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| REGISTRATION REPORT Part B  Section 3: Mammalian Toxicology  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

|  |  |
| --- | --- |
| **When** | **What** |
| August 2023 | Initial version submitted by the applicant for Art. 43 |
| May 2024 | Version evaluated by zRMS PL |
| November 2024 | Version revised by zRMS PL |
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IIIM 7 TOXICOLOGICAL STUDIES

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 7.1 Acute toxicity studies

The following studies were performed on Foray® 76B: acute LD50 oral (rat), acute LD50 dermal (rat), acute LC50 inhalation (rat), skin irritation (rabbit), and eye irritation (rabbit). The sensitization of the skin was conducted with Foray® 76B a formulation similar to Foray® 75B (composition provided in Part C). The results are summarised in Table 7.1-1 and individual study summaries are provided (IIIM 7.1.1 to 7.1.6). Although these studies were not evaluated as part of the EU review of the *Bacillus thuringiensis* subsp. *kurstaki*, strain ABTS-351, they have been evaluated for the existing authorisation of Foray® 76B and are considered acceptable.

Table 7.1-1: Summary of acute toxicity studies on Foray® 76B

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Study type / Report** | **Species** | **Test item** | **Dose level** | **Result** | **Classification** |
| Acute oral toxicity / IIIM 7.1.1/01 | Rat | ABG-6431 (Foray® 76B; formerly referred to as Foray® 75B) | 5050 mg/kg bw | LD50 > 5050 mg/kg | Not required |
| Acute dermal toxicity/ IIIM 7.1.2/01 | Rat | ABG-6431  (Foray® 76B; formerly referred to as Foray® 75B) | 2500 mg/kg bw | LD50 > 2500 mg/kg | Not required |
| Acute inhalation toxicity / IIIM 7.1.3/01 | Rat | ABG-6431  (Foray® 76B; formerly referred to as Foray® 75B) | 3.22 mg/L  for 4 h | LD50 > 3.22 mg/L (maximum attainable exposure) | Not required |
| Dermal irritation / IIIM 7.1.4/01 | Rabbit | ABG-6431  (Foray® 76B; formerly referred to as Foray® 75B) | 0.5 g/animal | Not irritating | Not required |
| Eye irritation / IIIM 7.1.5/01 | Rabbit | ABG-6431  (Foray® 76B; formerly referred to as Foray® 75B) | 0.1 g/animal | Moderately irritating | Not required |
| Skin sensitisation  Local Lymph Node Assay  IIIM 7.1.6/01 | Mice | ABG-6431  (Foray® 76B; formerly referred to as Foray® 75B) | 25%  50%  100% | Not sensitising | Not required |

Foray® 76B containing 206.5 g/L *Bacillus thuringiensis* subsp. *kurstaki* (strain ABTS-351) has a low toxicity in respect to acute oral, dermal and inhalation toxicity. Foray® 76B was not irritating to the rabbit skin, moderately irritating to rabbit eyes and was non-sensitising to mice in a Local Lymph Node Assay. Considering all submitted data and the classification of the ingredients, Foray® 76B does not require classification for acute effects.

According to the criteria given in Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008, the following classification and labelling with regard to toxicological data is proposed for the preparation:

**Hazard pictograms:** None

**Signal words:** None

**Hazard statements:** None

**Precautionary statements**:

**P261** - Avoid breathing dust/fume/gas/mist/vapors/spray.

**P280 -** Wear protective gloves, protective clothing and eye or face protection.

**P363 -** Wash contaminated clothing before reuse

**P302 + P352-** IF ON SKIN: Wash with plenty of water

**P501** - Dispose of contents and container in accordance with all local, regional, national and international regulations.

**Supplemental label elements:**

**EUH 208:** Contains 1,2-benzisothiazol-3(2H)-one (BIT). May produce an allergic reaction

**EUH 210:** Safety data sheet available on request.

**EUH 401:** To avoid risks to human health and the environment, comply with the instructions for use

Contains *Bacillus* *thuringiensis* subsp. *kurstaki*. Microorganisms may have the potential to provoke sensitising reactions.

**SP 1 -** Do not contaminate water with the product or its container (Do not clean application equipment near surface water/Avoid contamination via drains from farmyards and roads)**.**

IIIM 7.1.1 Acute oral toxicity

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.1.1/01 |
| Author(s) year | XXXX (1991) |
| Title | An acute oral toxicity study in rats |
| Report No. | 8162-91 |
| Test facility | XXXX |
| Published | No |
| Test guidelines | EPA Guidelines 81-1 |
| Deviations | Not applicable |
| GLP | Yes |

**Material and Methods:**

The test material consisted of Foray® 75B FC Batch No. BBN7001.

A single dose of 5050 mg/kg of undiluted test material was administered by oral intubation to five male and five female albino rats. Observations for mortality and signs of pharmacologic and/or toxicological effects were made at least three times on the day of treatment and at least once daily thereafter for 14 days. Individual body weights were recorded just prior to treatment and on days 7 and 14. A gross necropsy was conducted on each animal at termination of the study.

**Findings:**

No mortalities were observed. No effects on body weight and no abnormalities upon macroscopic examination were recorded. All animals appeared normal for the duration of the study.

**Conclusion/endpoint:**

The acute lethal oral dose of Foray® 75B FC in rats was found to be greater than 5050 mg/kg bw when administered undiluted to albino rats. The preparation does not warrant classification as being toxic or harmful on the basis of its acute oral toxicity.

|  |  |
| --- | --- |
| Study Comments: IIIM 7.1.1/01 | The applicant’s evaluation is considered acceptable. The study meets the requirements of Part B of Regulation (EU) No 284/2013, and no further data are considered required for this endpoint.  It is noted that Foray® 76B was formerly referred to as Foray® 75B. |
| Agreed endpoint: IIIM 7.1.1/01 | The acute oral LD50 value was estimated to be higher than 5050 mg/kg bw in male and female rats. Therefore, Foray® 76B is considered of low acute oral toxicity; no classification is required. |

IIIM 7.1.2 Acute percutaneous (dermal) toxicity

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.1.2/01 |
| Author(s) year | XXXX (1993a) |
| Title | Foray® 76B assessment of acute dermal toxicity in rats |
| Report No. | 92843 |
| Test facility | XXXX |
| Published | No |
| Test guidelines | OECD 402, EPA 81-2 and EEC, No. L 251/103 |
| Deviations | None |
| GLP | Yes |

**Material and Methods:**

The test substance consisted of 2500 mg Foray® 76B/kg bw, the dose volume was 2.2 mL/kg bw. In an acute dermal toxicity study, five male and five female SPF Wistar rats were exposed to Foray® 76B by the dermal route. The day before application of the test article the hair was removed from the back and flanks with an electric clipper. The day after the test article was applied onto an area of 5 x 6cm skin, which then was covered with a 4-layer gauze pack. The gauze packs were fixed with tape wound round the trunk. The rats were caged individually for the 24 hours of exposure time after which the dressings were removed. The skin and surrounding hair were sponged with soap and lukewarm water. The animals were then caged in groups of 2 or 3 during the rest of the 14-day observation period. Animals were observed 1, 3, and 6 hours after application and then daily. Body weights were recorded weekly. All animals were killed on day 14, the end of the observation period.

**Findings:**

No mortalities were observed. No clinical signs of toxicity were noted. No effects on body weight and no abnormalities upon macroscopic examination were recorded.

**Conclusion:**

The acute lethal dermal dose of Foray® 76B in rats was found to be greater than 2500 mg/kg bw. The preparation does not warrant classification as being toxic or harmful on the basis of its acute dermal toxicity.

|  |  |
| --- | --- |
| Study Comments: IIIM 7.1.2/01 | The applicant’s evaluation is considered acceptable. The study also meets the requirements of Part B of Regulation (EU) No 284/2013. Furthermore, microorganisms are not expected to penetrate the intact skin. No further data are therefore required for this endpoint. |
| Agreed endpoint: IIIM 7.1.2/01 | The acute dermal LD50 value was estimated to be higher than 2500 mg/kg bw in male and female rats. Therefore, Foray® 76B is considered of low acute dermal toxicity; no classification is required. |

IIIM 7.1.3 Acute inhalation toxicity to rats

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.1.3/01 |
| Author(s) year | XXXX (1991) |
| Title | Foray® 76B (formerly identified as Foray® 75B) acute inhalation toxicity study in rats with MPCA |
| Report No. | 8163-91 |
| Test facility | XXXX |
| Published | No |
| Test guidelines | US EPA Guideline 81-3 |
| Deviations | Not applicable |
| GLP | Yes |

**Material and Methods:**

Five male and five female albino rats were exposed to an aerosol generated from the undiluted liquid test material for a period of four hours. During the exposure period, the animals were individually housed in stainless steel cages within a 500 L New York University design, stainless steel, dynamic flow inhalation chamber. The concentration of test material in the exposure atmosphere was determined gravimetrically twice per hour (taken from the breathing zone of the animals), and nominally at the end of the exposure. Particle size (taken from the breathing zone of the animals) was determined twice during the exposure, using an Andersen cascade impactor, at a rate of 28.3 L/minute for a duration of 5minutes. The animals were returned to their stock laboratory cages at the termination of the exposure period.

Observations for mortality and pharmacologic and/or toxicological signs were made frequently on the day of exposure and at least once daily thereafter for 14 days. Individual body weights were recorded just prior to the inhalation exposure and on Days 7 and 14. A gross necropsy was conducted on each animal at termination of the study.

**Findings:**

Noanimals died during the study. The acute inhalation LC50 for Foray® 75B FC is greater than 3.22 mg/L (3.13 x l09CFU/L) when administered undiluted as an aerosol to albino rats. Prominent in-life observations included activity decrease, alopecia, piloerection and polyuria.

**Conclusion:**

An attempt was made to reach a concentration of 5.0 mg/L with 25% of particles under 1 micron. However, due to the nature of the test material, a test level could not be generated containing at least 25% of particles under 1 micron to calculate an LC50. The maximum attainable exposure concentration was 3.22 mg/L.

|  |  |
| --- | --- |
| Study Comments: IIIM 7.1.3/01 | The applicant’s evaluation is considered acceptable. The study also meets the requirements of Part B of Regulation (EU) No 284/2013. No further data are therefore required for this endpoint. |
| Agreed endpoint: IIIM 7.1.3/01 | The acute inhalation LC50 value was estimated to be higher than 3.22 mg/L in male and female rats. Although the tested concentration is lower than the limit value of 5 mg/L required in EU but is the maximum attainable exposure. Therefore it is concluded that Foray® 76B is considered of low acute inhalation toxicity; no classification is required. |

IIIM 7.1.4 Skin irritation

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.1.4/01 |
| Author(s) year | XXXX (1993b) |
| Title | Foray® 76B assessment of the skin irritant effect in rabbits |
| Report No. | 92834 |
| Test facility | XXXX |
| Published | No |
| Test guidelines | OECD 404, EPA Guideline 81-5, EEC B.4, No. L 251/106 |
| Deviations | None |
| GLP | Yes |

**Material and Methods:**

In this primary dermal irritation study, six SPF albino female rabbits were weighed and an area of 10 x 10 cm on the back was clipped as closely as possible with an electric clipper. The clipped area was divided into 4 fields. The 2 anterior fields were used for testing the experimental preparation. To each of twelve gauze patches (2.5 x 2.5 cm) 0.5 mL of the test article was applied, and the patches were placed on the appropriate test site at the back of each rabbit. After an exposure time of 4 hours the tape and patches were removed, and the treated skinwas cleaned with soap and lukewarm water. The skin reactions were read 30 minutes later according to an OECD 404 scale. Readings were also made 60 minutes as well as 24, 48, and 72 hours and on days 4-8 after application of the test substance. At each reading the skin was also examined for any lesion and other signs of toxic effects.

**Findings:**

Very slight erythema was noted in two rabbits 30 min and 1 h after exposure. At the 24-hour observation very slight erythema was observed in 5 rabbits and slight erythema in 1 rabbit. At the 48 hours observation very slight erythema was observed in all 6rabbits, still in 3rabbits at the 72-hour observation and in 2 rabbits at the observations on day 4 and 5*.* One rabbit still showed very slight erythema at the observations on day 6and 7, but at the observation on day 8 the skin of this rabbit appeared normal and the study was terminated. No oedema was observed at any of the observations.

