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| REGISTRATION REPORT Part B  Section 6: Ecotoxicological Studies  Detailed summary of the risk assessment |
| Product code:  FORAY® 76B (ABG-6431)  Active Substance:  *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351  206.5 g/L |
| Central Zone  (zRMS: Poland) |
| CORE ASSESSMENT |
| Applicant: XXXX  Submission Date: August 2023  Evaluation date: May 2024  **MS Finalisation date: September 2024** |

Version history

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| **When** | **What** |
| August 2023 | Initial version submitted by the applicant for Art. 43 |
| May 2024 | Version evaluated by zRMS PL |
| September 2024 | Final version by zRMS |
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IIIM 10 RATIONALE TO WAIVE ADDITIONAL TESTING, BASED ON ADEQUACY OF INFORMATION PROVIDED FOR MPCA, TO PERMIT AN ASSESSMENT OF THE IMPACT OF THE MPCP ON NON-TARGET ORGANISMS

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).

The concentration of the technical MPCA (fermentation solids, spores and insecticidal toxins) in Foray® 76B is 206.5 g/L. Both Foray® 76B and DiPel® DF consist of MPCA *Btk* ABTS-351. The analyses of DiPel® Technical Powder showed quantity of total protoxin range from 11.2 - 12.7% (w/w), which result in an estimated protoxin content in Foray® 76B between 2.1 – 2.3% (w/w) (please refer to Part C).

The ecotoxicological risk posed by *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 was evaluated during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on studies with the MPCA *Btk* ABTS-351 and the MPCPs DiPel® DF and Foray® 76B. Both DiPel® DF and Foray® 76B consist of the MPCA *Btk* ABTS-351 and co-formulants all of which either occur naturally in the environment and/or have a low hazard potential and therefore are used as food additives. Since the co-formulants of the products are expected neither to alter the environmental fate of *Btk* ABTS-351 (Part B Section 5) nor to affect pathogenic or infective properties of *Btk* ABTS-351 (RAR, 2020 Vol. 3, B.9), available studies with DiPel® Technical, DiPel® DF and Foray® 76B are considered suitable for the assessment of potential risks from *Btk* ABTS-351 and Foray® 76B (code: ABG-6431) to non-target organisms.

Relevant EU agreed endpoints and conclusions of the risk assessments drawn during the previous EU Renewal of *Btk* ABTS-351 are as follows (EFSA Journal 2021;19(10):6879 and RAR, 2020):

