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| REGISTRATION REPORT Part B  Section 2: Analytical Methods  Detailed summary of the risk assessment |
| Product code:  FORAY® 76B (ABG-6431)  Active Substance:  *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351  206.5 g/L |
| Central Zone  **(zRMS: Poland)** |
| CORE ASSESSMENT |
| Applicant: XXXX  Submission Date: August 2023  Evaluation date: May 2024  **MS Finalisation date: September 2024** |

Version history

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| **When** | **What** |
| August 2023 | Initial version submitted by the applicant for Art. 43 |
| May 2024 | Initial RR |
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Table of Contents

[IIIM 5 METHODS OF ANALYSIS, MANUFACTURING, QUALITY CONTROL AND POST-REGISTRATION MONITORING OF THE MICROBIAL PEST CONTROL PRODUCT 4](#_Toc142473970)

[IIIM 5.1 Quality control and post-registration monitoring methods 5](#_Toc142473971)

[IIIM 5.1.1 Methods to detect, isolate, and enumerate the microorganism. Methods to differentiate a mutant or genetically-modified microorganism from the parent strain 5](#_Toc142473972)

[IIIM 5.1.2 Methods to detect spontaneous change in major characteristics of microorganism 6](#_Toc142473973)

[IIIM 5.1.3 Methods to define content of microorganism in appropriate terms (same as IIIM 1.7.1), incl. standardisation, sensitivity, reproducibility, statistical validity, and representative data to validate the bioassay 6](#_Toc142473974)

[IIIM 5.1.4 Methods to identify contaminant microorganism in MPCP 6](#_Toc142473975)

[IIIM 5.1.5 Methods to show control to a specified and acceptable level, of microbial impurities and of any other impurities of toxicological concern, including toxic metabolites, which are known or suspected to be present at any stage of the manufacturing process 9](#_Toc142473976)

[IIIM 5.1.6 Methods to show presence of any human and mammalian pathogens 9](#_Toc142473977)

[IIIM 5.2 Storage stability test and determination of shelf life (methods of analysis) 9](#_Toc142473978)

[IIIM 5.3 Production process for MPCP, describing techniques used to ensure a uniform product and procedures when hazardous contamination is detected in a batch. List starting and intermediate materials, with source and purity of each 9](#_Toc142473979)

[IIIM 5.4 Method for determination of residues 10](#_Toc142473980)

[Appendix 1 – List of data submitted in support of the evaluation 11](#_Toc142473981)

IIIM 5 METHODS OF ANALYSIS, MANUFACTURING, QUALITY CONTROL AND POST-REGISTRATION MONITORING OF THE MICROBIAL PEST CONTROL PRODUCT

This registration report is submitted to the Ministry of Agriculture and Rural Development (Poland) as zonal Rapporteur Member State (zRMS) and cMS (DE, HU, RO) in August 2023 to support the authorisation of the plant protection product (PPP) Foray® 76B (product code ABG-6431) in the EU Central Zone under Article 43 of Regulation (EC) No. 1107/2009. The formulation Foray® 76B is an aqueous suspension concentrate (SC) containing 206.5 g/L the active substance *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351. The content of *B. thuringiensis* subsp*. kurstaki* strain ABTS-351 in Foray® 76B range between 1.17 x 1013 CFU/L and 1.69 x 1013 CFU/L (nominal concentration of 1.51 x 1013 CFU/L). It is currently authorised across the EU for use as an insecticide to control lepidopteran defoliating caterpillars on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens).

*B. thuringiensis* subsp. *kurstaki* strain ABTS-351 was first assessed for approval for use as PPP in the EU in 2008 by Denmark as Rapporteur Member State (RMS). It was included in Annex I of Directive 91/414/EC as a new active substance on 01 May 2009. Application for renewal of the active substance was submitted to Denmark (RMS) and the Netherlands (co-RMS) in 2016 under Regulation (EC) No. 1107/2009, replacing Directive 91/414/EC. EFSA Conclusion on the peer review of risk assessment of *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 was published on 22 October 2021 (EFSA Journal 2021;19(10):6879). No critical areas of concern were identified in the EFSA Conclusion. Renewal of approval of *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 was granted on 23 May 2023 (entry into force 1 July 2023); Commission Implementing Regulation (EU) 2023/999.