Table IIIM 7.1.4-1 Erythema scores in rabbits

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Animal No. | **Weight** | **Left or Right Test Field** | **Observation interval** | | | | | | | | | | **ERYTHEMA:  Mean per rabbit per test field 24h+48h+72h** |
| **Hours** | | | | | | | | | |
| **1/2** | **1** | **24** | **48** | **72** | **96** | **120** | **144** | **168** | **182** |
| 6544 | 2.4 | L | 0 | 0 | 1 | 1 | 1 | 0 | 0 |  |  |  | **1.00** |
| R | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |  |  | **0.00** |
| 6545 | 2.0 | L | 1 | 1 | 1 | 1 | 0 |  |  |  |  |  | **0.67** |
| R | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| 6546 | 2.4 | L | 0 | 0 | 1 | 1 | 0 |  |  |  |  |  | **0.67** |
| R | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| 6547 | 2.6 | L | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | **1.00** |
| R | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | **1.33** |
| 6549 | 2.2 | L | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |  |  | **1.00** |
| R | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |  |  | **0.67** |
| 6550 | 2.6 | L | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| R | 0 | 0 | 1 | 1 | 0 |  |  |  |  |  | **0.67** |

L: Left test field

R: Right test field

Table IIIM 7.1.4-2 Oedema scores in rabbits

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Animal No. | **Weight** | **Left or Right Test Field** | **Observation interval** | | | | | | | | | | **OEDEMA:  Mean per rabbit per test field 24h+48h+72h** |
| **Hours** | | | | | | | | | |
| **1/2** | **1** | **24** | **48** | **72** | **96** | **120** | **144** | **168** | **182** |
| 6544 | 2.4 | L | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |  |  | **0.00** |
| R | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |  |  | **0.00** |
| 6545 | 2.0 | L | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| R | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| 6546 | 2.4 | L | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| R | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| 6547 | 2.6 | L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **0.00** |
| R | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **0.00** |
| 6549 | 2.2 | L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |  | **0.00** |
| R | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |  | **0.00** |
| 6550 | 2.6 | L | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |
| R | 0 | 0 | 0 | 0 | 0 |  |  |  |  |  | **0.00** |

L: Left test field

R: Right test field

**Conclusion:**

According to the CLP legislation (Regulation (EC) No 1272/2008, the test article, Foray® 76 B shall not be classified as skin irritating.

|  |  |
| --- | --- |
| Study Comments: IIIM 7.1.4/01 | The applicant’s evaluation is considered acceptable. The study also meets the requirements of Part B of Regulation (EU) No 284/2013. No further data are therefore required for this endpoint. |
| Agreed endpoint: IIIM 7.1.4/01 | Foray® 76B is weakly irritating to rabbit skin; no classification is warranted. |

IIIM 7.1.5 Eye irritation

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.1.5/01 |
| Author(s) year | XXXX (1991) |
| Title | Eye irritation study in rabbits with the end product Foray® 75B |
| Report No. | 90113 |
| Test facility | XXXX |
| Published | No |
| Test guidelines | FIFRA's Pesticide Assessment Guidelines, Subdivision M: Toxicology Guidelines for Microbial Pest Control Agents, paragraph 152A-14 |
| Deviations | Not applicable |
| GLP | Yes |

**Materials and Methods:**

In this acute eye irritation study 0.1 mL of Foray® 75B were instilled into one eye of each of six healthy adult male New Zealand white rabbits. The untreated eye served as a control. Observations were recorded at 1, 24, 48, 72 hours and 4, 5, 6, and 7 days after the instillation of the test substance. The eyes were examined using a clinic lamp to ensure uniform lighting. At each observation time, lesions of the conjunctiva, iris and cornea were scored separately using a numerical system.

**Findings:**

Findings on 1, 24, 48, 72 hours and days 4 and 7 are reported. Since there were no reactions 7 days after the instillation of test substance, no further clinical observations were made. No corneal or iridal reactions were noted at any of the readings. One hour post application mild redness of the conjunctiva was observed in all six rabbits, accompanied by small amounts of discharge in three of the animals. Twenty-four hours post application mild redness was present in three animals accompanied by discharge in animal No. 11. Forty-eight hours post application diffuse crimson red colouration was present in animal No. 2 and mild redness in animal No. 7, 11, and 16. In animal No. 11 the redness was accompanied by discharge. Seventy-two hours post application and on day 4 mild redness was observed in four animals, this reaction cleared within the next day in three of the above-mentioned animals but persisted until day 7 in animal No. 2. No reactions were seen at day 7.

Table 7.1.5-1 Eye irritation scores of rabbits exposed to 0.1 mL Foray® 75B at 1 hour to 7 days after instillation

|  |  | Conjunctiva | | | | Iritis | Corneal  opacity | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Readings | Rabbit | Oedema | | Redness | |
|  | nos. | Degree | Area |
|  | 2 | 0 | d | 1 |  | 0 | 0 | 0 |
|  | 7 | 0 | d | 1 |  | 0 | 0 | 0 |
|  | 11 | 0 | d | 1 |  | 0 | 0 | 0 |
| 1 Hour | 12 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 14 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 16 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 2 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 7 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 11 | 0 | d | 1 |  | 0 | 0 | 0 |
| 24 Hours | 12 | 0 |  | 0 |  | 0 | 0 | 0 |
|  | 14 | 0 |  | 0 |  | 0 | 0 | 0 |
|  | 16 | 0 |  | 0 |  | 0 | 0 | 0 |
|  | 2 | 0 |  | 2 |  | 0 | 0 | 0 |
|  | 7 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 11 | 0 | d | 1 |  | 0 | 0 | 0 |
| 48 Hours | 12 | 0 |  | 0 |  | 0 | 0 | 0 |
|  | 14 | 0 |  | 0 |  | 0 | 0 | 0 |
|  | 16 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 2 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 7 | 0 |  | 1 |  | 0 | 0 | 0 |
|  | 11 | 0 |  | 1 |  | 0 | 0 | 0 |
| 72 Hours | 12 | 0 |  | 0 |  | 0 | 0 | 0 |
|  | 14 | 0 |  | 0 |  | 0 | 0 | 0 |
|  | 16 | 0 |  | 1 |  | 0 | 0 | 0 |
| d: Any amount different from normal (does not include small amounts observed in inner canthus of normal animals) | | | | | | | | |
| D: Discharge with moistening of the lids and hairs just adjacent to the lids | | | | | | | | |
| D+: Discharge with moistening of the lids and hairs and considerable area around the eye | | | | | | | | |

**Table 7.1.5-2 Eye irritation scores of rabbits exposed to 0.1 mL Foray® 75B – mean scores per rabbit (24h+48h+72h)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Rabbit nos. | **Mean per rabbit 24h+48h+72h** | | | |
| **Conjunctival oedema** | **Conjunctival redness** | **Iritis** | **Corneal opacity** |
| 2 | 0.00 | 1.33 | 0.00 | 0.00 |
| 7 | 0.00 | 1.00 | 0.00 | 0.00 |
| 11 | 0.00 | 1.00 | 0.00 | 0.00 |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 |
| 14 | 0.00 | 0.00 | 0.00 | 0.00 |
| 16 | 0.00 | 0.67 | 0.00 | 0.00 |

**Table 7.1.5-3 Reversibility of eye irritation scores of rabbits exposed to 0.1 mL Foray® 75B**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Conjunctiva | | | | Iritis | | Corneal  Opacity | | |
| Readings | Rabbit |  |  |  |  |
|  | nos. | Oedema | | Redness | | Degree | | Area |
| Day 4 | 2 | 0 |  | 1 |  | 0 |  | 0 |  | 0 |
| 7 | 0 |  | 1 |  | 0 |  | 0 |  | 0 |
| 11 | 0 |  | 1 |  | 0 |  | 0 |  | 0 |
| 12 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |
| 14 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |
| 16 | 0 |  | 1 |  | 0 |  | 0 |  | 0 |
| Day 7 | 2 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |
| 7 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |
| 11 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |
| 12 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |
| 14 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |
| 16 | 0 |  | 0 |  | 0 |  | 0 |  | 0 |

**Conclusion:**

The instillation of the End Product Foray® 75B, batch BBN 7001 to the conjunctival sac of 6 rabbits caused mild temporary reactions. Under the test conditions employed Foray® 75B, batch BBN 7001 may consequently be considered as “weakly irritating” to the rabbit eye.

|  |  |
| --- | --- |
| Study Comments: IIIM 7.1.5/01 | The applicant’s evaluation is considered acceptable. The study also meets the requirements of Part B of Regulation (EU) No 284/2013. No further data are therefore required for this endpoint.  Please note that Foray® 76B was formerly referred to as Foray® 75B. |
| Agreed endpoint: IIIM 7.1.5/01 | Foray® 76B was found to be weakly irritating to rabbit eyes, where mild temporary reactions to Foray® 76B were found to resolved within 7 days after application. |

IIIM 7.1.6 Skin sensitisation

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.1.6/01 |
| Author(s) year | XXXX (2011) |
| Title | Foray® 76B (ABG-6431) – Local Lymph Node Assay (LLNA) in Mice |
| Report No. | 32053 |
| Test facility | XXXX |
| Published | No |
| Test guidelines | U.S. E.P.A. OPPTS 870.2600  OECD Test No. 429 |
| Deviations | None |
| GLP | Yes |

**Materials and Methods:**

Twenty-five µL of three concentrations (25%, 50%, and 100%) of the test substance in 1% (w/w) mixture of pluronic L92 surfactant in distilled water or the vehicle alone were topically applied to twenty healthy test mice (5 mice/group) for three consecutive days. Three days after the last application, the mice were given a 20 µCi IV injection of 3H-methyl thymidine. Five hours later, the draining (auricular) lymph nodes were harvested and prepared for analysis in a scintillation counter. The results are presented in disintegrations per minute per mouse (dpm/mouse). Each animal’s ears were also evaluated for erythema and edema prior to each application and again on Day 6, prior to the IV injection. A positive control group (five animals) was maintained under the same environmental conditions and treated with a 25% (w/w) mixture of alpha-Hexylcinnamaldehyde Technical (HCA) in a 1% (w/w) mixture of pluronic L92 surfactant in distilled water in the same manner as the test animals.

**Findings:**

Lymph node assessment

Approximately five hours after the injection, the draining auricular lymph nodes from all animals were excised. The lymph nodes were pooled for each individual mouse. A single cell suspension of lymph node cells (LNC) was prepared in PBS by gently massaging the lymph nodes between the frosted ends of two microscope slides over a collection vessel. The slides were then rinsed briefly with PBS into the vessel. The contents of the vessel were transferred to a centrifuge tube and washed with an excess of PBS and centrifuged for approximately 10 minutes at 1750 rpm, with a g-force of 200 g. This process was carried out twice. In both cases, the supernatant was decanted and discarded following each centrifugation. After the second wash, approximately 5 mL of 25% trichloroacetic acid (TCA, Lot #: 101712) was then added to the sediment and the tube was vortexed briefly. The DNA was then precipitated in the TCA at approximately 6.5-7.2°C overnight (approximately 18 hours).

Following the overnight precipitation of the DNA, the tubes were centrifuged again for approximately 10 minutes at 1750 rpm and the supernatant was discarded. The resulting precipitate was re-suspended using 1 mL of TCA and transferred to 10 mL of scintillation fluid. Incorporation of 3H-methyl thymidine was measured by B-scintillation counting and expressed as disintegrations per minute, minus background dpm.

The mean and standard deviation of dpm per group were calculated. A stimulation index (SI) was derived for each experimental group by dividing the mean dpm of each experimental group by the mean dpm of the negative vehicle control group. Statistically significant increases in cell proliferation in the test concentration groups compared to the vehicle control group and/or SIs of greater than or equal to 3.0 generally indicate a positive response.