|  |  |
| --- | --- |
| **Section** | **Relevant endpoints and risk assessment** |
| **Effects on terrestrial vertebrates** | |
| Effects on birds | LD50 > 5.7 x 1010 CFU/kg bw/day  No treatment related mortalities or adverse effects were observed over 30 days in Mallard duck and Northern bobwhite quail following short-term (5 days) oral exposure to *Btk* ABTS-351. Investigation of infectivity was not performed in the studies. Open literature studies confirm general absence of adverse effects of *Btk* ABTS-351 on birds. |
| Effects on mammals | LD50 > 426 mg MPCA/kg bw (2.75 x 109 CFU/animal)  No treatment related mortalities or adverse effects were observed in rats following short-term oral exposure to *Btk* ABTS-351. No signs of infectivity and pathogenicity of *Btk* ABTS-351 were observed in the available studies. |
| Risk assessment | A low risk of *Btk* ABTS-351 and CryP to terrestrial vertebrates was concluded, since (1) no evidence for toxicity and pathogenicity was observed in the available studies, (2) *Btk* ABTS-351 and CryP are rapidly degraded on foliage, (3) oral exposure to crystalline proteins is not relevant due to their hydrolysation in the avian and mammalian digestive tract, and (4) terrestrial vertebrates are considered to be continuously exposed to low levels of *Bacillus thuringiensis* in the environment, while no adverse effects of *Bacillus thuringiensis* on terrestrial vertebrates have been reported. |
| **Effects on aquatic organisms** | |
| Effects on fish | 32 d NOEC ≥ 2.87 × 109 CFU/L for rainbow trout and bluegill sunfish  No adverse effects of *Btk* ABTS-351 on mortality and behaviour and no signs of pathogenicity and infectivity were observed in the studies with rainbow trout and bluegill sunfish following 32d exposure to *Btk* ABTS-351. |
| Effects on freshwater invertebrates | 10 d EC50 < 100 mg/L for *Daphnia magna* (based on mortality)  21 d EC50 < 1 x 109 CFU/L for *Daphnia magna* (based on mortality)  21 d EC50 = 2.3 x 108 CFU/L for *Daphnia magna* (based on reproduction)  10 d LC50 > 1.00 × 1010 CFU/kg sediment for harpacticoid copepod  30 d EC50 > 1.7 x 107 CFU/L + 2.87 x 109 CFU/g food for grass shrimp  Daphnids were adversely affected by the high solids concentration in the test media which may have interfered with the daphnids’ filtering system, thereby affecting growth, reproduction, and survival. These effects were not considered to reflect inherent toxicity or pathogenicity of *Btk* ABTS-351 in aquatic invertebrates. No adverse effects of *Btk* ABTS-351 on grass shrimp and harpacticoid copepod were observed. In addition, a series of open literature studies shows that *Btk* ABTS-351 is not toxic, pathogenic, or infectious in aquatic invertebrates. Therefore, the EU agreed endpoint was established as EC50 > 1.0 x 109 CFU/L in a weight-of-evidence approach. |
| Effects on algae | 72 h ErC50 = 5.94 × 108 CFU/L for *Pseudokirchneriella subcapitata*  No adverse effects on algae were observed in the available study that could be attributed to biological activity of *Btk* ABTS-351. |
| Effects on aquatic plants | No study on the effects of *Btk* ABTS-351 and CryP to aquatic plants other than algae is available nor considered necessary due to the highly specific insecticidal MoA of *Btk* ABTS-351. |
| Risk assessment | A low risk of *Btk* ABTS-351 and CryP to aquatic organisms was concluded, since (1) adequate studies were available showing low toxicity to aquatic organisms, (2) no signs of pathogenicity or infectivity were observed in the available studies, and (3) the quantitative risk assessment resulted in sufficiently high margins of safety. |
| **Effects on arthropods** | |
| Effects on bees | 48 h LD50 (oral) > 222.4 µg f.p./bee (2.60 × 106 CFU/bee) for adult honey bee  48 h LD50 (contact) > 185.0 µg f.p./bee (2.16 × 106 CFU/bee) for adult honey bee  14 d LD50 (oral) > 4042 µg MPCA/bee (3.6 × 105 IU/bee) for adult honey bee  17 d LD50 > 100 µg MPCA/larva (5513 IU/larva) for honey bee larva  *Btk* ABTS-351 showed no adverse effects on mortality, behaviour, development and emergence of the honeybee *A. mellifera* in the available studies. No signs of pathogenicity were observed, while infectivity was not assessed. In addition, open literature studies showed that *Btk* ABTS-351 has no adverse effects on mortality, pollen collection, hive activity, hive weight, and brood development of honeybees. |
| Risk assessment | A low risk of *Btk* ABTS-351 and CryP to bees was concluded, since (1) adequate studies were available showing low toxicity to honeybees, (2) no signs of pathogenicity or infectivity were observed in the available studies, and (3) the quantitative risk assessment resulted in a sufficiently high margin of safety. |
