 of TS}} \times \text{Potency of RS}$$

Note 5 **Detection of *Staphylococcus aureus* in product**

Equipment and materials

Balance, with tare, capable of weighing 10 g to within ±1%.
Incubator, controlled to within the range 30-35ºC.
Soybean Casein Digest Broth (BioMerieux P2300 or equivalent).
Sterile, pre-poured Mannitol Salt agar plates (BioMerieux M1018 or equivalent*).*

Procedure

Observe aseptic precautions and handling throughout.

Weigh out not less than 10 g of sample into 90 ml soybean casein digest broth. Swirl the contents to ensure an even suspension of the sample is achieved.

Incubate the suspension at 30-35°C for 18 to 24 hours.

Shake the sample preparation after it is removed from the incubator.

Aseptically transfer a portion of the sample preparation onto a pre-poured plate of Mannitol Salt Agar and streak for isolation.

Incubate the plate, inverted, at 30-35°C for 18 to 72 hours.

Examine the plate for the presence of yellow or white colonies, surrounded by a yellow zone, as they may indicate the presence of *Staphylococcus aureus.* Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

i. Presumptive colonies of *Staphylococci* may be confirmed using either a microbial identification system or by performing other appropriate biochemical and cultural tests to confirm the presence of *Stapyhylococcus aureus*.
ii. Optional: Perform gram stains on a typical suspect colony. *Staphylococci* are gram positive, cocci that occur in 'grape like' clusters.

Note 6 **Detection of *Salmonella* in product**

Equipment and materials

Balance, with tare, capable of weighing 10 g to within ±1%.
Incubator, controlled to within the range 30-35 ºC.
Soybean Casein Digest Broth (*BioMerieux P2300* or equivalent).
Sterile Rappaport Vassiliadis Salmonella Enrichment Broth (BioMerieux 42073, or equivalent).
*Xylose Lysine Deoxycholate Agar (*BioMerieux M1031, or equivalent).

Procedure

Observe aseptic precautions and handling throughout.

Weigh out not less than 10 g of sample into 90 ml soybean casein digest broth. Swirl the contents to ensure an even suspension of the sample is achieved.

Incubate the suspension at 30-35°C for 18 to 24 hours.

Shake the sample preparation after it is removed from the incubator.

Aseptically transfer 0.1 mL of the sample preparation to 10 mL of Rappaport Vassiliadis Salmonella Enrichment Broth. Incubate at 30-35°C for 18 to 24 hours.

Aseptically transfer a portion of the sample preparation onto a pre-poured plate of Xylose Lysine Deoxycholate Agar and streak for isolation.

Incubate the plate, inverted, at 30-35°C for 18 to 48 hours.

Examine the plate for the presence of red, well-developed colonies (with or without black centers), as they may indicate the presence of *Salmonella*. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

i. Presumptive colonies of *Salmonella* may be confirmed using either a microbial identification system or by performing other appropriate biochemical and cultural tests to confirm the presence of *Salmonella.*

ii. Optional: Perform gram stains on a typical suspect colony. *Salmonella* are gram negative, non-spore forming, rods.

Note 7 **Detection of *Pseudomonas aeruginosa* in product**

Equipment and materials

Balance, with tare, capable of weighing 10 g to within ±1%.
Incubator, controlled to within the range 30-35 ºC.
Soybean Casein Digest Broth (BioMerieux P2300 or equivalent).
Sterile, pre-poured Cetrimide agar plates, (BioMerieux M1067, or equivalent*).*

Procedure

Observe aseptic precautions and handling throughout.

Weigh out not less than 10 g of sample into 90 ml soybean casein digest broth. Swirl the contents to ensure an even suspension of the sample is achieved.

Incubate the suspension at 30-35℃ for 18 to 24 hours.

Shake the sample preparation after it is removed from the incubator.

Aseptically transfer a portion of the sample preparation onto a pre-poured plate of Centrimide Agar and streak for isolation.

Incubate the plate, inverted, at 30-35℃ for 18 to72 hours.

Examine the plate for growth, as colonies may indicate the presence of *Pseudomonas aeruginosa.* Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

i. Presumptive colonies of *Pseudomoas aeruginosa* may be confirmed using either a microbial identification system or by performing other appropriate biochemical and cultural tests to confirm the presence of *Pseudomoas aeruginosa.*
ii. Optional: Perform gram stains on a typical suspect colony. *Pseudomonas* are gram negative, non-spore forming, rods.

Note 8 **Detection of *Escherichia coli* in product**

Equipment and materials

Balance, with tare, capable of weighing 10 g to within ±1%.
Incubator, controlled to within the range 30-35ºC.
Incubator, controlled to within the range 42-44ºC.
Soybean Casein Digest Broth (BioMerieux P2300 or equivalent).
Sterile MacConkey Broth (BioMerieux P3155 or equivalent).
Sterile MacConkey Agar (BioMerieux M1017 or equivalent).

Method

Observe aseptic precautions and handling throughout.