When the AIR 4 dossier was submitted for EU renewal of *B. thuringiensis* subsp*. kurstaki* strainABTS-351, an application to demonstrate technical equivalence of *B. thuringiensis* subsp*. kurstaki* strainABTS-351produced at a new manufacturing site for XXXX, was also submitted to Denmark. Technical equivalence was granted in January 2018.

DiPel® DF (product code ABG-6404) is the representative formulation used to support the application for renewal of approval of *B. thuringiensis* subsp. *kurstaki* strain ABTS-351, thus have been evaluated during the approval process. The representative uses are outdoor vegetables (cabbage) and indoor vegetables (tomato).

IIIM 5.1 Quality control and post-registration monitoring methods

IIIM 5.1.1 Methods to detect, isolate, and enumerate the microorganism. Methods to differentiate a mutant or genetically-modified microorganism from the parent strain

During the EU active substance peer review phase, multiple methods of detecting *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 were submitted and considered acceptable. Unequivocal identification and characterisation of strain ABTS-351, as described in Part C (IIIM 5.1.1/01), was possible using the whole genome sequencing (WGS) technology.

A genomotyping study (van der Vossen *et al*., 2008; Part C IIIM 5.1.1/02) can also be used for high-resolution differentiation between strains of the same subspecies, as well as for identification of strain ABTS-351.

Additionally, van der Vossen *et al*., 2015 (Part C IIIM 5.1.1/03) describes a quantitative polymerase chain reaction (qPCR) method used to detect *B. thuringiensis* species in a background of *B. cereus* species. The method is not able to accurately detect and quantify *B. thuringiensis* species at strain level as primer/probes specificity is low. However, it can be used to complement other methods described above.

As the insecticidal activity does not correlate with the weight of bacterial technical powder or the amount of *B.* *thuringiensis* subsp*. kurstaki* strain ABTS-351 in CFU/mL, expression of the content of the active ingredient in terms of weight or CFU/mL may not be accurate. Therefore, the active ingredient has to be standardized by bioassays that indicate the potency of its insecticidal properties. Dulmage *et al*. (1971) first developed a bioassay (IIIM 5.1.1/04) for comparison of the potency of insecticidal activity of *B. thuringiensis* strains. The method has now become a standard method use to determining *B. thuringiensis* toxin potency, thus estimation of its concentration. It is also widely acceptable by regulatory authorities.

Described in Part C (IIIM 5.1.1/05) is the validation of the bioassay based on the quantal dose response of four-day post-hatch *Trichoplusia ni* larvae, which is used to determine the content of the microorganism in the preparation.

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| Data point addressed | IIIM 5.1.1/04 |
| Author(s) (year) | Dulmage, H.T., Boening, O.P., Rehnborg, C.S., and Hansen, G.D. (1971) |
| Title | A proposed standardized bioassay for formulations of *Bacillus thuringiensis* based on the international unit |
| Report number | Journal of Invertebrate Pathology, Vol. 18, Issue 2, p. 240-245 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |

**Abstract**

Since standardization of preparations of Bacillus thuringiensis containing the δ-endotoxin cannot be accomplished by spore count alone, a bioassay is needed. However, before a bioassay can enjoy widespread use, there must be a generally accepted bioassay procedure. A bioassay based on the international unit (IU) and using the cabbage looper, Trichoplusia ni, is proposed to fulfil this requirement. In this assay, the product is incorporated into an alfalfa meal-based diet, and 15–25 mg larvae are allowed to feed on the diet for 5 days. At the end of the test period, activity is measured by determining the LD50 of the test material and comparing it with that of a standard preparation. Potency is then expressed as IU/mg.

IIIM 5.1.2 Methods to detect spontaneous change in major characteristics of microorganism

*Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 is a wild type strain and not a mutant or genetically modified. In theory, a strain alteration can occur within a strain through genetic mutation or plasmid loss. However, in practice, due to mass-transfer inoculations, the possibility that gene mutation(s) might occur during the limited time of a fermentation, and that the mutant population would reach detectable levels, is very low. Spontaneous mutations have been found to occur in *B. thuringiensis* bacteria at the levels of one mutation per 107 to 1011 cells[[1]](#footnote-2), and many of these mutations are deleterious or lethal to the cell. Furthermore, the chance for a mutation to be manifested as an altered crystal protein is even lower because the crystal protein is only one of thousands of gene products of the *B. thuringiensis* microorganism. At such low frequency, non-mutated cells would mask any surviving mutant that occurs during the fermentation process.