| Effects on terrestrial arthropods other than bees | 14 d ER50 > 2.38 x 1011 IU/ha for *Typhlodromus pyri*  21 d ER50 > 2.38 x 1011 IU/ha for *Aphidius rhopalosiphi*  8 d EC50 > 62 g MPCA/L for *Metaseiulus occidentalis*  8 d EC50 < 6.2 g MPCA/L for *Tetranychus urticae* (EU endpoint not in line with study data)  *Btk* ABTS-351 showed no adverse effects on survival and reproduction of *A. rhopalosiphi*, and only a slight effect on survival (i.e., 14.1% corrected mortality) and no effect on reproduction of *T. pyri* were observed in the available studies. Exposure of *T. urticae* to 62 g MPCA/L resulted in 31.94% mortality of adult gravid host prey mites, while no mortality of protonymphs or effects on larva hatching was observed. Exposure of *M. occidentalis* to 6.2 and 62 g MPCA/L resulted in 10.29 - 12.3% mortality, while no effects on number of eggs laid were observed compared to control. Hatching rate of *M. occidentalis* was reduced in all treatment groups (i.e., 0.62 - 62 g MPCA/L) compared to control, while no dose-response relationship was apparent. Additional studies are available with *Chysoperla carnea* and *Trichogramma cacoeciae*, which did not provide reliable endpoints due to high control mortality. However, no effects were observed on larval mortality, time to pupation, pupation rate, pupal mortality, total mortality and egg production of *C. carnea*. In the study with *T. cacoeciae*, survival and fecundity were significantly lower in the test item groups compared to the control, despite high mortalities and decreasing fecundity in all test groups. No adverse effects were observed for hatching rate, sex ratio, emergence, physical appearance, and behaviour of *T. cacoeciae*.  In addition, a series of open literature studies indicate that *Bacillus thuringiensis* subsp. *kurstaki* has no detrimental effects on insects of the orders Orthoptera, Dermaptera, Heteroptera, Coleoptera, Diptera, and Hymenoptera following various routes of oral and contact exposure. Some adverse effects have been described for test species of the orders Hymenoptera and Lepidoptera. However, the observed adverse effects on Hymenoptera resulted from exposure to high levels of *Btk* ABTS-351 that would not be expected under realistic conditions, while non-target lepidopteran species are expected to recover quickly due to multiple life cycles per year. |
| Risk assessment | EFSA considered the risk assessment as not finalised due to insufficient data on infectivity and pathogenicity of *Btk* ABTS-351 in non-target arthropods. However, the RMS concluded a low risk of *Btk* ABTS-351 and CryP to non-target arthropods, since available strain specific data and available knowledge on *Bacillus thuringiensis* subsp. *kurstaki* indicate that *Btk* ABTS-351 is not toxic, pathogenic, or infective in insects other than the target pest. |
| **Effects on soil organisms** | |
| Effects on other terrestrial invertebrates | 30 d NOEC ≥ 1.1 × 1010 CFU/kg dry soil for *Eisenia fetida*  Since *Btk* ABTS-351 showed no adverse effects on mortality, body weight and behaviour in a 30-d laboratory study with *Eisenia fetida*, *Btk* ABTS-351 was considered to not exhibit pathogenicity and infectivity in earthworms. Findings were supported with results of two open literature studies showing that *Btk* ABTS-351 has no adverse effects on earthworms in forest soil. |
| Risk assessment | A low risk of *Btk* ABTS-351 and CryP to earthworms was concluded, since (1) no toxicity or pathogenicity were observed in the available studies, (2) the quantitative risk assessment resulted in a sufficiently high margin of safety, (3) and earthworms are recognized to have adequate immune systems to cope with microorganisms. |
| Effects on soil microorganisms | NOEL = 0.226 μL product/10 g soil for soil respiration and nitrification  A study is available on the effects of *Btk* ABTS-351 on soil respiration and nitrification following application of 0.226 µL product/10 g soil and 226 µL product/10 g soil. The low-test rate corresponds to 1.42 x 108 CFU/kg dry soil. No adverse effects on nitrification were observed after 8 weeks. Soil respiration was significantly higher in the high-test item group compared to control after 8 weeks, while no significant differences compared to control were detected for the low-test item group. |
| Risk assessment | A low risk of *Btk* ABTS-351 and CryP to soil microorganisms was concluded, since (1) the quantitative risk assessment resulted in a margin of safety, (2) and microbial communities in soil are well adapted to their habitat and show good resilience and recovery towards stressors. |
| **Effects on non-target plants** | |
| Risk assessment | In line with Commission Regulations (EU) 283/2013 and 284/2013, no risk assessment was performed for non-target terrestrial plants. |