Weigh out not less than10 g of sample into 90 ml soybean casein digest broth. Swirl the contents to ensure an even suspension of the sample is achieved.

Incubate the suspension at 30-35℃ for 18 to 24 hours.

Shake the sample preparation after it is removed from the incubator.

Aseptically transfer 1 mL of sample preparation into 100 mL of MacConkey Broth. Incubate at 42-44℃ for 24 to 28 hours.

Aseptically transfer a portion of the broth onto a pre-poured plate of MacConkey agar and streak for isolation.

Incubate the plate, inverted, at 30-35°C for 18 to 72 hours.

Examine the plate for the presence of pink to rose red colonies (usually surrounded by a zone of precipitated bile), as they may indicate the presence of *Escherichia coli*. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

i. Presumptive colonies of *E.coli* should be confirmed using either a microbial identification system or by performing other appropriate biochemical and cultural tests to confirm the presence of *E. coli*.
ii. Optional: Perform gram stains on a typical suspect colony. *E. coli* are gram negative, non-spore forming, rods.

Note 9 Measurement of dustiness must be carried out on the sample "as received" and, where practicable, the sample should be taken from a newly opened container, because changes in the water content of samples may influence dustiness significantly. The optical method, MT 171.2, usually shows good correlation with the gravimetric method, MT 171.1, and can, therefore, be used as an alternative where the equipment is available. Where the correlation is in doubt, it must be checked with the formulation to be tested. In case of dispute the gravimetric method shall be used.

Note 10 Tests for bacterial contaminants (clauses 4.1-4.4) are not specified after storage of the product for 14 days at 54ºC, because this regime is unlikely to reveal the extent of potential proliferation that might occur under normal storage conditions.

Note 11 Samples representing “before” and “after” the storage stability test should be tested concurrently after the test, in order to minimize the variation occurring in assays of the biopotency. Material for the “before” test sample should be stored in a sealed container at 2-8°C, for the duration of the test, prior to bioassay. If the container is stored for this purpose in a refrigerator or freezer, it must be equilibrated to room temperature and dried externally before opening, to avoid contaminating the granules with atmospheric moisture which could affect the results of tests such as biopotency and dustiness.

PART TWO

EVALUATION REPORTS

Bacillus thuringiensis subsp. *israelensis* strain AM65-52 + *Bacillus sphaericus* strain ABTS-1743

Bacillus thuringiensis subspecies *israelensis* strain AM65-52 + *Bacillus sphaericus* strain ABTS-1743

FAO/WHO EVALUATION REPORT 770+978/2015

Recommendation

The Meeting recommended that the new specification for *Bacillus thuringiensis* subspecies *israelensis* (Bti) strain AM65-52 + *Bacillus sphaericus* (Bs) strain ABTS-1743 granules (GR), proposed by Valent BioSciences and as amended, should be adopted by WHO.

Appraisal

The Meeting considered data and information submitted by Valent BioSciences in 2013, 2014 and 2015 in support of the development of a new specification for a fine granules formulation (FG) containing *Bacillus thuringiensis* subspecies *israelensis* (Bti) strain AM65-52 and *Bacillus sphaericus* (Bs) strain ABTS-1743.

As the FG formulation code is no longer included in the current version of the CropLife International catalogue of pesticide formulation types nor in the 2010 revision of the FAO/WHO Manual on development and use of specifications for pesticides, the Meeting decided that the formulation type should be GR (granules). Valent BioSciences did not agree as the formulation is registered and sold in many countries with the FG code. As fine granules formulations are covered by granules formulations, the Meeting and the manufacturer agreed that the formulation is well a GR but, for commercial purpose, the name of the formulation can refer to the FG formulation code.

WHO specifications have been published previously in 2007 and 2012 for WG and GR formulations containing *Bacillus thuringiensis* subspecies *israelensis* strain AM65-52, on basis on data submitted by Valent BioSciences. No WHO specification on *Bacillus sphaericus* strain ABTS-1743 was published yet. Nevertheless, *Bacillus sphaericus* 2362 serotype H5a5b, strain ABTS-1743 was approved by EU in March 2015 as an active substance for use in biocidal products.

Both *Bacillus thuringiensis* subspecies *israelensis* strain AM65-52 and *Bacillus sphaericus* strain ABTS-1743 are bacterial larvicides consisting of a mixture of crystalline inclusions (insecticidal proteins), cells and spores (28 kDa, 70 kDa and 135 kDa for the Bti and of 51 kDa and 42 kDa for the Bs). *Bacillus sphaericus* toxin is much slower acting than *Bacillus thuringiensis* toxin. The new formulation (VectoMax GR) is a bacterial larvicide formulation intended for direct application to mosquito larval habitats by machine or hands. Bs ABTS-1743 is proposed to be associated with Bti AM65-52 to manage future resistance development. The specification is therefore limited to WHO.

VectoMax GR was tested and evaluated by WHOPES who recommend it in 2016 as a mosquito larvicide (WHO 2016).