To ensure that no modifications occur to the production culture during growth and fermentation, all production runs are inoculated with sub-samples of the original strain. Serial transfers from the original strain are minimized. Mass transfers of the culture are used to avoid selection of a rare mutant. To ensure no contaminants are present, stringent sterility controls and precisely controlled growth conditions are used throughout the production process. Strain purity checks are routinely done at every transfer stage. The culture maintenance process previously described was developed to minimize the number of transfers and the possibility of genetic drift, and loss of plasmids during growth. Details of procedures typically used to prevent any spontaneous modification in *B. thuringiensis* susbp. *kurstkaki* (and other related strains)aredescribed in Part C under IIIM 5.1.2/01 (Smith, 1990). Smith (1990) has been evaluated during several phases of the EU approval process of *B. thuringiensis* subsp. *kurstaki* ABTS-351 (and similar strains) and is considered acceptable.

IIIM 5.1.3 Methods to define content of microorganism in appropriate terms (same as IIIM 1.7.1), incl. standardisation, sensitivity, reproducibility, statistical validity, and representative data to validate the bioassay

The content of *B. thuringiensis* subsp. *kurstaki*, strain ABTS-351 can be enumerated through multiple methods which are listed below:

* Total bacterial spore count methods as described in Part C (confidential information) under IIIM 1.7.1/01 and IIIM 5.1.3/01.
* Quantification of crystal proteins as described in Part C (confidential information) under IIIM 1.7.1/03, IIIM 5.1.3/03 and 5.1.3/04.
* Bioassay potency tests as described in Part C (confidential information) under IIIM 1.7.1/02 and IIIM 1.7.2.3/01.

IIIM 5.1.4 Methods to identify contaminant microorganism in MPCP

Bacterial pathogens may arise due to microbial contamination during the production of the technical material. However, the preparation of *Btk* (strain ABTS-351) is a pure culture fermentation process carried out under strict aseptic conditions. The integrity of the organism is routinely checked at key stages during manufacture, using standard microscopic and agar plating methods. Post fermentation handling of the organism is not performed under sterile conditions, although equipment is routinely cleaned under GMP control.

Methods to identify microbial contaminants included in the OECD Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products (ENV/JM/MONO(2011)43) have been provided in Part C IIIM 5.1.3 and Part C IIIM 1.7.2.3. The methods are standard microbiological techniques which can be used to identify the considered microbial contaminants in any microbial pest control product. However, the methods below are provided from the storage stability testing to check the bioburden before and after the storage period.

|  |  |
| --- | --- |
| Data point addressed | IIIM 5.1.4/01 |
| Author(s) (year) | Comb, A.L. (2012) |
| Title | ABG-6431 Storage stability |
| Report number | ZAB0150 |
| Test facility | Huntingdon Life Sciences Ltd. |
| Published | No |
| Test guideline | OPPTS 830.6317, OPPTS 830.6320 |
| Deviations | No |
| GLP | Yes |

**Materials and Methods:**

**Examination for contaminating microorganisms**

A portion (10 g) of the test substance was mixed with 90 mL of Buffered Peptone Water (BPW - Oxoid CM509).

For the determination of bacterial contamination, an aliquot (1 mL) of the BPW test substance broth was placed into empty duplicate sterile Petri dishes and pour plates prepared by adding molten Tryptone Soya Agar (TSA - Oxoid CM 131). The plates were incubated at 30 to 35°C for up to 5 days and then the numbers of colonies of the test organism present were recorded. In addition, the count plates were examined for the presence of contaminating bacteria amongst the colonies of test organism and the number of colonies of contaminating bacteria were recorded.

For the determination of fungal contamination, plate counts were prepared as described above except using Rose Bengal Chloramphenicol Agar (Merck - 1.00467.0500) and incubated at 20 to 25°C for up to 5 days. The numbers of colonies of contaminating fungi present were then recorded.