This document reviews the ecotoxicological studies for *Btk* ABTS-351 and the formulated product Foray® 76B (product code ABG-6431). A full risk assessment according to Uniform Principles which demonstrates the ecotoxicological safety of the product is provided. In cases where country specific assessments for some data requirements are provided, this document should be read in conjunction with the relevant addenda.

For the implementation of the uniform principles according to Part II of the Annex to Regulation (EU) No 546/2011, the conclusions of the review report on *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351, and in particular Appendices I and II thereof, as finalised shall be taken into account. In this overall assessment, any relevant concerns in the review report have been addressed within the current submission.

Appendix 1 of this document contains the list of references included for support of the evaluation.

Appendix 2 of this document is the table of intended uses for Foray® 76B (product code ABG-6431).

Information on the detailed composition of Foray® 76B (product code ABG-6431) can be found in the confidential dossier of this submission (Registration Report - Part C).

**Critical use patterns of Foray® 76B for the environmental risk assessment**

The following table documents the grouping of the intended uses to support application of the risk envelope approach (according to SANCO/11244/2011). Crop groups were assigned according to EFSA/2009/1438 or Part B Section 5 (Environmental Fate). For details on the intended uses of Foray® 76B see Appendix 2 and Part A.

**Table 10-1: Critical use patterns of Foray® 76B for the environmental risk assessment**

| **Crop group** | **Intended uses** | **Relevant use parameters** | **Covered applications** |
| --- | --- | --- | --- |
| **Effects on terrestrial vertebrates (IIIM 10.1)** | | | |
| Deciduous forest | 1-2 x 3.0 L product/ha  (1-2 × 4.53 × 1013 CFU/ha) a | Maximum application rate:  1-2 x 619 g MPCA/ha, 14 d interval | All uses based on max. single application rate |
| Pine trees, deciduous and coniferous forest, shrubs, ornamental plants | 1-4 x 2.5 L product/ha  (4 × 3.77 × 1013 CFU/ha) a | Maximum application rate:  4 x 516 g MPCA/ha, 5 d interval | All uses based on multiple application |
| **Effects on aquatic organisms (IIIM 10.2)** | | | |
| Pine trees, deciduous and coniferous forest, shrubs, ornamental plants | 1-4 x 2.5 L product/ha  (1-4 × 3.77 × 1013 CFU/ha) a | Highest PEDSW b  (i.e., 1.87 × 107 CFU/L)  Highest PECSW b  (i.e., 66.97 µg CryP/L) | All uses |
| **Effects on arthropods other than bees (IIIM 10.4)** | | | |
| Deciduous forest | 1-2 × 3.0 L product/ha (undiluted)  (1-2 × 619 g MPCA/ha) | Max. single application rate  - 6.59 × 1010 IU/ha c (619 g MPCA/L) | All uses |
| **Effects on earthworms (IIIM 10.5) and soil microbial activity (IIIM 10.6)** | | | |
| Pine trees, deciduous and coniferous forest, shrubs, ornamental plants | 1-4 x 2.5 L product/ha  (Annual dose: 1.69 × 1014 CFU/ha) d | Highest PEDSOIL b  (i.e., 2.26 × 108 CFU/kg d.s.)  Highest PECSOIL b  (i.e., 350 µg CryP/kg d.s.) | All uses |

Cspray = Maximum spore density in spray solution based on application rate, minimum application volume, and maximum spore density in formulation (i.e., 1.69 × 1013 CFU/L product); PEDSW / PEDSOIL = Worst-case spore density in surface water/soil calculated based on yearly total dose application as one single application assuming no degradation; PECSW / PECSOIL = Worst-case concentration of CryP in surface water/soil calculated in Part B Section 5.

a Based on nominal spore density of 1.51 × 1013 CFU/L product as proposed in GAP and Part C.

b Based on 0% interception, seasonal dose without degradation, and maximum spray drift of 33.2% for surface water entry.

c Based on max. single application rate and nominal potency of 19600 IU/mg product as specified in Part C.

d Based on maximum spore density of 1.69 × 1013 CFU/L product as stated in Part C.

**Toxins/metabolites from microbial pest control agent (MPCA)**

*Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 has the genetic capability to form the crystals of proteinaceous insecticidal δ-endotoxins Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab (also referred to as crystalline proteins, cry proteins, insecticidal crystal proteins (ICPs), parasporal crystals, parasporal protein-crystal, parasporal crystalline inclusions; hereafter referred to as CryP). Furthermore, *Btk* ABTS-351 has the potential to form a non-haemolytic (Nhe) and haemolytic (Hbl) enterotoxin complex (which also includes CytK2) though the operon related to Hbl is incomplete (indicating haemolysin action is not functional). *Btk* ABTS-351 has been reported to produce Nhe enterotoxins, with its vegetative cells secreting vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip). CryPs are formed during the stationary phase of the growth cycle of *Bacillus thuringiensis* and can be released into the alkaline midgut of susceptible insect larvae (e.g., Lepidoptera) upon ingestion of *B. thuringiensis*. Most CryPs are protoxins, that are solubilised under the alkaline conditions of the insect midgut where they are proteolytically activated by proteases to become activated Cry toxins. The activated Cry toxins then bind readily to specific receptors on the apical brush border of the midgut microvillae of susceptible insects, leading to cell disruption and consequently death of the insect. Therefore, the pathogenicity of *Bacillus thuringiensis* to insects requires activation of protoxins and highly specific receptor binding sites (i.e., cadherin receptors) in the host. *Btk* ABTS-351 is specific to several species of insects of the order Lepidoptera and no cases of infectivity in other animal organisms or in plants are reported. The crystal proteins of *B. thuringiensis* must be ingested to be effective against the target insect. Spore germination and proliferation of vegetative cells into haemocoel is possible and may result in septicemia and subsequently in mortality of the insect larva.

It is not known to what extent products containing *Btk* ABTS-351 will produce CryP or vegetative cells following application. However, CryP (except Cry1Ia and Cry2Ab) constitute components in products containing *Btk* ABTS-351 within and outside spores and are responsible for the insecticidal mode of action of the MPCA. Analyses of DiPel® Technical Powder showed that the quantity of total protoxin ranges from 11.2 - 12.7% (w/w), which results in an estimated protoxin content in Foray® 76B between 2.1 – 2.3% (w/w; refer to Part C). Since the concentrations of the CryP in the formulated product is known, it is considered appropriate to estimate the potential exposure on environmental compartments based on the CryP content in the formulated product (in line with approach followed in latest EU Renewal of *Btk* ABTS-351; details in EFSA Journal 2021;19(10):6879 and RAR, 2020). Commercial products containing *Btk* strain ABTS-351 have been shown not to contain β-exotoxins or enterotoxins.