General information on the manufacturing process of the Bti and Bs were provided by the manufacturer. Regarding Bti, the manufacturer stated that the strain is the same than that published in the WHO specifications 770/WG and 770/GR. The culture fermentation of Bti strain AM65-52 and Bs strain ABTS-1743 is performed under aseptic conditions to maintain culture purity. Each batch is tested to ensure batch purity, viability and protein crystal production. Microscopic examinations are performed to ensure that no microbial contamination is present. The manufacturer stated that the active ingredients in VectoMax GR are from pure culture fermentation grown under aseptic conditions. Details were not provided but the Meeting considered that it was not necessary to require more data on Bti and Bs strains. No specification was provided for the Bs strain as previously agreed for the Bti strain.

The manufacturing process of the GR formulation consists of mixing concentrates of Bti strain AM65-52 and Bs strain ABTS-1743 (previously characterised) with formulants using a special production process and leading to combined active ingredients and formulants into particles. Concentrates are adjusted based on the biopotency to follow the clauses given in the specifications. Methods for the determination of the biological activity (biopotency) using respectively *Aedes aegypti* larvae and *Culex quinquefasciatus* were well described. A GLP five-batch analysis was provided (mean results are 72 Bs ITU/mg and 210 Bti ITU/mg) and considered acceptable. VectoMax GR is formulated within a closed system and quality of all constituents is checked before manufacturing. In the specification, a global biopotency limit of minimum 50 Bs ITU/mg is specified. No limit for Bti is given. The Meeting initially required specific limits of biopotency for Bs and Bti. The manufacturer replied this was never required at national levels for registration and separate limits of biopotency have not been proposed in order to not duplicate assays for registration. The most relevant test for this combination product is the *Culex quinquefasciatus* bioassay (Bs ITU/mg). The Meeting finally agreed with the manufacturer being aware that testing for biopotency can only be conducted in a very limited number of laboratories and individual contributions to biopotency cannot be determined by one biotest only.

Data on the content of different pathogens in the formulation have been provided (no detection of *Staphylococcus aureus*, *Salmonella* species and *Pseudomonas aeruginosa* or < 10 CFU/g of *Escherichia coli*).

As the water content in this formulation is important to maintain the product quality, water was proposed as a relevant impurity. A clause of 60 g/kg for water was provided. The value of 60 g/kg is higher than the target given in the manufacturing process. The manufacturer justified this limit in order to prevent the growth of bacterial contaminants which was considered as acceptable. The test method is a standard CIPAC method.

The data for the physical and chemical properties were in accordance with the requirements of the 2010 revision of the FAO/WHO Manual on development and use of specifications for pesticides and supported the specification for this new WHO specification for a granular formulation. Tests were performed using CIPAC methods.

The manufacturer provided data on the stability of the formulation stored at 20ºC for 24 months and at 54°C for 1 and 2 weeks. After 2 years storage, no modification of the physical and chemical properties and of the biopotency were observed, as well as the appearance after 2 weeks at 54°C. Nevertheless, biopotency (expressed as

Bs ITU/mg) slowly decreased after 1 week and then after 2 weeks at 54°C although it remained above the specified minimum limit of 70% of the average found before storage.

Moreover, for the nominal size range, to be in line with the CIPAC method MT 170, the Meting proposed a size range of 500 to 2000 µm instead of 840 to 2000 µm.

The Meeting proposed also to revise the limit for dustiness from "Essentially non-dusty" to "The formulation shall have a maximum collected dust of 30 mg by the gravimetric method or a maximum dust factor of 25 by the optical method", as recommended in the amendments to the FAO/WHO specification Manual published on the FAO and WHO websites.

Toxicological and ecotoxicological data based on acute toxicity, irritation, sensitization and ecotoxicity using Bs strain ABTS-1743 having same profile than the strains used in the GR formulation were provided by the manufacturer. Bs strain ABTS-1743 is not acute toxic, very slightly skin irritant and no pathogenicity is observed. EPA stated that based on extensive testing, no harmful effects are expected to occur to the public with this active ingredient but, because there is the potential for skin and eye irritation, applicators are warned to avoid direct contact with the granules or a concentrated spray mix. No data on genotoxicity and mutagenicity have been provided. Moreover, various tests revealed no expected harm to non-target organisms.

Bti, strain AM65-52 + Bs, strain ABTS-1743 overview of uses

The most important differences between the toxins of Bs and Bti are speed of action and persistence in natural larval habitats. Bs toxin is much slower-acting than Bti toxin. Larval mortality can take several days but is usually expressed within 48 hours of ingestion, while Bti provides quick mortality of larvae. Initial results can be seen within 2-24 hours with Bti. Bs toxin also provides longer residual in natural larval habitats than Bti. Combining Bti + Bs allows for quick kill seen with Bti (which provides end-users with the ability to quickly respond to rapid population growth) with the residual properties of Bs (which minimizes number of applications needed thus reducing operational costs).