A summary of results for vehicle, test, and positive control animals is presented in Table below.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Dose Level | Group # | Group Mean DPM | SI | Sensitization Response |
| Vehicle Control | 1 | 513.19 | - | N/A |
| 25% Test Substance | 2 | 1028.41 | 2.00 | Not a Sensitizer |
| 50% Test Substance | 3 | 1039.26 | 2.03 | Not a Sensitizer |
| 100% Test Substance | 4 | 1193.25 | 2.33 | Not a Sensitizer |
| 25% HCA-Positive Control | 5 | 2819.11\* | 5.49 | Positive - valid study |

N/A= Not Applicable

\* Statistically significant difference from vehicle control at p < 0.001 by Kruskal-Wallis Test

Although six mice lost body weight during the study, all animals appeared active and healthy throughout the study.

Very slight erythema was noted for most sites between Days 2 and 6 when dosed with the vehicle or at a 25%, 50% or 100% concentration of the test substance in 1% pluronic L92 surfactant in distilled water. A stimulation index (SI) of greater than 3.0 was not observed in any of the test groups treated with 25%, 50% and 100% Foray® 76B (ABG-6431).The positive control (HCA) at 25% produced a dermal sensitization response in mice (SI=5.49). Therefore, the LLNA test system was valid for this study with Foray® 76B (ABG-6431).

**Conclusions:**

Based on these findings and on the evaluation system used, Foray® 76B (ABG-6431) is not considered to be a contact dermal sensitizer at concentrations equal to or less than 100%.

The positive response observed in the concurrent positive control validation study with alpha‑Hexylcinnamaldehyde Technical (HCA) validates the test system used in this study.

|  |  |
| --- | --- |
| Study Comments: IIIM 7.1.6/01 | The applicant’s evaluation is considered acceptable. The study also meets the requirements of Part B of Regulation (EU) No 284/2013. No further data are therefore required for this endpoint. |
| Agreed endpoint: IIIM 7.1.6/01 | Foray® 76B was considered not sensitising to the mouse following exposure via the skin. However, microbial active substances are generally considered to be potential skin sensitizers. Therefore, all products containing microbial active substances must carry a standard label phrase indicating the potential to provoke sensitising reactions. |

IIIM 7.2 Operator, workers, residents and bystander exposure: monitoring data (if available)

*Bacillus thuringiensis* acts highly specifically and is not pathogenic to mammals. This has been shown in many tests on toxicity, pathogenicity and infectiveness to vertebrates, all without adverse effects.

No harmful effects have been observed on personnel in research or industrial mass production, over a production period of more than 20 years. Because all components of the preparation used are of negligible toxicity as well, a toxic effect of Foray® 76B on the operator, worker, or bystander can be excluded. For the same reasons no maximum allowable concentration (MAC) in drinking water was calculated.   
There is a long history (over three decades) of safe use of commercial *B. thuringiensis*-based products in crop protection without unacceptable risks to operators reported. Although microorganisms are unexpected to penetrate intact skin as it is an effective barrier for microorganisms, it is recommended on the label operators wear gloves, goggles, and protective clothing (e.g., coveralls) during preparation and application of Foray® 76B. As Foray® 76B is a suspension concentrate, significant exposure by inhalation during mixing and loading is not expected, especially as the product label recommends that operators use appropriate protective equipment during preparation and application of the product.

Furthermore, literature evidence suggests exposure of greenhouse workers to products containing *B. thuringiensis* spores did not result in the occurrence of respiratory symptoms or adverse effect on lung function, although an increase in IgE antibody levels was observed (Bernstein *et al*., 1999[[1]](#footnote-2); Doekes *et al*., 2004[[2]](#footnote-3); Baelum *et al*., 2012[[3]](#footnote-4)).

However, in the 2020 EFSA conclusion of the renewal of the approval for *B.* *thuringiensis* subsp. *kurstaki* strain ABTS-351, it was noted that potential adverse health effects following repeated exposure by inhalation of *B. thuringiensis* subsp. *kurstaki* strain ABTS-351and the Cry proteins could not be excluded for the intended representative uses. This was based on the results from published non-GLP studies by Barfod *et al.* (2010) and Mezzomo *et al*. (2015b) which were considered for evaluation of EU renewal of approval of several *B. thuringiensis* strains, including *B.* *thuringiensis* subsp. *kurstaki* strain ABTS-351.

A further rebuttal of the effects reported by Barfod *et al.* (2010) is provided below. Although this information is specific for the active substances and products evaluated in Barfod *et al.* (2010), the conclusions are considered to be relevant for assessing the risk of Foray® 76B, containing 206.5 g/L *B. thuringiensis* subsp. *kurstaki* strain ABTS-351.

In Barfod *et al.* (2010), products containing *B. thuringiensis* subsp. *israelensis* strain AM 65-52 (*Bti*) and *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 (*Btk*) were administered by aerosol inhalation and intratracheal instillation to mice for 60 min/day for 5 days/week over a period of 2 weeks, at one dose level (the aerosol inhalation dose of 5 × 104 CFU per mouse per exposure was chosen to “mimic occupational exposure”). In addition, both products were intratracheally instilled with a bolus dose. This study, non-GLP and not test guideline compliant, showed no changes in lung function parameters or indications of airway irritation 70 days after the end of the exposure period. However, after 70 days, some degree of interstitial lung inflammation was detected in 3 out of 17 mice after aerosol treatment and all 20 mice following intratracheal instillation with the product containing *Bti*, whereas less significant effects were observed in mice treated with the product containing *Btk*. In the commentary of the RAR for the *Btk*, the RMS (Denmark) noted that histopathological pictures of lung tissue taken from control animals were only shown in low magnification compared to treated animals, and that the “subchronic inflammation” observed in this study was most likely due to the prolonged presence of *Bt* spores or other ingredients/residues from the products in the lungs, triggering and maintaining the inflammatory response. As a result of these inconclusive study results, inhalation exposure assessment could not be finalised without additional information. Use of respiratory protective equipment (RPE) was recommended pending the availability of additional information to suggest that effects observed in the test mice are not expected following exposure to *B. thuringiensis* subsp. *kurstaki* strain ABTS-351spores or DiPel® ES (ABG-6158).

An assessment, presented in Appendix 2 of Part C (because it includes details of co-formulants), investigated factors which may have contributed to the reported inflammatory effects in mice detailed by Barfod *et al*. (2010), following repeated aerosol exposure (over a period of 2 weeks) and intratracheal instillation of products containing Bti and Btk. The most pronounced inflammatory changes were noted in animals given VectoBac (Gnatrol® SC, Bti) which only contains 11.61% (w/w) of *Bti* and approx. 88% (w/w) of co-formulants. Therefore, a review of inhalation toxicity data of the co-formulants in Gnatrol® SC, XenTari® WG, and DiPel® DF was performed. Assessment of the co-formulants did not identify any constituent that, at the given concentration and formulation-type, may be of concern.

Moreover, various deficiencies in the study conduct and reporting of results were discussed. These include the use of only one dose level, inconsistent or absence of control data, insufficient magnification of histological data (photomicrographs) and use of an animal species which may be particularly susceptible to inflammatory responses as discussed in Part C, Appendix 2. Other deficiencies in Barfod *et al.,* (2010), contributing to results which are irrelevant for occupational risk assessment of a plant protection product have also been discussed in Part C, Appendix 2. These include the administration of a considerably higher dose of the test material in the intratracheal instillation experiment and confounding factors associated with instillation, *e.g.* concentrated and uneven deposition within the lung leading to overload and resulting inflammatory response.

Overall, several factors indicate that the repeat dose results presented by Barfod *et al.* (2010) are inherently flawed and do not provide a compelling indication of toxicological effect on the lungs and that the findings generated following intratracheal instillation are considered to be irrelevant for occupational risk assessment due to exposure conditions unrepresentative of the occupational setting.

Although Mezzomo *et al.* (2015b) was not specifically used by the applicant to support the renewal of *B.* *thuringiensis* subsp. *kurstaki* strain ABTS-351, Authorities appear to have extended the conclusions of the study for their assessments of the genotoxic potential of Cry proteins produced by *B.* *thuringiensis* subsp. *kurstaki* strain ABTS-351 and its implication of non-dietary exposure assessments. Mezzomo *et al.* (2015b) investigated the genotoxicity potential of a selection of Cry proteins in a mouse micronucleus study following intraperitoneal administration. Positive results were observed with the spore-crystal complex containing Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa. These results were considered equivocal as several uncertainties were noted. Particularly, it was unclear whether or not, the Cry proteins had been solubilised or activated prior to administration. It may seem that the Cry proteins had not been solubilised, given the information provided in Mezzomo *et al.* (2015b), as well as the information provided in Mezzomo *et al.* (2013). However, the mortalities observed in Mezzomo *et al.* (2015b), suggested that some kind of activation may possibly have occurred. Since an alkaline condition (pH ≥10) is required for solubilisation and activation of Cry proteins but the human gut is acidic, EFSA review concluded that genotoxicity of the insecticidal proteins is not a concern for dietary exposure. However, a possible concern for non-dietary exposure could not be excluded.

It is the opinion of the applicant that the Mezzomo *et al.* (2015b) investigated a compromised test system and that results presented in the paper cannot be reliably interpreted due to deficiencies in methodology, performance and reporting to implicate *Bt* spore crystals as a potential genotoxin. Furthermore, it cannot be ruled out that the *Bt* spore crystals may have been partly solubilised and activated prior to injection at high dose into the test mice, as the normal physiological condition is unlikely to be favourable for the solubilisation and activation of the spore crystals. Further details related to the abovementioned arguments are included in Appendix 3 below.

Considering the arguments presented above, the recommendation stated in the EFSA conclusion that operators and workers must use RPE when during activities potentially requiring close contact with Foray® 76B during mixing and loading is unmerited. Inhalation exposure during dilution and loading activities is likely to be low as Foray® 76B is a suspension concentrate. However, spray application may also generate particles of inhalable size, therefore the additional use of respiratory protective equipment (RPE; disposable filtering face piece respirator to at least EN149 FFP3 or equivalent) is recommended. For other applications, including mixing and loading activities, drench or automated irrigation, the use of PPE as recommended below in IIIM 7.2.1.2 is considered sufficient.

**RMS PL**:

As indicated in the Conclusions of Peer review of the pesticide risk assessment of the active substance Bacillus thuringiensis subsp. kurstaki strain ABTS-351 (EFSA Journal 2021;19(10):6879) regarding non-dietary exposure to Bacillus thuringiensis subsp. kurstaki strain ABTS-351, since toxicity/infectivity after repeated exposure by inhalation could not be concluded, and a genotoxic potential of the Cry proteins could not be excluded by nondietary exposure, the risk assessment by inhalation for residents and bystanders cannot be concluded, except for permanent greenhouses (issue not finalised). In the absence of a quantitative risk assessment, the use of respiratory protective equipment for the operators and workers might be considered to reduce the exposure via inhalation. The RMS (Denmark and the Netherlands ) disagreed with this view, being of the opinion that Bacillus thuringiensis subsp. Kurstaki preparations are not to be considered of health concern for operators, workers, bystanders and residents.

zRMS PL agrees and support the opinion of Denmark and the Netherlands, which were the rapporteur and co-rapporteur Member States preparing the registration report for Bacillus thuringiensis subsp. kurstaki strain ABTS-351 (EFSA Journal 2021;19(10):6879), that Bacillus thuringiensis subsp. Kurstaki preparations are not to be considered as a health concern for operators, workers, bystanders and residents. However, based on the precautionary principle it is recommended to use the respiratory protective equipment (RPE; disposable filtering face piece respirator to at least EN149 FFP3 or equivalent) for the operators when exposure to sprayed product is possible during application of the product with aerial (airborne equipment) and ground agricultural equipment (orchard sprayer) as proposed by the applicant.