**Potential exposure of non-target organisms to *Btk* ABTS-351 and CryPs**

*Bacillus thuringiensis* occurs naturally and ubiquitously in the environment. It is a common component of the soil microflora and has been isolated from numerous habitats in different countries worldwide. *B. thuringiensis* subsp. *kurstaki* has been used for decades for control of Lepidopteran pests in agricultural settings and is the most widely used sub-species used for control of pest insects of crops and forests. *B. thuringiensis* subsp. *kurstaki* strain ABTS-351 is also widely authorised for use as a plant protection product in European countries. Unlike most insect pathogenic microbes, *Bacillus thuringiensis* is recognised to be a poor infectious agent that rarely recycles. Optimum growth conditions for *Bacillus thuringiensis* subsp. *kurstaki* are 28°C to 30°C and pH 6.8–7.2 (EFSA Journal 2021;19(10):6879).

In soil, spores of *Bacillus thuringiensis* subsp. *kurstaki* can remain viable for many years and can germinate in the rhizosphere of some plants. Available data indicate that spore germination does not occur in bulk soil with limited nutrient levels. Repeated application of *Btk* ABTS-351 is expected to lead to an accumulation of spores in the soil environment, but multiplication in bulk soil is considered unlikely. Under unfavourable natural conditions, the microorganism undergoes sporulation, leading to the formation of endospores and CryP (EFSA Journal 2021;19(10):6879). CryP are not expected to persist or accumulate in soil.

Available data indicate that *Bacillus thuringiensis* is present in surface water and that the species is likely capable of growing in freshwater environments under nutrient/oxygen-rich conditions (EFSA Journal 2021;19(10):6879), while crystalline proteins are not considered to persist or accumulate in water and are degraded rapidly (details in RAR, 2020 Vol. 3 B.8). However, since *Bacillus thuringiensis* is a soil bacterium, itis not expected to find optimal conditions for growth in aquatic environments, and proliferation is not likely to occur in freshwater systems. Vegetative cells and spores of *Btk* ABTS-351 may survive but will be subject to sunlight, predation and natural competition by in the diverse microbiota of natural waters. Therefore, it is expected that sporulation and germination will not occur and thus CryP will not be synthesized or released to significant extent. This is further supported by a series of open literature studies (details in Part B Section 5) showing that *Bacillus thuringiensis* is not a dominant member of the aquatic microbial community under nutrient-rich conditions (simulated by organic matter influx) and is dominated by various other microbiota which are better adapted to aquatic environments such as species of the phyla Proteobacteria, Bacteriodetes, Verrucomicrobia, Actinobacteria, and Chloroflexi.

On edible plant commodities, viable counts of *Btk* ABTS-351 were shown to decline following application and to not persist or multiply on edible plant commodities (fruiting vegetable and leafy crops). Open literature studies suggest inactivation and decline of viable spores due to environmental factors such as solar radiation, rainfall, plant growth and temperature (EFSA Journal 2021;19(10):6879). The microorganism is not translocated in the plant. *Btk* ABTS-351 has a half-life of less than 24 h on foliage (RAR, 2020 Vol. 3 B7). Following spray application, δ-endotoxins are rapidly degraded, and endospores are rapidly inactivated when exposed to UV radiation (EFSA Journal 2021;19(10):6879).

While *Btk* ABTS-351 and CryP are not expected to be persistent in or accumulate to high levels in relevant environmental compartments, very conservative exposure estimates were modelled for *Btk* ABTS-351 and CryP based on a series of worst-case assumptions (e.g., yearly total dose application as one single application assuming no degradation; details in Part B Section 5) and used below to assess the risk from *Btk* ABTS-351 and CryP to non-target organisms.

IIIM 10.1 Effects on terrestrial vertebrates

|  |  |
| --- | --- |
| Evaluator  Comments: | The presented approach was accepted.  The submitted justification was accepted.  The risk for terrestrial vertebrates (birds and mammals) is acceptable. |

**Effects on birds**

The effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on terrestrial vertebrates were evaluated during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on studies with the microbial pest control agent (MPCA) *Btk* ABTS-351. The representative microbial pest control product (MPCP) during this EU review was DiPel® DF. No toxicity and pathogenicity data are available from studies with the formulated product Foray® 76B (product code: VBC-60013) nor considered necessary (see rationale in Section IIIM 10). Details on the available studies are available in EFSA Journal 2021;19(10):6879 and related documents (RAR, 2020). The relevant endpoints used for the present risk assessment are shown below.