Bacterial mosquito larvicides have gained considerable acceptance in recent years. The value of Bti and Bs to mosquito control programs worldwide is well established (Becker et al 2003). Bti and Bs each offer unique advantages relative to chemical insecticides. Both Bti and Bs offer relative safety to humans and non-target organisms (Siegel and Shadduk 1990b, WHO 1999, Lacey and Merritt 2003). By combining Bti and Bs in a specific toxin ratio into every micro particle, mosquito control professionals can take advantage of each microbial larvicide's strengths, while significantly reducing the limitations that each possesses.

Species: *Bacillus sphaericus*

Strain: ABTS-1743

Serotype: H5a5b

Genus: Bacillus

Family: Bacillaceae

Phylum: Firmicutes

Bs Identity

Scientific name

Bacillus sphaericus strain ABTS-1743.

Shorthand terms

Bs: All subspecies of *Bacillus sphaericus* (serotype H5a5b)

Bs, strain ABTS-1743: The strain to which CIPAC code 978 applies and the subject of the current evaluation

CIPAC number

978

Identity tests

Identification is based upon the following tests.

(i) Microscopic examination: gram-positive rods, presence of a spherical spore within a visible swelling at one end of the cell, prior to lysis.

(ii) SDS-PAGE analysis provides a profile of the two Bs crystalline endotoxin proteins of sizes 51kDa and 42kDa.

(iii) Genomotyping analysis determines the overall genetic relatedness to highly similar strains within the same species (Note 1).

Definition of active ingredient

The active ingredient of Bs strain ABTS-1743 is defined as a mixture of the cells, spores, and the crystalline inclusion associated with the spores.

Measurement of active ingredient activity

The content of active ingredient activity (biopotency) is measured and expressed as Bs International Toxic Units (BsITU) per mg of product. Bioassay with early L3 third instar larvae of *Culex quinquefasciatus. R*esults expressed as international toxic units (ITU)/mg product, relative to a reference ABTS-1743 material. Note: The only reference standard currently available is Valent BioSciences Corporation Bs strain ABTS-1743, Lot # 089-273-W501, which has a biopotency of 1639 BsITU/mg.

Note 1 : To identify to strain level, genomotyping methodology may be used.

Bacterial strains can be characterized by comparing their genomic DNA to an array of genomic DNA fragments originating from a mixture of different strains of the same species. Background information on the potential of the technology and its resolution is given in the following references (Salama et al. 2000, Leavis et al. 2007, Vlaminckx et al. 2007). This current hybridization technology has permitted the assaying of thousands of nucleic acid sequences in a single reaction on a solid substrate. Such a massively parallel system offers the opportunity of diagnostic applications for strain identification through a comparative process.

Composition and properties

Table 1. Composition and properties of Bti strain AM65-52 + Bs strain ABTS-1743 formulated as FG

Manufacturing process, data on components, impurities and contaminants	Confidential information supplied and held on file by WHO.
Declared minimum biopotency	50 Bs ITU/mg
Relevant impurities ≥ 1 g/kg and maximum limits for them	Water, 60 g/kg
Relevant impurities < 1 g/kg and maximum limits for them	none
Relevant microbial contaminants and maximum limits for them	*Staphylococcus aureus*, not detected *Salmonella* species, not detected *Pseudomonas aeruginosa*, not detected *Escherichia coli*, not more than 100 colony-forming units/g
Stabilizers or other additives and maximum limits for them	None

Bacterial larvicide hazard summary

The standard tests of sub-acute to chronic toxicity, used for synthetic chemicals, are not entirely appropriate for bacterial larvicides, which are regulated primarily on the basis of pathogenicity studies (for example, PMRA Canada: DIR 2001-2. EU: Annex 6b of Directive 91/414/EEC). The toxicology of microbial pesticides is considered but pathogenicity potential, infectivity and the pattern of clearance are at least as important.

Many data on the hazards of Bt, Bti and Bs are available from the open literature.

Bacillus thuringiensis ssp. *israelensis* (Bti) and *Bacillus sphaericus* (Bs) have been evaluated by the US EPA. Furthermore, Bti has been evaluated by the IPCS (IPCS 1999) and Bti, strain AM65-52 was reviewed for re-registration by the US EPA in 2006. Bti strain AM65-52 has completed review by the European Commission and Bs strain ABTS-1743 review is scheduled for completion by late 2015.

The IPCS concluded that commercial Bt products do not contain metabolites that are considered hazardous to humans and the environment. Moreover, WHO and the EU (under Council Directive 67/548/EEC) have given no hazard classification to Bti. The US EPA has exempted Bti and Bs from a requirement for tolerances, it has concluded that Bt and Bs products pose no threat to groundwater.

Bti + Bs formulations

Sustainability of Bs strain ABTS-1743 as a public health pesticide requires it is co-formulated with Bti strain AM65-52 to manage future resistance development. The combination of Bti strain AM65-52 + Bs strain ABTS-1743 FG is registered in Brazil, France, Italy, Spain, Turkey and the USA and planned for Africa and Asia.