**Workers**

No appropriate models are currently available to accurately estimate worker exposure from application of microorganisms. Typically, worker exposure values are estimated for plant protection products containing chemical active substances using models and the outcome is compared to an appropriate toxicological endpoint (*e.g.* AOEL). However, in the case of *B. thuringiensis* subsp. *kurstaki* strain ABTS-351, derivation of AOEL is not applicable based on the lack of toxicity, infectivity and pathogenicity. Therefore, quantitative assessment of worker exposure is considered not required for Foray® 76B. Nevertheless, it is recommended that unprotected workers should be excluded from areas during spray application and until the product has dried on the foliage. Workers present in areas during spray application should use appropriate PPE (coveralls, gloves and face masks). The use of PPE is not required for workers present in areas being treated by drench or drip application. Although microorganisms are unlikely to penetrate intact skin, it is considered good practice for workers monitoring efficacy, checking the forest following application or handling soil treated with Foray® 76B to wear suitable PPE (gloves and coveralls) to reduce the potential for dermal contact with *B.* *thuringiensis* subsp. *kurstaki* strain ABTS-351.

**RMS PL**: In agreement with EFSA recommendations (EFSA Journal 2021;19(10):6879) in the absence of a quantitative risk assessment, the use of respiratory protective equipment (RPE; disposable filtering face piece respirator to at least EN149 FFP3 or equivalent) for the workers in case when they could be exposed to spray of the pesticide is recommended to reduce the exposure via inhalation.

**Residents**

Due to lack of appropriate models for microorganisms, quantitative assessment of resident exposure to *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 is considered not applicable. However, it is recommended that residents are excluded from areas during spray application.

**Bystanders**

Due to lack of appropriate models for microorganisms, quantitative assessment of bystander exposure to *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 is considered not applicable. However, it is recommended that bystanders are excluded from areas during spray application.

Overall, it is considered that Foray® 76B can be used without potential health risks to operators, workers, residents, or bystanders, subject to the use of protective equipment specified on the product label.

Summaries of selected studies referred to above are presented below:

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.2.1/01 |
| Authors(s) year | XXXX (2010) |
| Title: | Sub-chronic lung inflammation after airway exposures to *Bacillus thuringiensis* biopesticides in mice |
| Report No. | *BMC Microbiology* Vol. 10, p. 233-242 |
| Test facility | Not applicable |
| Published | Yes |
| Test guidelines: | Not applicable |
| Deviations | Not applicable |
| GLP: | No |

**Material:** Bacterial suspensions from the commercially available insecticides Vectobac® (*Bt israelensis*) and Dipel® (*Bt kurstaki*), both from XXXX (XXXX); Group of 10 BALB/cJ mice (Taconic M&B, Ry, Denmark).

**Method:** Bacterial suspensions were prepared from the commercially available insecticides Vectobac® (*Bt israelensis*) and Dipel® (*Bt kurstaki*), both from XXXX (XXXX). Groups of ten BALB/cJ mice (Taconic M&B, Ry, Denmark) were intratracheally instilled with one bolus (3.5 × 105 CFU *Btk* or 3.4 × 106 CFU *Bti* per mouse) of either biopesticide or sterile water as control. After 4 hours, 24 h, 4 days, and 70 days mice were sacrificed, bronchoalveolar lavage fluid (BALF) was collected, and CFU and inflammatory cells were assessed. For each mouse, 200 cells were counted and differentiated. Groups of nine BALB/cJ mice were (whole-body) exposed to an aerosol of 5 × 104 CFU per mouse for 60 min/day and 5 days/week over a period of 2 weeks. Histology was performed 70 days after exposure.

**Findings:** A significant neutrophilic influx was seen 24 hours post exposure for both biopesticides. Four days after instillation, the neutrophil number was normalised, and macrophages represented the predominant cell type in BALF. Seventy (70) days after instillation, the inflammation was interstitially located with few inflammatory cells present in the lung lumen. Bacteria in CFU counts of BALF were still present 70 days post exposure in 8 of 10 mice treated with Vectobac® (3.4 × 106 CFU Bti) and 1 out of 9 mice treated with Dipel® (3.5 × 105 CFU Btk) with an average of 150 and 2850 CFU/BALF, respectively. In the mice exposed by inhalation to DiPel® aerosols, one mouse out of 10 had CFU recovered (630 CFU/BALF), while no CFU was recovered from mice exposed to Vectobac® aerosol. All 20 mice that received high doses of Vectobac® or DiPel® by intratracheal instillation showed tissue changes 70 days after exposure. The group given Vectobac® developed the most pronounced changes. These were localized in focal areas adjacent to the larger blood vessels. Interstitial inflammation was apparent as small patches, affecting approximately 5% of the lung surface. Slight interstitial inflammation was observed after Vectobac® instillation. Instillation of Dipel® resulted in fewer and less intense changes. In 3 of the remaining 17 mice receiving aerosol exposure, some patches of interstitial inflammation were observed 70 days after end of the repeated exposures to Vectobac®, whereas exposure to DiPel® gave rise to less significant effects.

**Conclusion:** Acute exposure to *Bt* based biopesticides induced an influx of neutrophilic granulocytes in BALF, which was reversible after 4 days and represents a typical inflammatory response to an external stimulus. 70 days post exposure, slight tissue changes as a sign of interstitial inflammation were observed in both Vectobac® and Dipel® groups.

|  |  |
| --- | --- |
| Study Comments: IIIM 7.2.1/01 | This published non-GLP study is acceptable with restriction due to limited description of the study results and insufficient data on control animals).  The result of the study indicate that there was no inflammation of respiratory tract of mice within 4 hours after intratracheal instillation of any of two commercial biopesticides based on the Bt. subspecies kurstaki and israelensis. However there was an inflammatory response 24 hours post i.t. instillation seen as increase in number of neutrophils, which was lowered after 4 days post exposure.  There was no acute airway irritation during 60 minutes inhalation exposure of mice to these biopesticides what may suggest that inhalation exposure to these pesticides will not evoke a warning signal, making the exposure for workers insidious.  The study indicate that instilled or even inhaled Bt spores may be present in the lung and extracted by bronchoalveolar lavage fluid even 70 days after administration  Instillation of biopesticide Dipel® containing Bt. subspecies kurstaki at high doses resulted 70 days after exposure in small focal areas in lungs with accumulation of inflammatory cells interstitially, inflammation was observed also peripherally even to the level of the pleura.  The results of the study suggest that repeated exposure to biopesticide aerosols may lead to sub-chronic lung inflammation, thus respiratory protection of operators is recommended |
| Agreed endpoint: IIIM 7.2.1 | Not applicable |

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.2.1/02 |
| Authors(s) year | Rosenblum Lichtenstein J.H., Molina R.M., Donaghey T.C., and Brian J.D. (2006) |
| Title: | Strain differences influence murine pulmonary responses to *Stachybotrys chartarum* |
| Report No. | *Am J Respir Cell Mol Biol,* Vol. 35, p.415–423 |
| Test facility | Not applicable |
| Published | Yes |
| Test guidelines: | Not applicable |
| Deviations | Not applicable |
| GLP: | No |

**Material:** Three strains of 8.5-week-old (±1 week) male mice: C57BL/6J, C3H/HeJ, and BALB/c were examined. All are TLR2 and only C3H/HeJ is TLR4. Both TLR2 and TLR4 have been shown to mediate responses to the fungi *Aspergillus fumigatus* and *A. niger*. In an additional group of BALB/c mice, airway inflammation was induced with OVA to test whether or not underlying allergic inflammation exacerbated pulmonary injury and inflammation in response to *S. chartarum*. A toxin-producing strain of *S. chartarum* was grown. Spores were vacuumed from the surface of 14- to 21-d agar cultures and suspended to concentrations of 105–107 spores per milliliter.

**Method:** All mice were intratracheally instilled (2.5 mL/kg) with *S. chartarum* spores suspended in saline (105–107 spores/mL), for final doses of 6.25 × 103 to 6.25 × 105 spores (62.5 µL) per 25-g mouse. At least five mice for each combination of strain and dose of spores were studied (0, 1 million, 3 million, and 10 million spores/mL). In addition, five BALB/c mice were instilled at doses of 100,000 and 300,000 spores/mL to evaluate the effects of lower doses on BALB/c mice. Six BALB/c and five C57BL/6J mice were each instilled with 5 × 105 spores to verify that the two strains received comparable spore doses. Spore numbers were calculated based on counts per unit volume of sample and total volume of lung homogenate. Also, bronchoalveolar lavage (BAL) was performed.

**Findings:** There is a significantly greater response as seen in the BAL parameters to the same *S. chartarum* doses in the BALB/c mice when compared with the C57BL/6J and C3H/HeJ mice (*P* < 0.0001). TNF-α, keratinocyte-derived cytokine (KC), and macrophage inflammatory protein (MIP)-2 all show significantly higher levels in both BALB/c mice and C57BL/6J mice at 6 h when compared with baseline. The magnitude of this increase is significantly higher in the BALB/c mice than in the C57BL/6J mice. All three mouse strains show a significant dose response in the albumin levels (a measure of capillary permeability in the lungs) in response to *S. chartarum* (*P <* 0.0001). But BALB/c mice show the greatest response with increasing dose, with a 20-fold increase in albumin levels from baseline to the highest dose. While all three strains show a significant dose response in haemoglobin levels (a measure of alveolar haemorrhage) (*P <* 0.0001), BALB/c mice show the largest increase, with 5-fold higher levels than in C57BL/6J and C3H/H3J mice at the highest *S. chartarum* dose. BALB/c mice show more infiltration and degranulation of neutrophils at higher doses than did C57BL/6J and C3H/HeJ mice. BALB/c mice show the largest increase in the number of polymorphonuclear leukocytes (PMNs) present 24 h after instillation of the highest dose of *S. chartarum*, and C57BL/6J mice show a modest increase (spores to PMNs slopes significantly different at *P <* 0.0001 for C57BL/6J versus BALB/c). The PMN increase in C3H/HeJ mice is significantly less than in BALB/c. BALB/c mice show the largest increase in myeloperoxidase (MPO), with levels 14-fold higher than the C57BL/6J mice and 2-fold higher than the C3H/HeJ mice at the highest dose of *S. chartarum*. In addition, the lungs of BALB/c mice showed more pulmonary pathologic changes after instillation with the 1 million spores/ml dose of *S. chartarum.* BALB/c mice produced 5.2 times higher MIP-2 and KC peak levels than the C57BL/6J mice in response to *S. chartarum* at 6 hours after instillation (P = 0.005 and p = 0.002, respectively, t test). Moreover, BALB/c mice produce 13 times more TNF-α than the C57BL/6J mice at the 6-h time point (*P =* 0.0007, *t* test). A significant increase in KC, MCP-1, MCP-3, MIP-1α, MIP-1β, MIP-1γ, MIP-2, RANTES, IL-1α, IL-1β, IL-3, IL-6, IL-18, leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (MCSF), and TNF-α was noted in the BALB/c mice but not in the C57BL/6J mice exposed to the same dose of *S. chartarum*.

**Conclusion:** BALB/c mice are more sensitive to pulmonary exposure to *S. chartarum* than are C57BL/6J and C3H/HeJ mice. More vigorous cytokine and chemokine release increased capillary permeability and BAL leukocyte numbers were observed, as well as increased release of white cell constituents in the lungs of BALB/c mice.

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| --- | --- |
| Study Comments: IIIM 7.2.1/02 | This study is non-GLP. The study provides supporting information and indicates that BALB/c mice are more sensitive to pulmonary exposure to *S. chartarum* than are C57BL/6J and C3H/HeJ mice. |
| Agreed endpoint: IIIM 7.2.1 | Not applicable |

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.2.1/03 |
| Authors(s) year | XXXX |
| Title: | Female mice are more susceptible to the development of allergic airway inflammation than male mice |
| Report No. | *Clin. Exp. Allergy.* 35: 1496-1503 |
| Test facility | Not applicable |
| Published | Yes |
| Test guidelines: | Not applicable |
| Deviations | Not applicable |
| GLP: | No |

**Method:** The authors tested the hypothesis that female mice are more susceptible to the development of allergic asthma than male mice and studied allergic immune responses in the lung. Effects of allergic airway inflammation, *i.e.* methacholine (MCh) responsiveness, serum IgE, and cytokines, and the number of the different leucocytes in lungs of male and female BALB/c mice, twice-sensitized to ovalbumin (OVA) and subsequently challenged with OVA (OVA-mice) or phosphate-buffered saline (PBS-mice) aerosols on days 24–26, 30, and 31 were compared.