**Table 10.1-1: Available data on adverse effects of** ***Btk* ABTS-351 on birds**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test substance** | **Exposure system** | **Endpoint** | **Reference** |
| Northern  bobwhite quail  (*Colinus virginianus*) | *Btk*  ABTS-351 a | Oral toxicity and pathogenicity b | LD50 > 2857 mg MPCA/kg bw/d  (5.7 × 1010 CFU/kg bw/d) | EFSA Journal 2021;19(10):6879 |
| Mallard  (*Anas platyrhynchos*) | Oral toxicity and pathogenicity b | EFSA Journal 2021;19(10):6879 |

CFU= Colony Forming Units

a Studies performed with DiPel® technical material, i.e., the source of *Btk* ABTS-351 in Foray® 76B

b 5-day administration, 30-day observation period

The available studies with mallard duck and bobwhite quail over 30 days did not show any treatment-related mortalities or signs of toxicity and pathogenicity following oral 5-d exposure to *Btk* ABTS-351 at 2857 mg/kg bw/d (equivalent to approx. 5.7 × 1010 CFU/kg bw/d). Infectivity was not investigated in the studies.

In addition, open literature studies are available from the previous EU Review for *Btk* ABTS-351 which were considered to provide supportive information by the RMS (details in RAR, 2020 Vol. 3 B.9). In the study by Buckner *et al.* (1974), adverse effects of *Bacillus thuringiensis* var. *kurstaki* on breeding bird populations were assessed in a field trial following application of the formulated products DiPel® and Thuricide. Since no statistically significant differences were detected between the populations of treated and untreated plots, it was concluded that *Bacillus thuringiensis* var. *kurstaki* has no effect on bird populations. In the study by Sopuck *et al.* (2002), the potential adverse effects of *Bacillus thuringiensis* var. *kurstaki* on songbirds were evaluated in the course of a control program for the gypsy moth (*Lymantria dispar*) in gary oak (*Quercus garryana*) dominated habitats. Since no differences in songbird numbers were detected following spray application of Foray® 48B compared to unsprayed control areas, the study was considered to indicate the absence of direct adverse effects of *Bacillus thuringiensis* var. *kurstaki* on songbirds (RAR, 2020 Vol. 3 B.9). The RMS concluded that the available laboratory studies and open literature studies sufficiently demonstrate that *Btk* ABTS-351 is not toxic, pathogenic or infective in birds (EFSA Journal 2021;19(10):6879 and RAR, 2020 Vol. 3 B.9).

**Infectivity and pathogenicity of *Btk* ABTS-351 in birds**

The available effect studies with mallard duck and bobwhite quail did not show any treatment-related mortalities or signs of infection or pathogenicity over a period of 30 days. Since infectivity was not particularly assessed in the available studies, they do not allow for specific conclusions on the infectivity of *Btk* ABTS-351 in birds. However, since the avian gastrointestinal tract does not provide optimum growth conditions for *Btk* ABTS-351 (refer to section below on toxicity of CryP to birds), and *B. thuringiensis* subsp. *kurstaki* has been used for decades for control of Lepidopteran pests in agricultural settings and forests in European countries, while no negative effects on birds have been reported, it was concluded during the latest EU Renewal of *Btk* ABTS-351 (RAR, 2020 Vol. 3 B.9) that *Btk* ABTS-351 is not expected to exhibit infectivity in birds.

**Risk assessment for birds**

Main exposure of birds to the MPCA/MPCP occurs *via* consumption of residues on contaminated food such as vegetation, insects and earthworms, and *via* uptake of contaminated drinking water.

According to PRAPeR M2 (Expert Meeting on microorganisms in 2009) and Ctgb (Evaluation Manual for the Authorisation of Biopesticides according to Regulation (EC) No 1107/2009, v. 1.4, 2022), the guidance document EFSA/2009/1438 on “The Risk Assessment for Birds and Mammals” is intended for chemical substances and is considered less relevant for plant protection products containing microorganisms. Note that this does not necessarily mean that risk assessment schemes available for chemicals do not provide approaches and methods that may (in part) be useful to support the assessment of the risk posed by microorganisms to terrestrial vertebrates.