Bacterial larvicide methods of analysis and testing

The method for determination of bacterial larvicide active ingredient content is bioassay of activity towards mosquito larvae. Bacterial larvicides are determined as biopotency, comparing mosquito larval mortality produced by the product under test with the mortality produced by a corresponding reference standard expressed in ITU/mg.

Prior to the first global introduction of a commercial Bs based product in the 1990s, it was necessary for a new biopotency method to be established for quality control. Unfortuntely, the internationally accepted Bti bioassay method developed in the 1980s utilized *Aedes aegypti* larvae, a species that is not susceptible to Bs (sometimes refered to as “refractory” towards Bs). As such, a new bioassay method using *Culex quinquefasciatus* was developed (unit identifier = BsITU/mg) and has been used by the international community for Bs based larvicides for over 20 years. *Culex quinquefasciatus* is recognized as the standard species for assessing potency of Bs based products internationally. As such, it requires different bioassay methods relative to Bti bioassays to account for the genus differences between *Aedes* and *Culex*. With the introduction of commercial Bti + Bs based products in 2008 (now registered in Brazil, Nigeria, Turkey, Italy, France, Spain and the United States; others pending), international regulatory authorities have agreed that only a single

bioassay test should be utilized (reference Bti + Bs labels) and that the most relevant test for this combination product is the *Culex quinquefasciatus* bioassay (unit identifier = BsITU/mg).

Identification of the active ingredient depends upon a series of tests, in addition to the quantitative test mentioned above. Microscopic examination is a simple and rapid initial screen for identification, used to establish that the bacterial cells are gram-positive rods, and the presence of a spherical spore within a visible swelling at one end of the cell, prior to lysis for Bs and gram-positive rods, motile rods, with the presence of spores and protein crystals providing evidence for Bti.

Both Bti and Bs is identifiable by SDS-PAGE of the crystal proteins produced by it. However, only genomotyping analysis can identify bacterial larvicides to the strain level.

Test methods for the bacterial contaminants, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* species and *Escherichia coli*, involve standard bacteriological techniques.

Test methods for determination of physical/chemical properties of the formulation were CIPAC, as indicated in the specification.

Bti + Bs physical properties

The physical properties, the methods for testing them and the limits proposed for the formulation, comply with the requirements of the FAO/WHO Manual (FAO/WHO 2010).

Containers and packaging

Packaging must be impervious to moisture and light.

Bacterial larvicide expression and measurement of the active ingredient

The active ingredient Bti+Bs is defined as a combination of endotoxin protein crystals and fermentation solids and solubles. The content of active ingredient (biopotency) is measured and expressed as Bs International Toxic Units (BsITU) per mg of product. Biopotency is measured by comparing mosquito larval mortality produced by a product under test with mortality produced by a lyophilized reference powder, using early L3 larvae of *Culex quinquefasciatus*. Note: The only reference standard currently available is Valent BioSciences Corporation strain Bs, strain ABTS-1743, Lot # 089-273-W501, which has a biopotency of 1639 BsITU/mg.

ANNEX 1

HAZARD SUMMARY PROVIDED BY THE PROPOSER

Note: Valent BioSciences Corp. provided written confirmation that the toxicological and ecotoxicological data included in the following summary were derived from specially produced technical Bs ABTS-1743 having impurity and microbial contaminant profiles corresponding to those of the granule formulation, although the technical grade active ingredient is not normally isolated as such.

Table A. Toxicology profile of *Bacillus sphaericus* strain ABTS-1743 technical material, based on acute toxicity, irritation and sensitization

Species	Test	Duration and conditions or guideline adopted	Result	Reference
Rat, Albino (7m, 7f)	Acute Oral	Fasted rats, gavage, US EPA FIFRA, subdivision F, 81-1, CFR 152-30 ITU/mg not recorded*	LD_{50} > 5000mg/kg; Oral administration of *Bacillus sphaericus* did not result in multiplication of the microorganism in the tissue of the rat.	024-025
Rabbit, New Zealand Albino (5m, 5f)	Acute Dermal	US EPA FIFRA, subdivision F, 81-2, CFR 152-31 ITU/mg not recorded*	LD_{50} > 2000 mg/kg; No clinical signs were observed during the course of the study. No treatment related lesions were observed upon gross necropsy or histological examination of the skin.	024-026
Rat, Albino (5m, 5f)	Acute Inhalation	US EPA FIFRA, subdivision F, 81-3, CFR 152-32; 4 hr exposure ITU/mg not recorded*	LC_{50} > 0.09 mg/L; No clinical signs were observed during the course of the study. At necropsy, two test animals exhibited discoloration of the lungs.	420-2507
Rabbit, New Zealand Albino (3m, 3f)	Skin Irritation	US EPA FIFRA, subdivision F, 81-5, CFR 152-34 ITU/mg not recorded*	Very slight (3 of 6 at 1 hour, 1 of 6 at 24 hours) to well-defined erythema (1 of 6 at 24 hours) was present. Very slight edema (1 of 6) was present at the 1 hour observation interval. The primary irritation index of 0.3 out of a possible 8.0 was obtained from the 1, 24, 48 and 72 hour observations and was used to give a descriptive rating of slightly irritating.	2709-96
Rabbit, New Zealand Albino (male & female; 3 washed & 6 unwashed)	Eye Irritation	US EPA FIFRA, subdivision F, 81-4, CFR 152-35 ITU/mg not recorded*	The maximum group mean scores for the 30 second washed and unwashed groups occurred at the 24-hour (both groups) and the 48-hour (30 second washed group only) readings were 2.7 and 11.2, respectively. Signs of eye irritation were absent in all rabbits on test by 10 days post-instillation. Instillation into the eyes of rabbits resulted in slight to moderate, reversible irritation. Washing of the eyes following ocular exposure appears to diminish the resultant irritation.	024-024