**Findings:** OVA challenge significantly increased MCh responsiveness, numbers of eosinophils, CD4+ T cells, CD4+/CD25+ T cells, B cells, and levels of T helper (Th)2 cytokines, total, and OVA-specific IgE. There was, however, also an effect of gender, with female mice responding to OVA challenges with higher numbers of eosinophils, CD4+ T cells, B cells, and levels of IL-4, IL-13, IFN-γ, total, and OVA-specific IgE than male mice. In contrast, female PBS-mice had significantly lower percentages of regulatory CD4+/CD25+ T cells than males (females 4.2±0.2% vs. males 5.3±0.1% of CD4+ T cells, *P*<0.05).

**Conclusion:** Female mice develop a more pronounced type of allergic airway inflammation than male mice after OVA challenge. The reduced percentage of regulatory T cells in the lungs of female PBS-mice may indicate that the level of these cells in the lung during the sensitization phase is important for the development and/or progression of an allergic immune response after multiple OVA challenges.

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| --- | --- |
| Study Comments: IIIM 7.2.1/03 | This study is non-GLP. The study provides supporting information and indicates that female BALB/c mice are more susceptible to the development of allergic airway inflammation than male mice. |
| Agreed endpoint: IIIM 7.2.1 | Not applicable |

|  |  |
| --- | --- |
| Data point addressed | IIIM 7.2.1/04 |
| Authors(s) year | XXXX (1994) |
| Title: | Subacute inhalation toxicity study of ‘VectoBac®’ 12 AS in Wistar rats |
| Report No. | 6779 |
| Test facility | XXXX |
| Published | No |
| Test guidelines: | CIB-RC for microbial pesticides |
| Deviations | No data available |
| GLP: | Yes |

**Abstract:** The repeated dose inhalation toxicity of VectoBac® 12 AS was evaluated in male and female Wistar rats. Four rats/sex were exposed for 4 hours/day (nose-only) to a nominal concentration of 3.8 x 106 CFU/L on 14 consecutive days. Four rats/sex were exposed to a 0.9% saline solution atmosphere and served as vehicle controls and a further four rats/sex were exposed to a clean air atmosphere and served as untreated controls. Rats were observed for mortality and clinical signs at hourly intervals during exposure and on a daily basis post exposure. Bodyweights were recorded at weekly intervals. Food consumption and rectal temperatures were recorded daily. Blood samples were collected from all treated rats on Day 15 and from two rats/sex from the control groups on Day 29, for the investigation of haematological and clinical chemistry parameters. Two rats/sex from each group were subjected to gross necropsy on Day 15 and the remaining animals were necropsied on Day 29. Organ weights were recorded, and tissues preserved for subsequent histopathological examination. The achieved concentration was 1.18 - 1.84 x 106 spores/L. All rats survived the duration of the study; there were no signs of toxicity and no treatment-related effects on body weight, food consumption, body temperature, haematological and clinical chemistry parameters. There were no treatment related effects on organ weights and no gross pathological or histopathological changes.

**Conclusion:** Under the conditions of the study, consecutive daily 4-hour exposures for 14 days to VectoBac® 12 AS (1.18-1.84 x 106 spores/L) was without adverse clinical findings. The NOAEL for this study is therefore identified as 1.84 x 106 spores/L. This endpoint was agreed in the EFSA Conclusion and is still considered to be valid.

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| Study Comments: IIIM 7.2.1/04 | This GLP-study provides supporting information and reports no treatment-related effects on organ weights and no gross pathological or histopathological changes following daily 4-hour exposure to VectoBac® 12 AS containing Bacillus thuringiensis israelensis (achieved concentration of up to 1.84 × 106 spores/L) over a period of 14 days. |
| Agreed endpoint: IIIM 7.2.1 | Not applicable |

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| Data point addressed | IIIM 7.2.1/05 |
| Authors(s) year | XXXX (2015b) |
| Title: | Haematotoxicity and genotoxicity evaluations in Swiss mice intraperitoneally exposed to *Bacillus thuringiensis* (var *kurstaki)* spore crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac, or Cry2Aa |
| Report No. | *Environmental Toxicology* 31(8):970-978 |
| Test facility | Not applicable |
| Published | Yes |
| Test guidelines: | Not applicable |
| Deviations | Not applicable |
| GLP: | No |

**Material:** *Bt* spore crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa were obtained in lyophilized form from the Germplasm Bank of the Brazilian Agricultural Research Corporation (Embrapa).

**Methods:** Groups of three Swiss mice/sex (10-12 weeks old) were administered a single intraperitoneal dose of the lyophilised *Bt* spore crystals Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa. Based on the results of a pilot study, all spore crystals were administered at a dose level of 27 mg/kg bw. Cry1Aa, 1Ab and 2Aa were also administered at 136 and 270 mg/kg bw. Control groups received water (negative controls) or cyclophosphamide (positive controls) intraperitoneally. All mice were terminated at 24 hours after dosing. Blood smear slides were also prepared for visual assessments. After euthanasia by cervical dislocation, bone marrow cells were surgically removed, the slides for the micronucleus (MN) test were prepared and the genotoxic potential of *Bt* spore crystals was evaluated.

Findings: Mortality: In the pilot study, 28.57% of mice intraperitoneally exposed at 270 mg/kg died within 24 h after administrations of Cry1Aa and Cry2Aa, and the same occurred with 42.85% of the animals exposed to Cry1Ab, meaning that 270 mg/kg was the maximum tolerated dose. Cry1Ac was the most toxic in the i.p. route, being completely lethal at 136 mg/kg. Therefore, for this spore crystal toxin, 27 mg/kg was the only dose used, with no deaths recorded.

Haematotoxicity: A significant reduction in lymphocyte count was observed after treatment with Cry1Aa 270 mg/kg (p=0.015), Cry1Ab 136 (p=0.026) and 270 mg/kg (p=0.009) and Cry2Aa 136 (p=0.009) and 270 mg/kg (p=0.041); all of them were below the reference range. Cry1Ab (270 mg/kg bw) also caused a significant increase in neutrophils + monocytes (p=0.041). Although statistically non-significant compared to the negative control, Cry1Aa (136 mg/kg bw) caused a reduction below the reference range for the total white blood cell (WBC) count, due mainly to lymphocytes and neutrophils + monocytes. This finding was associated with an increased eosinophil count. The majority of treatments also increased platelet counts and/or platelet distribution width values.

Micronucleus test: Compared to the negative control, a significant increase in MN-NCE was observed after treatment with CP and all *Bt* spore crystals; the same occurred with MN-PCE, except for Cry1Aa 27 mg/kg, and Cry2Aa 27 and 270 mg/kg. Except for the treatment with Cry1Ab 27 mg/kg, all treatments with spore crystals also caused a significant reduction in %PCE.

**Table 7.2.1.1-1: Micronucleus frequency**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Dose group** | | **MnNCE**1 | **MnPCE**1 | **% PCE** |
| **Negative control** | | 0.17 | 2.33 | 70.13 |
| **Cry1Aa** | **27 mg/kg bw** | 2.17\*\* | 3.33 | 41.69\*\* |
| **136 mg/kg bw** | 3.67\*\* | 5.17\* | 49.32\*\* |
| **270 mg/kg bw** | 4.20\*\* | 7.00\*\* | 39.68\*\* |
| **Cry1Ab** | **27 mg/kg bw** | 3.57\*\* | 8.71\*\* | 56.63 |
| **136 mg/kg bw** | 6.50\*\* | 12.17\*\* | 51.91\*\* |
| **270 mg/kg bw** | 6.17\*\* | 9.67\* | 46.28\*\* |
| **Cry1Ac** | **27 mg/kg bw** | 2.50\*\* | 5.67\*\* | 51.36\*\* |
| **Cry2Aa** | **27 mg/kg bw** | 3.83\*\* | 4.33 | 42.66\*\* |
| **136 mg/kg bw** | 4.67\*\* | 5.50\*\* | 37.39\*\* |
| **270 mg/kg bw** | 2.33\*\* | 5.00 | 34.71\*\* |
| **Positive control** | | 14.33\*\* | 26.83\*\* | 66.56 |

1*frequency: units not reported*

\**significantly different to negative control (p<0.05);* \*\**p<0.01*

**Conclusion:** Theresults suggested that these MCAs, intraperitoneally administered, presented toxicity for lymphocytes when in higher doses, which varied according to the type of spore crystal studied, besides promoting cytotoxic and genotoxic effects for the erythroid lineage of bone marrow, mainly at the highest doses. The profile of such adverse side effects can be related to their high level of exposure, which is not commonly found in the environment. Furthermore, studies using purified proteins are necessary to show whether the observed effects are really specific to Cry toxins.

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| Study Comments: IIIM 7.2.1/05 | In this non-GLP study, a compromised test system was investigated. The results presented in this paper cannot be reliably interpreted due to deficiencies in methodology, performance and reporting to implicate Bt spore crystals as a potential genotoxin. Furthermore, it cannot be ruled out that the Bt spore crystals may have been partly solubilized and activated prior to injection at high dose into the test mice, as the normal physiological condition is unlikely to be favourable for the solubilization and activation of the spore crystals. |
| Agreed endpoint: IIIM 7.2.1 | Not applicable |

IIIM 7.3 Operator, workers, residents and bystander exposure: reporting of hypersensitivity incidents before and after registration

No cases on hypersensitivity have been reported in production or application of Foray® 76B.

IIIM 7.4 Safety data sheet for each additive

Foray® 76B does not contain ingredients in concentrations of toxicologically critical concern. The properties of non-active ingredients and their toxicological data are provided in confidential Part C.

IIIM 7.5 Supplementary information on all data points in part 7: effects on human health, if it is recommended that MPCP be tank-mixed with an adjuvant or another pest control product

Foray® 76B is not intended for combinations with other adjuvants or pest control products. Furthermore, due to the nature of this biological insecticide, no influence on the toxicological profile of *Bacillus thuringiensis* is to be anticipated from interactions with chemical or other biological plant protection products.

IIIM 7.6 Summary and evaluation of health effects

Foray® 76B containing 206.5 g/L (1.51 x 1013 CFU/L) of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 has a low toxicity in respect to acute oral, dermal and inhalation toxicity. Foray® 76B was not irritating to the rabbit skin, moderately irritating to rabbit eyes and was non-sensitising to mice in a Local Lymph Node Assay. Considering all submitted data and the labelling of the active substance, Foray® 76B does not require classification for acute effects according to the CLP legislation (Regulation (EC) No 1272/2008). Furthermore, Foray® 76B does not require classification as a skin or eye irritant according to CLP legislation. Based on the available data, there is no evidence of dermal sensitisation caused by Foray® 76B on mice.

Derivation of toxicological reference values (including an AOEL) is not applicable for *B. thuringiensis* subsp. *kurstaki* strain ABTS-351based on the lack of toxicity, infectivity or pathogenicity. Quantitative assessment of operator, worker, bystander, and resident exposure is therefore not required for Foray®76B. It is therefore concluded that the product can be used without potential health risks to operators, workers, or bystanders, subject to the use of protective equipment specified on the product label.

Overall, all submitted toxicological studies and supplemental information on the Microbial Pest Control Product, Foray® 76B indicate that it is non-toxic and non-infectious to mammals and imposes no health risk for operators and bystanders. Foray® 76B is therefore considered safe for use in a manner consistent with the recommendations on the label.