In the latest EU Renewal of *Btk* ABTS-351 (RAR, 2020 Vol. 3 B.9), the zRMS Denmark assessed the risk from dietary uptake to birds by comparing the maximum concentration in the application liquid (expressed in CFU/L) and the relevant avian endpoint (expressed in CFU/kg bw/d) on a numerical basis. Risk quotients > 1.0 were considered to indicate acceptable risks from MPCAs/MPCPs to birds through dietary exposure. However, this numerical comparison is not based on sound scientific principles or appropriate risk assessment schemes, because the units of the respective exposure and effect data are not comparable and this approach does not reflect the actual exposure of birds to the relevant components of Foray® 76B (i.e., spores and vegetative cells of *Btk* ABTS-351 and associated CryP) following the intended uses.

Indeed, no appropriate risk assessment scheme to address the risk from MPCAs/MPCPs to terrestrial vertebrates through dietary exposure and no guidance on interpretation of calculated risk quotients is currently available for microorganisms. Nevertheless, a quantitative risk assessment is presented below that attempts to describe the risk posed by *Btk* ABTS-351 to birds through exposure *via* dietary consumption. In addition, the risk from *Btk* ABTS-351 to birds through uptake of contaminated drinking is assessed based on the worst-case scenario for exposure *via* drinking water according to EFSA/2009/1438.

*Dietary exposure*

Birds may be exposed to vegetative cells, spores and/or associated toxins of *Btk* ABTS-351 through dietary consumption *via* two oral routes of exposure; (1) direct exposure to the MPCP Foray® 76B (containing spores of *Btk* ABTS-351 and associated toxins) by ingestion of freshly sprayed plant parts and/or insects shortly after the application, or (2) indirect exposure to spores, vegetative cells and CryP by ingestion of insects (e.g., lepidoptera larvae) that may have ingested *Btk* ABTS-351 following application of Foray® 76B.

Note that the formulated product Foray® 76B contains spores and associated toxins but no vegetative cells. Since it is not known to what extent the *Btk* ABTS-351 spores will produce vegetative cells following application of Foray® 76B, and no effect studies characterizing potential adverse effects of vegetative cells of *Btk* ABTS-351 in birds are available, the risk from vegetative cells of *Btk* ABTS-351 to birds cannot be quantitatively assessed. In addition, a quantitative risk assessment for oral exposure of birds to CryP is not considered necessary since CryPs are hydrolised and inactivated in the avian gastrointestinal tract (see Section “Risk posed by toxins/metabolites from *Btk* ABTS-351 in birds”).

To quantitatively relate the potential exposure of birds to spores of *Btk* ABTS-351 directly after application of Foray® 76B to the endpoint of the available avian pathogenicity studies, a quantitative risk assessment based on the screening assessment scheme according to EFSA/2009/1438 is shown below. The presented screening assessment compares the endpoint of the avian pathogenicity studies (which showed that 5-day exposure of birds to *Btk* ABTS-351 spores at 2857 mg MPCA/kg bw/d does not cause adverse effects in birds over a period of 30 days) with calculated peak exposures of birds to the MPCA (containing spores of *Btk* ABTS-351) on food items directly after the intended spray applications of Foray® 76B. Exposure estimates are calculated using short-term residue unit doses (RUD; 90% percentile), which reflect the worst-case peak exposure of birds to chemicals according to EFSA/2009/1438. Since spores of *Btk* ABTS-351 in Foray® 76B are considered in this approach as inert component of the formulated product, while actual spore densities on relevant food items of birds are likely to decrease (e.g., due to radiation) or remain stable in the worst-case (but probably do not increase), short-term RUD according to EFSA/2009/1438 are considered suitable in this case. Since there are no significant differences between chemicals and *Btk* ABTS-351 spores in terms of contamination of food items directly after spray application, the risk assessment below appropriately relates the endpoint of the avian studies and peak exposures of birds to spores of *Btk* ABTS-351 directly after application of Foray® 76B.

In a very conservative approach, the full screening assessment according to EFSA/2009/1438 is shown for all possible application and crop scenarios, considering the dietary consumption of all relevant model species in EFSA/2009/1438 and residue unit doses (RUD; 90% percentile) for their respective food items. This includes all possible worst-case combinations of model species, feeding habits and RUD values (e.g., RUD for vegetation that has been directly oversprayed reflecting worst-case exposures directly after application). The risk assessment is shown for application of 1 x 619 g MPCA/ha and 4 x 516 g MPCA/ha based on a 5-d interval between applications. For multiple applications, the default DT50 for chemicals in feed matrices according to EFSA/2009/1438 was used, while it is noted that the default DT50 was established for chemicals and may not be adequate to describe exposure levels for MPCAs over time. However, since *Btk* ABTS-351 has a short half-life on e.g., foliage (details in RAR, 2020 Vol. 3 B7 and Vol. 3 B8), and biological activity of spores is expected to decline within few hours to days, the default DT50 of 10 days is considered to reflect a conservative approach in this case.