Guinea Pig (Young adult, male)	Skin Sensitization	US EPA FIFRA, subdivision F, 81-6, CFR 152-36 ITU/mg not recorded*	The initial test dose produced average skin irritation scores of 0.75, and 0.53 respectively. Average scores for the challenge dose were 1.25 and 0.78 respectively and represent an increase of 1.7 and 1.5 times that of the initial test dose response. The challenge dose elicited a response characterized by very slight to well defined erythema and very slight edema.	024-027

* Doses based on spore count or mg product.

Table B. Additional toxicology profile for *Bacillus sphaericus* strain ABTS-1743 technical material, based on single administration

Species	Test	Duration and conditions or guideline adopted	Result	Reference
Rat, Sprague Dawley (15m, 15f)	Acute oral toxicity & pathogenicity	EPA FIFRA 40 CFR 152A-10. Single oral dose of approximately 10^8 CFU. ITU/mg not recorded*	All animals survived the 21-day test period, and treatment related toxicity was not apparent. There was no evidence of pathogenicity or infectivity. Clearance through the gastrointestinal tract was rapid. Body weights were not affected by test article treatments. Oral administration produced no apparent signs of toxicity, pathogenicity, or infection following a 21 day test period.	G-7161-222
Rat, Sprague Dawley (17m, 17f)	Acute intravenous toxicity & pathogenicity	EPA FIFRA 40 CFR 152A-13. Single intravenous injection of approximately 10^7 CFU. ITU/mg not recorded*	All animals survived the 21-day test period, and treatment related toxicity was not apparent. There was no evidence of pathogenicity or infectivity. There was no evidence of pathogenicity. With the exception of one male, in all tissues examined, the presence of the test microbe was substantially reduced by 35 days post-treatment. Body weights did not appear effected by test article treatment. Intravenous administration produced no apparent signs of toxicity, or pathogenicity following a 21 day test period. Clearance of the test microbe was evident by 21 days.	G-7161-224
Rat, Sprague Dawley (17m, 17f)	Intra-tracheal instillation	EPA FIFRA 40 CFR 152-33. Single intra-tracheal instillation of 10^8 CFU. ITU/mg not recorded*	Body weights did not appear to be affected by test article treatment. All test and control group animals survived to the scheduled sacrifice and no treatment –related signs of toxicity were observed at any point during the study. No test article infectivity was observed in control rats of either sex. There was no evidence of pathogenicity and clearance of the microbe was observable by 35 days post-treatment. The number of microbes found in the lungs was dramatically reduced by day 49. Microbes were found to a much lesser extent in the spleen and lymph nodes on day 1; and in the liver of one rat at day 7.	G-7161-225

* Doses based on spore count or mg product.

Table C. Ecotoxicology profile *of Bacillus sphaericus*, strain ABTS-1743 technical material.

Species	Test	Duration and conditions or guideline adopted	Result	Reference
Rainbow trout (*Oncorhynchus mykiss*)	96-hour Static Renewal	Rainbow trout were held in 18.9 L glass aquaria which contained 15 L of test solution. The test stock solution of 4.0 mg/ml was prepared by diluting 2.0 g of the *Bacillus sphaericus* Technical Powder in a 500ml volumetric flask. Three replicate of Rainbow trout were exposed at a test concentration of 15.5 mg/l. ITU/mg not recorded*	No mortalities or apparent adverse effects were observed among the rainbow trout. No physical signs of stress were observed among control or exposed fish. The NOEL was 15.5 mg/L. The 96 hour LC_{50} >15.5 mg/L.	2439-0786-6100-103
Bluegill sunfish (*Lepomis macrochirus*)	96-hour Static Renewal	Bluegill were held in 18.9 L glass aquaria which contained 15 L of test solution. The test stock solution of 4.0 mg/ml was prepared by diluting 2.0 g of the *Bacillus sphaericus* Technical Powder in a 500ml volumetric flask. Three replicate of bluegill were exposed at a test concentration of 15.5 mg/l. ITU/mg not recorded*	No mortalities or apparent adverse effects were observed among the bluegill. No physical signs of stress were observed among control or exposed fish. The NOEL was 15.5 mg/L. The 96 hour LC_{50} for bluegill >15.5 mg/L.	2439-0786-6100-100
Sheepshead minnow (*Cyprinodon variegates*)	96-hour Static Renewal	Sheepshead minnows were held in 18.9 L glass aquaria which contained 15 L of test solution. The study was conducted at the following concentrations of *Bacillus sphaericus* Technical Material: 7.8, 13, 22, 36, 60 and 100 mg/l. Solutions were very cloudy at the 100 mg/l concentration. Ten fish were exposed to each test concentration and control. ITU/mg not recorded*	No mortalities or apparent adverse effects were observed among the sheepshead minnow. Surfacing was the only abnormal effect observed during the 96-hour exposure period in the 36, 60 and 100 mg/l concentrations. The NOEL was 22 mg/L. The 96 hour LC_{50} >100 mg/L.	37730