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| RMS conclusions:  As indicated in the Conclusions of Peer review of the pesticide risk assessment of the active substance Bacillus thuringiensis subsp. kurstaki strain ABTS-351 (EFSA Journal 2021;19(10):6879) regarding non-dietary exposure to Bacillus thuringiensis subsp. kurstaki strain ABTS-351, since toxicity/infectivity after repeated exposure by inhalation could not be concluded, and a genotoxic potential of the Cry proteins could not be excluded by nondietary exposure, the risk assessment by inhalation for residents and bystanders cannot be concluded, except for permanent greenhouses (issue not finalised). In the absence of a quantitative risk assessment, the use of respiratory protective equipment for the operators and workers might be considered to reduce the exposure via inhalation. The RMS (Denmark and the Netherlands ) disagreed with this view, being of the opinion that Bacillus thuringiensis subsp. Kurstaki preparations are not to be considered of health concern for operators, workers, bystanders and residents.  zRMS PL agrees and support the opinion of Denmark and the Netherlands, the rapporteur and co-rapporteur Member States preparing the registration report for the pesticide active substance Bacillus thuringiensis subsp. kurstaki strain ABTS-351 (EFSA Journal 2021;19(10):6879), that Bacillus thuringiensis subsp. Kurstaki preparations are not to be considered of health concern for operators, workers, bystanders and residents. |

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 7.1.1/01 | XXXX | 1991 | Acute oral toxicity study in rats  XXXX  Report No.: 8162-91  GLP: Yes  Unpublished | N | XXXX |
| IIIM 7.1.2/01 | XXXX | 1993a | Foray® 76B assessment of acute dermal toxicity in rats  XXXX  Report No.: 92843  GLP: Yes  Unpublished | N | XXXX |
| IIIM 7.1.3/01 | XXXX | 1991 | Foray® 76B (formerly identified as Foray 75B) acute inhalation toxicity study in rats with MPCA  XXXX  Report No.: 8163-91  GLP: Yes  Unpublished | N | XXXX |
| IIIM 7.1.4/01 | XXXX | 1993b | Foray® 76B assessment of the skin irritant effect in rabbits  XXXX  Report No.: 92834  GLP: Yes  Unpublished | N | XXXX |
| IIIM 7.1.5/01 | XXXX | 1991 | Eye irritation study in rabbits with the end product Foray 75B, batch bbn 7001  XXXX  Report No.: 90113  GLP: Yes  Unpublished | N | XXXX |
| IIIM 7.1.6/01 | XXXX | 2011 | Foray® 76B (ABG-6431) – Local Lymph Node Assay (LLNA) in Mice  XXXX  Report No.: 32053  GLP: Yes  Unpublished | N | XXXX |
| IIIM 7.2.1/01 | XXXX | 2010 | Sub-chronic lung inflammation after airway exposures to *Bacillus thuringiensis* biopesticides in mice  BMC Microbiology Vol. 10 p. 233-242  GLP: No  Published | N | Open literature |
| IIIM 7.2.1/02 | Rosenblum Lichtenstein, J.H., Molina, R.M., Donaghey, T.C., and Brian, J.D. | 2006 | Strain differences influence murine pulmonary responses to *Stachybotrys chartarum*  *Am J Respir Cell Mol Biol,* Vol. 35, p. 415-423  GLP: No  Published | N | Open literature |
| IIIM 7.2.1/03 | XXXX | 2005 | Female mice are more susceptible to the development of allergic airway inflammation than male mice  *Clin. Exp. Allergy.* 35: 1496-1503  GLP: No  Published | N | Open literature |
| IIIM 7.2.1/04 | XXXX | 1994 | Subacute inhalation toxicity study of ‘VectoBac®’ 12 AS in Wistar rats  XXXX  Report No.: 6779  GLP: yes  Unpublished | N | XXXX |
| IIIM 7.2.1/05 | XXXX | 2015b | Haematotoxicity and genotoxicity evaluations in Swiss mice intraperitoneally exposed to *Bacillus thuringiensis* (var *kurstaki)* spore crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac, or Cry2Aa  *Environmental Toxicology* 31(8):970-978  GLP: No  Published | N | Open literature |

Appendix 2: GAP table

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **1** | **2** | **3** | **4** | **5** | **6** | | **7** | **8** | **9** | **10** | **11** | | **12** | **13** | **14** |
| **Use-No.** | **Member state(s)** | **Crop and/  or situation    (crop destination / purpose of crop)** | **F, Fn, Fpn  G, Gn, Gpn  or  I** | **Pests or Group of pests controlled    (additionally: developmental stages of the pest or pest group)** | **Application** | | | | | **Application rate** | | | | **PHI  (days)** | **Remarks:     e.g. g safener/synergist per ha** |
| Method / Kind | Timing / Growth stage of crop & season | | Max. number  a) per use  b) per crop/ season | Min. interval between applications (days) | kg or L product / ha  a) min / max. rate per appl.  b) max. total rate per crop/season | | g or kg as/ha   a) min / max. rate per appl.  b) max. total rate per crop/season | Water L/ha    min / max |
| 1 | IT | Coniferous and deciduous forest and green areas (trees and shrubs in parks and gardens) | F | Lepidoptera caterpillars L1 to L4  *Choristoneura sp*. - CHONSP,  *Geometridae* - 1GEOMF,  *Hyphantria cunea* - HYPHCU  *Malacosoma neustria* - MALANE,  Stilpnotia salicis - LEUOSA,  *Euproctis chrysorrhoea* - EUPRCH,  *Lymantria dispar* - LYMADI,  *Lymantria monacha* - LYMAMO,  *Thaumetopoea pityocampa* - THAUPI,  *Thaumetopoea processionea* - THAUPR,  *Dendrolimus pini* - DENDPI,  *Dendrolimus superans* - DENDSU,  *Tortrix viridana* - TORTVI | Ground spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | | a) 1 - 4  b) 4 | 5 days | a) 2 - 2.5 L/ha  b) 10 L/ha | | a) 0.413 - 0.516 kg a.s/ha  b) 2.06 kg a.s./ha | 0 - 500 L/ha | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha  Aerial application only by emergency permits (Avio). |

| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | | **12** | **13** | **14** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Use-No.** | **Member state(s)** | **Crop and/  or situation    (crop destination / purpose of crop)** | **F, Fn, Fpn  G, Gn, Gpn  or  I** | **Pests or Group of pests controlled    (additionally: developmental stages of the pest or pest group)** | **Application** | | | | **Application rate** | | | | **PHI  (days)** | **Remarks:     e.g. g safener/synergist per ha** |
| Method / Kind | Timing / Growth stage of crop & season | Max. number  a) per use  b) per crop/ season | Min. interval between applications (days) | kg or L product / ha  a) min / max. rate per appl.  b) max. total rate per crop/season | | g or kg as/ha   a) min / max. rate per appl.  b) max. total rate per crop/season | Water L/ha    min / max |
| 2 | ES | Coniferous forest, Deciduous forest, Palm trees, shurbs and small ornamental trees | F | Lepidoptera caterpillars L1 to L4  Procesionaria, *Thaumetopoea processionea* - THAUPR  Procesionaria del pino, *Thaumetopoea pityocampa* - THAUPI  Lagarta, *Lymantria spp*. - LYMASP  Oruga del zurrón, *Euproctis chrysorrhoea* - EUPRCH  Tortrix, *Tortrix viridana* - TORTVI | Spray (ground and aerial application) | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 1 - 4  b) 4 | 5 days | a) 2 - 2.5 L/ha  b) 10 L/ha | | a) 0.413 - 0.516 kg a.s/ha  b) 2.06 kg a.s./ha | Aerial application: no dillution  Ground application: 0 - 500 L/ha | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 3 | ES | Coniferous forest, Deciduous forest, Palm trees, , shurbs and small ornamental trees | F | Lepidoptera caterpillars L1 to L4 | Spray (ground and aerial application) | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 1 - 4  b) 4 | 5 days | a) 1.5 - 2.5 L/ha  b) 10 L/ha | | a) 0.31 - 0.52 kg a.s/ha  b) 2.06 kg a.s./ha | Aerial application: no dillution  Ground application: 0 - 500 L/ha | - | Application rate in CFU:  a) 2.26 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 4 | LT | Forest | F | Lepidoptera caterpillars  *Lymantria monacha* - LYMAMO  *Dendrolimus pini* - DENDPI  *Tortrix viridana* - TORTVI | Spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 2  b) 2 | 7 days | a) 2 - 2.5 L/ha  b) 5 L/ha | | a) 0.413 - 0.516 kg a.s/ha  b) 1.03 kg a.s./ha | - | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |

| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Use-No.** | **Member state(s)** | **Crop and/  or situation    (crop destination / purpose of crop)** | **F, Fn, Fpn  G, Gn, Gpn  or  I** | **Pests or Group of pests controlled    (additionally: developmental stages of the pest or pest group)** | **Application** | | | | **Application rate** | | | **PHI  (days)** | **Remarks:     e.g. g safener/synergist per ha** |
| Method / Kind | Timing / Growth stage of crop & season | Max. number  a) per use  b) per crop/ season | Min. interval between applications (days) | kg or L product / ha  a) min / max. rate per appl.  b) max. total rate per crop/season | g or kg as/ha   a) min / max. rate per appl.  b) max. total rate per crop/season | Water L/ha    min / max |
| 5 | PL | Pine trees | F | *Lymantria monacha* - LYMAMO  *Dendrolimus pini* - DENDPI | Spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 1 - 4  b) 4 | 5 days | a) 2.5 L/ha  b) 10 L/ha | a) 0.52 kg a.s/ha  b) 2.06 kg a.s./ha | - | - | Application rate in CFU:  a) 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 6 | PL | Deciduous forest | F | *Operophtera brumata* - CHEIBR  *Tortrix viridana* - TORTVI | Spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 1 - 4  b) 4 | 5 days | a) 2.5 L/ha  b) 10 L/ha | a) 0.52 kg a.s/ha  b) 2.06 kg a.s./ha | UVL application: 0-10 L/ha,  application of high pressure (10 bar): 200 L/ha,  application of low pressure (2-3 bar): 600 L/ha. | - | Application rate in CFU:  a) 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 7 | PL | Deciduous forest | F | *Euproctis chrysorrhoea* - EUPRCH | Spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 1 - 2  b) 2 | 14 days | a) 3 L/ha  b) 6 L/ha | a) 0.619 kg a.s/ha  b) 1.24 kg a.s./ha | UVL application: 0-10 L/ha,  application of high pressure (10 bar): 200 L/ha,  application of low pressure (2-3 bar): 600 L/ha. | - | Application rate in CFU:  a) 4.53 x 1013 CFU/ha  b) 9.06 x 1013 CFU/ha |

| **1** | **2** | | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Use-No.** | | **Member state(s)** | **Crop and/  or situation    (crop destination / purpose of crop)** | **F, Fn, Fpn  G, Gn, Gpn  or  I** | **Pests or Group of pests controlled    (additionally: developmental stages of the pest or pest group)** | **Application** | | | | **Application rate** | | | **PHI  (days)** | **Remarks:     e.g. g safener/synergist per ha** |
| Method / Kind | Timing / Growth stage of crop & season | Max. number  a) per use  b) per crop/ season | Min. interval between applications (days) | kg or L product / ha  a) min / max. rate per appl.  b) max. total rate per crop/season | g or kg as/ha   a) min / max. rate per appl.  b) max. total rate per crop/season | Water L/ha    min / max |
| 8 | | RO | Coniferous forest | F | Lepidoptera caterpillars L1 to L4  *Choristoneura spp.* - CHONSP  *Lymantria monacha* - LYMAMO  *Thaumetopoea pityocampa* - THAUPI  *Dendrolimus pini* - DENDPI | Spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 4 b) 4 | 5 days | a) 2 - 2.5 L/ha  b) 10 L/ha | a) 0.413 - 0.516 kg a.s/ha b) 2.06 kg a.s./ha | 0-10 L/ha (undiluted for ULV application) | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 9 | | RO | Deciduous forest | F | Lepidoptera caterpillars L1 to L4  *Hyphantria cunea* - HYPHCU  *Malacosoma neustria* - MALANE  *Stilpnotia salicis* - LEUOSA  *Euproctis chrysorrhoea* - EUPRCH  *Lymantria dispar* - LYMADI  *Thaumetopoea processionea* - THAUPR  *Tortrix viridana* - TORTVI  *Operophtera bru*mata - CHEIBR | Spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 4 b) 4 | 5 days | a) 2 - 2.5 L/ha  b) 10 L/ha | a) 0.413 - 0.516 kg a.s/ha b) 2.06 kg a.s./ha | 0-600 L/ha (undiluted for ULV application; high pressure application: 200L/ha and low pressure application 600 L/ha) | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |

| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Use-No.** | **Member state(s)** | **Crop and/  or situation    (crop destination / purpose of crop)** | **F, Fn, Fpn  G, Gn, Gpn  or  I** | **Pests or Group of pests controlled    (additionally: developmental stages of the pest or pest group)** | **Application** | | | | **Application rate** | | | **PHI  (days)** | **Remarks:     e.g. g safener/synergist per ha** |
| Method / Kind | Timing / Growth stage of crop & season | Max. number  a) per use  b) per crop/ season | Min. interval between applications (days) | kg or L product / ha  a) min / max. rate per appl.  b) max. total rate per crop/season | g or kg as/ha   a) min / max. rate per appl.  b) max. total rate per crop/season | Water L/ha    min / max |
| 10 | HU | Deciduous forest species  (also on public areas) | F | Lepidopteran foliage pests  *Lymantria dispar* - LYMADI  *Hyphantria cunea* - HYPHCU  *Euproctis chrysorrhoea* - EUPRCH  *Aporia crataegi* - APORCR  *Thaumetopoea processionea* - THAUPR  *Tortrix viridana* - TORTVI  *Geometridae -* 1GEOMF  *Tortricidae* - 1TORTF  *Gracillariidae* - 1GRACF | Spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 4 b) 4 | 5 days | a) 2 - 2.5 L/ha  b) 10 L/ha | a) 0.413 - 0.516 kg a.s/ha b) 2.06 kg a.s./ha | Ground spray: 600 - 1500 L/ha  Aerial spray: 60 - 80 L/ha | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 11 | HU | Pine species  (also on public areas) | F | Lepidopteran foliage pests  *Dendrolimus pini -* DENDPI *Rhyacionia buoliana* - EVETBU *Gracillariidae* - 1GRACF | Spray | When caterpillars are  visible following egg hatch & foliage growth sufficient for deposition | a) 4 b) 4 | 5 days | a) 2 - 2.5 L/ha b) 10 L/ha | a) 0.413 - 0.516 kg a.s/ha b) 2.06 kg a.s./ha | Ground spray: 600 - 1500 L/ha  Aerial spray: 60 - 80 L/ha | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |

| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Use-No.** | **Member state(s)** | **Crop and/  or situation    (crop destination / purpose of crop)** | **F, Fn, Fpn  G, Gn, Gpn  or  I** | **Pests or Group of pests controlled    (additionally: developmental stages of the pest or pest group)** | **Application** | | | | **Application rate** | | | **PHI  (days)** | **Remarks:     e.g. g safener/synergist per ha** |
| Method / Kind | Timing / Growth stage of crop & season | Max. number  a) per use  b) per crop/ season | Min. interval between applications (days) | kg or L product / ha  a) min / max. rate per appl.  b) max. total rate per crop/season | g or kg as/ha   a) min / max. rate per appl.  b) max. total rate per crop/season | Water L/ha    min / max |
| 12 | HU | Ornamental trees, bushes  (also on public areas) | F | Lepidopteran foliage pests  *Lymantria dispar* - LYMADI *Hyphantria cunea* - HYPHCU *Euproctis chrysorrhoea* - EUPRCH *Aporia crataegi* - APORCR *Thaumetopoea processionea* - THAUPR *Tortrix viridana* - TORTVI *Geometridae* - 1GEOMF *Tortricidae*  - 1TORTF *Gracillariidae -* 1GRACF *Dendrolimus pini* - DENDPI *Rhyacionia buoliana -* EVETBU | Spray | When caterpillars are  visible following egg hatch & foliage growth sufficient for deposition | a) 4 b) 4 | 5 days | a) 2 - 2.5 L/ha b) 10 L/ha | a) 0.413 - 0.516 kg a.s/ha b) 2.06 kg a.s./ha | Ground spray: 600 - 1200 L/ha  Aerial spray: 60 - 80 L/ha | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 13 | DE | Coniferous forest, Deciduous forest | F | Lepidoptera caterpillars  L1 to L3 | Ground spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 2  b) 2 | 14 days | a) 2 - 2.5 L/ha  b) 5 L/ha | a) 0.413 - 0.516 kg a.s/ha  b) 1.03 kg a.s./ha | 600 L/ha | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 14 | DE | Coniferous forest, Deciduous forest | F | Lepidoptera caterpillars  L1 to L3 | Aerial spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 2  b) 2 | 30 days | a) 2 - 2.5 L/ha  b) 5 L/ha | a) 0.516 kg a.s/ha  b) 1.03 kg a.s./ha | 70 L/ha | - | Application rate in CFU:  a) 3.77 x 1013 CFU/ha  b) 1.51 x 1014 CFU/ha |
| 15 | DE | Ornamental trees | F | Lepidoptera caterpillars  L1 to L3 | Ground spray | When caterpillars are visible following egg hatch & foliage growth sufficient for deposition | a) 1  b) 1 | NA | a) 2 - 2.5 L/ha  b) 5 L/ha | a) 0.413 - 0.516 kg a.s/ha  b) 0.516 kg a.s/ha | 600 L/ha | - | Application rate in CFU:  a) 3.02 - 3.77 x 1013 CFU/ha  b) 3.77 x 1013 CFU/ha |

|  |  |  |  |
| --- | --- | --- | --- |
| **Remarks**  **columns:** | 1 Numeration necessary to allow references  2 Use official codes/nomenclatures of EU Member States  3 For crops, the EU and Codex classifications (both) should be used; when relevant, the use situation should be described (e.g. fumigation of a structure)  4 F: professional field use, Fn: non-professional field use, Fpn: professional and non-professional field use, G: professional greenhouse use, Gn: non-professional greenhouse use, Gpn: professional and non-professional greenhouse use, I: indoor application  5 Scientific names and EPPO-Codes of target pests/diseases/ weeds or, when relevant, the common names of the pest groups (e.g. biting and sucking insects, soil born insects, foliar fungi, weeds) and the developmental stages of the pests and pest groups at the moment of application must be named.  6 Method, e.g. high volume spraying, low volume spraying, spreading, dusting, drench  Kind, e.g. overall, broadcast, aerial spraying, row, individual plant, between the plants - type of equipment used must be indicated. |  | 7 Growth stage at first and last treatment (BBCH Monograph, Growth Stages of Plants, 1997, Blackwell, ISBN 38263-3152-4), including where relevant, information on season at time of application  8 The maximum number of application possible under practical conditions of use must be provided.  9 Minimum interval (in days) between applications of the same product  10 For specific uses other specifications might be possible, e.g.: g/m³ in case of fumigation of empty rooms. See also EPPO-Guideline PP 1/239 Dose expression for plant protection products.  11 The dimension (g, kg) must be clearly specified. (Maximum) dose of a.s. per treatment (usually g, kg or L product / ha).  12 If water volume range depends on application equipments (e.g. ULVA or LVA) it should be mentioned under “application: method/kind”.  13 PHI - minimum pre-harvest interval  14 Remarks may include: Extent of use/economic importance/restrictions |

Appendix 3 - Further assessment of the genotoxic potential of the Cry insecticidal proteins by non-dietary exposure based on Mezzomo *et al.* (2015b) study in the context of the renewal of PPP microbial active substance, *Bacillus thuringiensis* subsp. *aizawai* strain ABTS – 1857

pH dependent solubilization and proteolytic activation of crystal proteins

For insecticidal toxins to be released, spore crystals of *Bt* require alkaline conditions of the midgut for solubilisation and activation (Palma *et al*., 2014). The insect midgut (approx. pH ≥10) is strongly alkaline (Dow, 1992) while the pH of mammalian venous blood and interstitial cells varies between 6 to 7.5 (Mezzomo *et al.*, 2015b). Specifically, the pH of bone marrow ranges between 7.1 to 7.5 (Calhoun *et al.*, 1998).

Solubilisation and proteolytic activation of *Bt* spore crystals does not occur below pH 9 (Knowles and Dow, 1993; Knowles, 1994; Naimov *et al*., 2008). Therefore, it is not expected that under normal physiological conditions, *Bt* spore crystal proteins will be capable of eliciting cytotoxic or genotoxic effects in mammalian cells even under the unlikely scenario that mammalian cells possess receptors with specificity for *Bt* Cry toxins.

Deficiencies in study design, performance and reporting of Mezzomo *et al.* (2015b)

The Mezzomo *et al.* study (2015b) does not comply with OECD TG 474 and applied methods are considered inadequate. For instance, the small group size (3 animals/sex/dose instead of 5) and lack of reference to historical (negative and positive) control compromise the validity of the results.

In addition, in the Mezzomo *et al.* study (2015b), lyophilised *Bt* spore crystals were introduced via a non-physiological route of administration (*i.e.* intraperitoneally), which whilst maximising the potential for systemic exposure, does not mimic in any way the potential routes of exposure in the human population.  In fact, Mezzomo and colleagues (Mezzomo *et al.*, 2015b), also acknowledge the limitation of their findings based on the exposure route - “*So, the i.p. injection may have induced secondary effects mediated by local toxicity rather than by genotoxicity, because this type of application is absorbed quickly and transported to the liver, being metabolized and activated in the alkaline environment prior reaching bone marrow”.* However, despite this argument, it is unlikely that *Bt* Cry proteins were activated in the liver due the pH of the liver being only slightly alkaline (pH ˂ 7.5; Griffiths, 1991). It may therefore be argued that it is unlikely that insolubilised and inactivated Bt spore crystals are solubilised and activated following intraperitoneal administration and transport through the liver and the rest of a mammalian body.

A further criticism of the Mezzomo *et al.* study (2015b) is the improbability of the reported increases in micronucleated (MN) normochromatic erythrocytes (NCE), including the positive control response, due to exposure to *Bt* spore crystals, which also suggests the entirety of the micronucleus data are unreliable. As indicated in OECD TG 474 (and its associated supporting references) the timing of animal dosing and bone marrow sampling is associated with the kinetics of erythrocyte formation and maturation. Following exposure to a genotoxic agent, chromosomal damage occurs in the nucleated erythroblasts. The resultant micronuclei are formed during the final round of mitosis and cell division. Maturation of the erythroblasts from their final cell division into erythrocytes in the systemic circulation takes approximately 36-48 hours. The initial stage, involving expulsion of the nucleus to form the enucleated polychromatic erythrocytes (PCE) occurs within 5-10 hours of final cell division. The PCE persist in the bone marrow for a further 10-30 hours, during which time the cellular RNA is degraded, and further maturation of the cells occurs to form the NCE. The NCE are released into the systemic circulation where they continue to mature into erythrocytes. Based on the cell maturation kinetics, the optimum sample time for micronucleus analysis of MN PCE (as defined by OECD TG 474) is 24 hours after a single dose administration. However, various sources recommend a minimum of two sampling times, at 24 and 48 hours after a single dose administration (MacGregor *et al.*, 1987; Hayashi *et al*., 1994; Mavournin *et al*., 1990). The majority of NCE observed 24 hours after animal dosing would have existed in the bone marrow as enucleated PCE at the time of animal exposure. Consequently, any micronuclei observed in the NCE population at 24 hours after animal treatment would have been formed prior to animal dosing. It is the opinion of the applicant that it is improbable the MN NCE observed in treated mice were induced by exposure to *Bt* spore crystals or cyclophosphamide (the positive control).

The observations of apparently treatment-related increases in MN NCE are considered by the applicant to be an artefact, most likely related to the slide staining and/or slide reading procedures. Mezzomo *et al.* (2015b) do not report the slide staining procedures they used, instead they reference the seminal paper of Schmid (1975) and one of their previous publications (Mezzomo *et al.*, 2013). Mezzomo *et al.* (2013) also only reference Schmid (1975), but they do indicate that slide scoring was performed using light microscopy. It is the opinion of the applicant that it is highly likely that Mezzomo *et al.* (2015b) used a Giemsa-based stain for assessment of the bone marrow smears. Giemsa does not specifically stain nucleic-acids and as a consequence can result in slide scoring artefacts, the most well reported being the staining of granules from mast cells present in rat bone marrow, which have the appearance of MN (OECD TG 474). One possible explanation for the apparent increase in MN in both the NCE and PCE populations in animals receiving an intraperitoneal injection of *Bt* spore crystals, is that *Bt* Cry proteins may have been present in the bone marrow smears and as a consequence became stained purple by the Giemsa stain used. However, this would not account for the apparently positive response in cyclophosphamide-treated animals. Whatever the cause of the artefactual results, it cannot be excluded that the issue also affected assessment of micronuclei in the PCE population. Consequently, it is the opinion of the applicant that the entirety of the micronucleus data reported by Mezzomo *et al.* (2015b) are unreliable and unsuitable for evaluation of the potential *in vivo* genotoxicity of *Bt* spore crystals.