For the determination of the test substance spore count, the BPW test substance broth was diluted in PS and then 1 mL pour plates were prepared in duplicate using TSA as described above. The plates were incubated at 30 to 35°C for up to 5 days and then the numbers of colonies of the test organism were counted and the spore count of the test substance was calculated.

**Absence of pathogens test**

A portion (10 g) of each lot of the test substance was mixed with 90 mL of Lactose Broth (LB – Oxoid CM137) and 90 mL of Tryptone Soya Broth (TSB - Oxoid CM129). The broths were then pre-incubated at 30 to 35°C for approximately 24 hours for LB and for approximately 48 hours for TSB. Each container was then shaken, and the quantity of pre-incubated test substance transferred as appropriate for the following tests:

***Escherichia coli***

Separate portions (90 mL) of Enterobacteria Enrichment Broth (EEB - Oxoid CM317) were inoculated with the appropriate pre-incubated LB test substance broth (10 mL) and incubated at 30 to 35°C for approximately 24 hours. The samples were sub-cultured onto MacConkey Agar (Oxoid - CM0113) and incubated at 30 to 35°C for at least 48 hours.

***Salmonella***

A portion (10 mL) of the appropriate pre-incubated LB test substance broth was mixed with 90 mL of Tetrathionate Broth (TB - Oxoid CM671) and incubated at 30 to 35°C for approximately 24 hours. The samples was sub-cultured onto Xylose Lysine Desoxycholate Agar (XLD - Oxoid CM469) and Brilliant Green Agar (BGA - Oxoid CM329) and incubated at 30 to 35°C for at least 48 hours.

***Pseudomonas aeruginosa***

The appropriate pre-incubated TSB test substance broth was sub-cultured onto Pseudomonas Isolation Agar (PIA - Difco 292710) and incubated at 30 to 35°C for at least 48 hours.

***Staphylococcus aureus***

The appropriate pre-incubated TSB test substance broth was sub-cultured onto Mannitol Salts Agar (MSA - Oxoid CM85) and incubated at 30 to 35°C for at least 48 hours.

Validation of the recovery of the 4 pathogens listed above was performed using the above procedures. For each pathogen, 1 mL of an inoculum suspension containing 100 CFU of organism was added to an appropriate broth containing 10 g of test substance, equivalent to a detection limit of 10 CFU/g. A control broth without the test substance present was also inoculated with 1 mL of inoculum suspension. The above procedures were followed, and the absence or presence of the pathogen determined.

**Results:**

The results for the microbiological examination prior to and following 15 months storage at both test temperatures (15°C and 25°C) are presented in the tables below.

**Screen for bacterial contaminants prior to and following 15 months storage**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Timepoint** | **Storage condition** | **Lowest dilution factor** | **Plate count** | | **Plate count x dilution factor (CFU/g)** |
| **Colonies/plate** | **Mean** |
| Initial | - | 101 | 11, 9 | 10 | 100 |
| 15 months | 15°C | 101 | 0, 0 | 0 | < 10 |
| 15 months | 20°C | 101 | 0, 0 | 0 | < 10 |

CFU = Colony Forming Unit

**Screen for fungal contaminants** **prior to and following 15 months storage**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Timepoint** | **Storage condition** | **Lowest dilution factor** | **Plate count** | | **Plate count x dilution factor (CFU/g)** |
| **Colonies/plate** | **Mean** |
| Initial | - | 101 | 0, 0 | 0 | < 10 |
| 15 months | 15°C | 101 | 0, 0 | 0 | < 10 |
| 15 months | 20°C | 101 | 0, 0 | 0 | < 10 |

CFU = Colony Forming Unit

**Screen for bacterial pathogens in colony forming units (CFU) per gram prior to and following 15 months storage**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Timepoint** | **Storage condition** | **Colony forming units (CFU) per gram** | | | |
| ***Escherichia coli*** | ***Salmonella*** | ***Pseudomonas aeruginosa*** | ***Staphylococcus aureus*** |
| Initial | - | Absent | Absent | Absent | Absent |
| 15 months | 15°C | Absent | Absent | Absent | Absent |
| 15 months | 20°C | Absent | Absent | Absent | Absent |

CFU = Colony Forming Unit

**Conclusion:** *Escherichia coli, Salmonella, Pseudomonas aeruginosa and Staphylococcus aureus* were not

detected in any of the test substance samples.