Mysidopsis bahia (Mysid Shrimp)	96-hour Static Renewal	*Bacillus sphaericus* at nominal concentrations of 6.5, 13, 25, 50 and 100 mg/l in solution renewed daily. A loading factor of 1 mysid per 200 ml of solution was used, with 5 replicates. Observations of test solution and organism behaviour, i.e quiescence, surfacing, loss of equilibrium, erratic behavoiur and organisms tending towards the bottom of the test chamber were performed at 0 and every 24 hours thereafter. ITU/mg not recorded*	After each renewal phase of the study, water quality measurements of dissolved oxygen performed on the 24 hour old 50 and 100 mg/l test concentrations had fallen to well below the critical levels required to sustain the organisms, therefore, it was concluded that the dissolved oxygen depletion was the probable cause of the high mortality rate observed. 96 hour LC_{50} = 71 mg/L NOEL after 96 hours based on the absence of mortality and/or abnormal behavior = 50 mg/L.	38126
Eastern Oysters (*Crassostrea virginica*)	Acute Toxicity under flow through conditions	Nominal concentrations of 7.3, 15, 58, and 116 mg/L were maintained by introducing approximately six aquarium volumes per day of newly prepared test solution via continuous flow serial diluter apparatus. Solutions containing >15 mg a.i./L were observed to be cloudy. ITU/mg not recorded*	Dissolved oxygen concentrations of the test solution were reduced by the presence of *Bacillus sphaericus* and were directly related to the concentration of test material in the exposure solutions. Following 96 hour exposure shell growth was reduced by 28, 59 and 90% among oysters in the highest treatment levels, 29, 58 and 116 mg/L respectively. There was statistically no significant reduction in shell growth reduction among oysters exposed to the two lowest nominal test concentrations. 96 hour LC_{50} = 42 mg/L. NOEL after 96 hours based on the absence of mortality and/or abnormal behaviour = 15 mg/L.	89-06-3017
Daphnia magna (water flea)	Acute Toxicity	48-hour static test system. Test vessels contained 1000 mL of test media, with a test medium depth of 12.8 cm. The study was conducted with a control and a single *Bs* treatment of 15.5 mg/L. ITU/mg not recorded*	After 48 hour exposure of *Daphnia magna* to *Bacillus sphaericus* at a nominal exposure concentration of 15.5 mg/L there were no immobile *Daphnia* or any apparent adverse effects. The 48-hour EC_{50} >15.5 mg/L (the highest concentration tested). The NOEC was observed to be 15.5 mg/L.	11493-1288-6115-110

Mallard duck (*Anas platyrhynchos*)	Avian Single Dose Oral Toxicity	Oral gavage single dose of 50 ml/kg of a 0.18g/ml saline suspension. ITU/mg not recorded*	No clinical signs were observed. No treatment related lesion was observed upon gross necropsy. No birds died during the course of the study. Thus the acute oral LD_{50} > 9g/kg. Oral administration of 6.4 times the adjusted host equivalent (9g/kg) of *Bacillus sphaericus* does not result in acute toxicity.	024-030
Mallard duck (*Anas platyrhynchos*)	Avian Injection Pathogenicity	The test material was suspended in distilled water and administered by intraperitoneal injection to groups of 30 birds at a dose level of 5 ml/kg of a 0.3 g/ml saline suspension. ITU/mg not recorded*	Intraperitoneal injection of 1.1 times the adjusted host equivalent of *Bacillus sphaericus* to mallard ducks results in an LD_{50} greater than 1.5 g/kg. The organism did not appear to grow to any great extent nor replicate in the tissues of mallard duck.	024-031
Mallard duck (*Anas platyrhynchos*)	Avian Dietary Pathogenicity and Toxicity	The test material in the diet at 1.0%, 7.5% and 20% w/w concentrations for thirty days. Controls: Basal diet containing 2% corn oil for thirty days. 40 Mallards were distributed without regard for sex into eight groups of 5 birds each. ITU/mg not recorded*	There did not appear to be a treatment related effect on feed consumption at any time interval. Following the 30 days of exposure, there was no apparent pathogenicity or effect upon survival of young mallards.	161-111