An additional deviation from OECD TG 474 is the definition of the maximum tolerated dose (MTD) and subsequent dose selection. Mezzomo *et al.* (2015b) define a Cry1Aa, Cry1Ab and Cry2Aa dose of 270 mg/kg an MTD following which 28.6% (Cry1Aa and Cry2Aa) and 42.9% (Cry1Ab) of dosed animals died. However, OECD TG 474 defines an MTD as the highest dose administered in the mammalian erythrocyte micronucleus test that will be tolerated without evidence of study-limiting toxicity (no death or distress necessitating humane euthanasia). As a considerable proportion of animals dosed at 270 mg/kg Cry1Aa, Cry1Ab and Cry2Aa died following exposure, the MTD was clearly exceeded. No mortality or clinical signs were reported in the animals examined for MN formation. Therefore, it cannot be excluded that none of the doses selected were below or at the MTD.

A further consideration is the fact the nature of the test material is not extensively described in Mezzomo *et al*. (2015b). Therefore, it cannot be ruled out that the Cry proteins may have been activated during the treatments steps during or leading to lyophilization. For example, activation of Cry proteins could potentially result from treatment of spores with strongly alkaline buffer solutions.

Potential for confounding systemic toxicity and homeostatic perturbation

Aside from the technical errors described above, the route of exposure and doses above the MTD may have compromised the typical homeostatic mechanisms in the test system (mice), causing what appears to be a genotoxic response, but is, in fact, more likely to be a typical immune response following the overload of *Bt* spore crystals administered. An immune response is corroborated by significant increases in neutrophils, monocytes and eosinophils also measured. Although not measured in Mezzomo *et. al.* (2015b), it is plausible that administration of such significant doses of Cry proteins directly into the systemic system of mice could lead to an increase in body temperature as part of the immune response.  There is evidence in the literature that an increase in temperature (hyperthermia) can enhance the induction of micronuclei (Asanami and Shimono, 1999; Shuey et al., 2006; Hintzsche, H. *et. al.*, 2012), which may not necessarily be because of toxic agents but solely caused by a temperature-induced genomic damage (Hintzsche, H. *et. al.*, 2012). However, as discussed above, observations of increases in MN formation is considered by the applicant to be an artefact.

Dosing in excess of the MTD is considered to have induced systemic toxicity which would have confounded any MN formation had it in fact occurred. Systemic toxicity may have manifested in haematotoxic effects indicated by a significant decrease in lymphocytes, a (non-significant) decrease in total white blood cells (WBC) and an increase in neutrophils + monocytes at any dose (no dose-response relationship was noted). Haemolysis was observed in cell lines of rat, mouse, sheep, horse and human erythrocytes exposed to alkali-solubilised crystal δ-endotoxin protein from *Bt* *var israelensis* at room temperature (in 50 mM-Na2CO3·HCl, pH 10.5) (Thomas and Ellar, 1983). However, no haemolytic response was noted when erythrocytes were incubated at a lower temperature or with proteins from *Bt* *var kurstaki*, which was used in Mezzomo *et al.* (2015b). The decrease of total WBC noted in Mezzomo *et al.* (2015b) was not significant and well above the lower bound of the haematological leukocyte range noted in male Swiss Webster mice (Santos *et al.*, 2016). A reduction in lymphocytes and an increase in neutrophils + monocytes may be interpreted as indication of haematotoxicity and would be considered to be evidence of systemic toxicity caused by dosing above the MTD.

Concerns related to non-dietary exposure

While genotoxicity of Cry proteins by dietary exposure was considered of no concern in the recent EFSA conclusion (2021), genotoxicity following non-dietary exposure could not be excluded as area of concern. One of the relevant routes of exposure is via inhalation. Similar to pH values reported for the blood and tissues reported above, the airway surface liquid covering a healthy airway epithelium has a pH of approximately 7.2 (Song *et al.*, 2006) and the air present in healthy lungs (measured as exhaled breath condensate) has a pH of 7.7 (Vaughan *et al.*, 2003). This implies that solubilization and proteolytic activation of *Bt* spore crystals are unlikely in the alveolar space due to an insufficiently alkaline environment.

Also, the toxic fragments of Cry proteins are expected to be too big to be readily absorbed in the alveolar space. Most of the Cry proteins have a long chain equivalent to 120-250 kDa molecular weight (Mezzomo *et al.*, 2015c). Following solubilization (at approx. pH ≥10) and activation, the toxic fragment of the protein has a molecular weight of about 60 kDa or higher (Nguyen and Russel, 2010; Bergamasco *et al.*, 2013; Mezzomo *et al.*, 2015c). Therapeutic proteins of a molecular weight of ≥40 kDa are expected to exhibit a 5% bioavailability of the dose via inhalation (Pfister *et al.*, 2014). These bioavailability values were generated with optimized aerosols and inhalation devices; these are prerequisites which are not applicable to a worker and bystander inhalation scenario. Taking this into account, the deposition of 120-250 kDa crystals (non-solubilized) in the alveoli is expected to be minimal and an inhalation bioavailability of 5% is considered to be conservative.

Conclusion

As a consequence of the abovementioned, it is the opinion of the applicant that the Mezzomo *et al.* (2015b) investigated a compromised test system and that results presented in this paper cannot be reliably interpreted due to deficiencies in methodology, performance and reporting to implicate *Bt* spore crystals as a potential genotoxin. Furthermore, it cannot be ruled out that the *Bt* spore crystals may have been partly solubilised and activated prior to injection at high dose into the test mice, as the normal physiological condition is unlikely to be favourable for the solubilisation and activation of the spore crystals.

**References**

Asanami S. and Shimono K (1999). The effect of hyperthermia on micronucleus by mutagens in mice. *Mutat. Res. Genet. Toxicol. Environ. Mutagen* 446(2), 149 -154

Bergamasco V.B., Mendes D.R.P., Fernandes O.A. et al (2013). *Bacillus thuringiensis* Cry1Ia10 and Vip3Aa protein interactions and their toxicity in Spodoptera spp. (Lepidoptera). *J. Invertebr. Pathol*. 112, 152-158

Calhoun C.M., Schnell T.D., and Mandigo R.W. (1998). Porcine Bone Marrow: Extraction procedure and characterization by bone type. *Meat Sci.* 50(4) 489 - 497

Dow J.A.T. (1992). pH gradients of Lepidopteran midgut. *J. exp. Biol.* 172, 355 -375

European Food Safety Authority (EFSA), Alvarez F, Arena M., Auteri D., et al (2021). Conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351. EFSA Journal 2021; 19(10):6879, 20 pp; doi: 10.2903/j.efsa.2021.6879

Griffiths J.R. (1991). Are cancer cells acidic? *Br. J. Cancer* 64, 425-427

Hayashi M., Tice R.R., MacGregor J.T et al. (1994). In vivo rodent erythrocyte micronucleus assay. Mutation Research/Environmental Mutagenesis and Related Subjects, Vol. 312/3, pp. 293-304

Hintzsche H., Riese T. and Stopper H. (2012). Hyperthermia-induced micronucleus formation in a human keratinocyte cell line. Mutat. Res. 738 – 739, 71-74

Knowles B.H. and Dow J.A.T. (1993). The crystal δ‐endotoxins of *Bacillus thuringiensis*: Models for their mechanism of action on the insect gut. *BioEssays* 15(7), 469 – 476

Knowles B.H (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins. *Adv. In Insect Phys.* 24, 275 – 308

MacGregor J.T., Heddle J.A., Hite M. (1987). Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. Mutation Research/Genetic Toxicology, Vol. 189/2, pp. 103-112

Mavournin K.H., Blakey D.H., Cimino M.C., Salamone M.F. and Heddle J.A. (1990). The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program, Mutation Research/Reviews in Genetic Toxicology, Vol. 239/1, pp. 29-80

Mezzomo B., Miranda-Vilela A., Freire I., Barbosa L., Portilho F., Lacava Z., Grisolia C. (2013). Hematotoxicity of Bacillus thuringiensis as spore-crystal Strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss Albino Mice. J Hematol Thromb Dis 1:104

Mezzomo B.P., Miranda-Vilela A.L., Barbosa L.C.P. *et al* (2015b). Hematotoxicity and genotoxicity evaluations in Swiss Mice intraperitoneally exposed to *Bacillus thuringiensis* (*var kurstaki*) spore crystals genetically modiﬁed to express individually Cry1Aa, Cry1Ab, Cry1Ac, or Cry2Aa. *Environ. Toxicol.* 31 (8), 970 -978

Mezzomo B.P., Miranda-Vilela A.L., Grisolia C.K. (2015c). Toxicological evaluation of a potential immunosensitizer for use as a mucosal adjuvant – *Bacillus thuringiensis* Cry1Ac spore-crystals: A possible inverse agonist that deserves further investigation. Toxins 7, 5348-5358

Naimov S., Boncheva R., Karlova R. *et al.* (2008). Solubilization, activation, and insecticidal activity of *Bacillus thuringiensis* Serovar thompsoni HD542 crystal protein. *Appl. Environ. Microbiol.* 75(23), 7145 – 7151

Nguyen J. and Russell S.C. (2010) Targeted proteomics approach to species-level identification of *Bacillus thuringiensis* spores by AP-MALDI-MS. *J. Am. Soc. Mass Spectrom*. 21, 993-1001

Palma L., Munoz D., Berry C. et al. (2014). *Bacillus thuringiensis* toxins: An overview of their biocidal activity. *toxins* 6, 3296 – 3325

Pfister T., Dolan D., Bercu J. *et al.* (2014). Bioavailability of therapeutic proteins by inhalation – worker safety aspects. *Ann Occup. Hyg.* 1-13.

Santos E.W., Olivera D.C. de, Hastreiter A. *et al.* (2016). Hematological and biochemical reference values for C57BL/6, Swiss Webster and BALB/c mice. *Braz. J. Vet. Res. Anim. Sci.*, *53*(2), 138-145.

Schmid W. (1975). The micronucleus test. Mutat Res 31:9–15

Shuey D.L, Gudi R., Krsmanovic L. and Gerson R.J. (2006). Evidence that oxymorphone-induced increases in micronuclei occur secondary to hyperthermia. *Toxicol. Sci.* 95(2), 369-375.

Song Y., Salinas D., Nielson D.W. et al. (2006). Hyperacidity of secreted fluid from submucosal glands in early cystic fibrosis. *Am. J. Physiol. Cell Physiol*. 290, C741-C749

Thomas W.E. and Ellar D.J. (1983). *Bacillus thuringiensis* *var israelensis* crystal δ-endotoxin: Effects on insect and mammalian cells in vitro and in vivo. *J. Cell Sci*. 60, 181-197

Vaughan J., Ngamtrakulpanit L., Pajewski T.N. et al. (2003). Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. *Eur. Respir. J*. 22, 889-894

1. Bernstein L *et al*. (1999). Immune Responses in Farm Workers after Exposure to Bacillus thuringiensis Pesticides; Environmental Health Perspectives 107 (7):575-582. [↑](#footnote-ref-2)
2. Doekes G *et al*. (2004). IgE sensitization to bacterial and fungal biopesticides in a cohort of Danish greenhouse workers: the BIOGART study. American Journal of Industrial Medicine 46(4):404-407. [↑](#footnote-ref-3)
3. Baelum J *et al.* (2012). Health effects of selected microbiological control agents: a 3-year follow-up study. Annals of Agricultural and Environmental Medicine 19(4):631-636. [↑](#footnote-ref-4)