IIIM 5.1.5 Methods to show control to a specified and acceptable level, of microbial impurities and of any other impurities of toxicological concern, including toxic metabolites, which are known or suspected to be present at any stage of the manufacturing process

Methods described in IIIM 5.1.4 above are suitable for ensuring microbial impurities in Foray® 76B are at an acceptable level. For other impurities such as β-exotoxin and enterotoxins potentially produced by *B. thuringiensis*, the methods described in Part C IIIM 5.1.5 are applicable. All studies have been evaluated during the EU review phase of *B. thuringiensis* subsp. *kurstaki* strain ABTS-351/DiPel® DF and were considered adequate.

The methods that exist to identify enterotoxins and β-exotoxins summarised lack full validation, however, as none of the non-microbial impurities listed are considered as relevant full validation is not considered necessary at present.

IIIM 5.1.6 Methods to show presence of any human and mammalian pathogens

Methods described in Part C IIIM 5.1.3/01 and Part C IIIM 1.7.2.3/01 can be used to demonstrate the presence of human and mammalian pathogens in Foray® 76B. Human and mammalian pathogens considered are based on the OECD Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products (ENV/JM/MONO(2011)43). The methods are standard microbiological techniques which can be used to show the presence of the considered microbial pathogens in any microbial pest control product.

IIIM 5.2 Storage stability test and determination of shelf life (methods of analysis)

The storage stability study (Comb, 20212) for Foray® 76B determines the activity of the product based on a biopotency. This method is suitable for determining the shelf-life of the product. Details of the methodology is provided in IIIM 5.1.1/01 above and Part C (IIIM 1.7.1//02 and IIIM 5.1.1/05).

IIIM 5.3 Production process for MPCP, describing techniques used to ensure a uniform product and procedures when hazardous contamination is detected in a batch. List starting and intermediate materials, with source and purity of each

The production process for the MPCP is described in Part C under IIIM 5.3.

IIIM 5.4 Method for determination of residues

The proposed uses of Foray® 76B (ABG-6431) are outdoor forestry uses on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) only, which do not form part of human and livestock diets. Therefore, assessments of the persistence and likelihood of multiplication in or on crops, feeding stuffs or foodstuffs, and further information on non-viable and viable residues are not required or presented.

There is no potential route of dietary exposure to viable and non-viable residues of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 from the proposed uses of Foray® 76B (ABG-6431) and no specific mitigation measures are required.

Although the use of Foray® 76B is not anticipated to be used on edible crops, a combination of techniques for identification and detection is described under IIIM 5.1.1 and IIIM 5.1.3. For example, the Total Viable Count method (described in Part C, IIIM 5.1.3/01), and qPCR method (van der Vossen *et al.*, 2015), described in Part C III 5.1.1/03 may be used to enumerate the quantity of *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 cells in other environments.

No monitoring methods for the metabolites are deemed necessary as no residue definition or MRLs is currently set for the metabolites.

**Evaluator**: The dRR is accepted.

In the future the dossier should be definitely submitted on new dRR templates (Section 5 instead of Section 2)

Appendix 1 – List of data submitted in support of the evaluation

| **Annex point** | **Author** | **Year** | **Title**  **Source (where different from company)**  **Company, Report No.**  **GLP or GEP status (where relevant)**  **Published or Unpublished** | **Data protection claimed Y/N** | **Owner** |
| --- | --- | --- | --- | --- | --- |
| IIIM 5.1.1/04 | Dulmage, H.T., Boening, O.P., Rehnborg, C.S., and Hansen, G.D. | 1971 | A proposed standardized bioassay for formulations of *Bacillus thuringiensis* based on the international unit  Journal of Invertebrate Pathology, Vol. 18, Issue 2, p. 240-245  GLP: No  Published | N | Open literature |
| III 5.1.4/01 | Comb, A.L. | 2012 | ABG-6431 Physico-Chemical Properties  Huntingdon Life Sciences Ltd., UK  Report-No.: ZAB0121  GLP: Yes  Unpublished | N | XXXX |

1. Prescott, L.M., Harley, J.P. and Klein, D.A. 1990. Microbiology. W.C. Brown. IA. pp 345. [↑](#footnote-ref-2)