Adult Honey Bees	Safety to Caged Bees	Newly emerged adult worker bees were collected and placed in wood-frame cages (approximately 11 × 9 × 7 cm), approximately 75 – 100 bees per cage. Dilutions of the test material were prepared in 20% sucrose solution (wt/wt) to give final concentrations of 10^4, 10^6 and 10^8 spores per mL. Experiment 1 – bees in three replicate cages per treatment were given water and sucrose *ad libitum*. Controls were given plain sucrose and three other sets were given *B. sphaericus*-contaminated sucrose solution. The duration was 15 days. Experiment 2 - bees were treated identically except that in addition each cage was given a supply of commercial pollen substitute *ad libitum*. The duration was 28 days. ITU/mg not recorded*	Isolation of viable *Bacillus sphaericus* organisms in the intestinal tract of most bees examined confirmed the ingestion of the test material by the bees. The number of colonies was not quantitated; however, it was observed that there was no evidence for multiplication of *Bacillus sphaericus* in the bee. Log-transformed dose had no effect on mortality in experiment 2 and a negative effect on mortality in experiment 1 (i.e. slightly lower mortality as dose increases). While the latter effect was statistically significant, its biological significance is doubtful. It is clear that under these experimental conditions, feeding *Bacillus sphaericus* technical material in sucrose concentrations of 10^4 to 10^8 spores per mL, had no effect on adult honey bee longevity.	Document 24
Algae (*Selenastrum capricornutum)*	Acute toxicity	A static test system was used to determine the acute toxicity to the green alga *Selenastrum capricornutum.* The study was conducted in glass Erlenmeyer flasks, each containing 100 mL of test media. The study was conducted with a control and nominal *Bs* test concentrations of 0.022, 0.22 and 2.2 mg/L. Three replicate test vessels were prepared for the control and each treatment. ITU/mg not recorded*	Based on nominal exposure concentrations the 120-h EC_{50} of *Bs* to the green alga *Selenastrum capricornutum* was observed to be > 2.2mg/L (the highest concentration tested). Therefore the no-observed effect concentration was observed to be 2.2 mg/L.	37657

* Doses based on spore count or mg product.

ANNEX 2: REFERENCES

Study number	Author(s)	Year	Study title. Study identification number. Report identification number. GLP [if GLP]. Company conducting the study
37730		1989	Acute Toxicity of *Bacillus sphaericus* Technical Material to Sheepshead Minnow (Cyprinodon variegatus)
37657		1989	Acute Toxicity of *Bacillus sphaericus* to *Selenastrum capricornutum*
38126		1990	Acute Toxicity of *Bacillus sphaericus* to My*sidopsis bahia*
024-024		1986	*Bacillus sphaericus* Primary Eye Irritation Study
024-025		1986	*Bacillus sphaericus* Acute Oral LD50 in Rats
024-026		1986	*Bacillus sphaericus* Acute Dermal Toxicity Study
024-027		1986	Hypersensitivity Study of *Bacillus sphaericus* in Albino Guinea Pigs
024-030		1986	*Bacillus sphaericus* Avian Single Dose Oral Toxicity Study in Mallard Ducks
024-031		1986	*Bacillus sphaericus* Avian Injection Pathogenicity Test in Mallard Ducks
161-111		1989	*Bacillus sphaericus* Technical Powder An Avian Dietary Pathogenicity and Toxicity Study in the Mallard
420-2507		1986	1986; Four hour Acute Dust Aerosol Inhalation Toxicity Study in Rats of *Bacillus sphaericus*
2709-96		1996	Primary Dermal Irritation Study in Rabbits
89-06-3017		1990	(*Bacillus sphaericus* technical material) – Acute Toxicity to Eastern Oysters (*Crassostrea virginica*) Under Flow-Through Conditions
G-7161-222		1989	Acute Oral Toxicity/Pathogenicity Study of *Bacillus sphaericus* Technical Powder in Rats
G-7161-224		1989	Acute Intravenous Toxicity/Pathogenicity Study of *Bacillus sphaericus* Technical Powder in Rats
G-7161-225		1989	Acute Pulmonary Toxicity/Pathogenicity Study of *Bacillus sphaericus* Technical Powder in Rats
2439-0786-6100-100		1986	*Bacillus sphaericus* Acute Toxicity of ABG-6184 to Bluegill (Lepomis macrochirus)
2439-0786-6100-103		1986	*Bacillus sphaericus* Acute Toxicity of ABG-6184 to Rainbow Trout (*Salmo gardneri*)
11493-1288-6115-110		1986	*Bacillus sphaericus* Acute Toxicity of ABG-6184 to Daphnids (*Daphnia magna*)
Document 24		1986	1986; Safety of *Bacillus sphaericus* for Caged Adult Honey Bees
VA-60035		2005	Five lot analysis – final report Study report : VA-04-001 GLP : yes
VBC-60035		2008	Two year storage stability Sponsor : Valent study number : VBC-60035
WHO		2016	Report of the Nineteenth WHOPES Working Group Meeting, WHO/HQ, Geneva, 08-11 February 2016. WHO, Geneva.