The RMS and the co-RMS strongly disagree with the applicant on applying the chemical risk assessment scheme for products based on microorganisms. However, this information has been provided for completeness.

**Table 10.1‑2: Risk from spores of *Btk* ABTS-351 to birds through dietary exposure directly following spray application of Foray® 76B**.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Intended use** | **Pine trees, deciduous/coniferous forest, shrubs, ornamental plants** | | | | |
| **Active substance/product** | *Btk* ABTS-351 / Foray® 76B | | | | |
| **Application rate [g MPCA/ha]** | 1 x 619 g MPCA/ha in deciduous forest,  4 x 516 g MPCA/ha (5-d interval) in pine trees, deciduous/coniferous forest, shrubs, and ornamental plants | | | | |
| **Relevant endpoint [mg MPCA/kg bw/d]** | 30-d LD50 > 2857 mg MPCA/kg bw/d | | | | |
| **Short-term screening assessment - 1 x 619 g MPCA/ha** | | | | | |
| **Crop scenario** | **Model species** | **SV90** | **MAF90** | **DDD90**  **[mg/kg bw/d]** | **MoS** |
| Bare soils and hop | Small granivorous bird | 24.7 | 1.00 | 15.66 | > 182.5 |
| Grassland | Large herbivorous bird | 30.5 | 1.00 | 18.87 | > 151.4 |
| Bush and cane fruit | Small frugivorous bird | 46.3 | 1.00 | 32.30 | > 88.4 |
| Orchards and ornamentals/nursery | Small insectivorous bird | 46.8 | 1.00 | 28.96 | > 98.7 |
| Vineyard | Small omnivorous bird | 95.3 | 1.00 | 58.97 | > 48.4 |
| Bulbs/onion like crops, cereals, fruiting/leafy vegetables, legume forage, maize, oilseed rape, potatoes, pulses, root/stem vegetables, strawberries, sugar beet and sunflower | Small omnivorous bird | 158.8 | 1.00 | 98.26 | > 29.1 |
| Cotton | Small omnivorous bird | 160.3 | 1.00 | 99.19 | > 28.8 |
| **Short-term screening assessment - 4 x 516 g MPCA/ha (5 d interval)** | | | | | |
| **Crop scenario** | **Model species** | **SV90** | **MAF90** | **DDD90**  **[mg/kg bw/d]** | **MoS** |
| Bare soils and hop | Small granivorous bird | 24.7 | 2.00 | 26.14 | > 109.3 |
| Grassland | Large herbivorous bird | 30.5 | 2.00 | 31.51 | > 90.7 |
| Bush and cane fruit | Small frugivorous bird | 46.3 | 2.00 | 53.93 | > 53.0 |
| Orchards and ornamentals/nursery | Small insectivorous bird | 46.8 | 2.00 | 48.35 | > 59.1 |
| Vineyard | Small omnivorous bird | 95.3 | 2.00 | 98.45 | > 29.0 |
| Bulbs/onion like crops, cereals, fruiting/leafy vegetables, legume forage, maize, oilseed rape, potatoes, pulses, root/stem vegetables, strawberries, sugar beet and sunflower | Small omnivorous bird | 158.8 | 2.00 | 164.06 | > 17.4 |
| Cotton | Small omnivorous bird | 160.3 | 2.00 | 165.61 | > 17.3 |

SV: shortcut value; MAF: multiple application factor; DDD: daily dietary dose; MoS: Margin of safety. Exposure estimates are rounded to 2 significant figures; MoS represent accurate values underlying the actual exposure estimates.

The screening assessment above indicates an acceptable risk to birds through dietary uptake of spores of *Btk* ABTS-351 directly following exposure of Foray® 76B with high margins of safety for all assessment scenarios presented in EFSA/2009/1438. In addition, actual exposure of birds to spores of *Btk* ABTS-351 by ingestion of freshly sprayed plant parts and/or insects shortly after the application of Foray® 76B is likely to be lower than considered for the risk assessment above, since *Btk* ABTS-351 has a short half-life on foliage and its biological activity degrades within hours (DT50 of < 24 h on foliage according to EFSA Journal 2021;19(10):6879). Moreover, *Btk* ABTS-351 is not translocated in plants. The high margins of safety calculated in the quantitative risk assessment above provide supportive evidence indicating a low risk posed by *Btk* ABTS-351 to birds.

No appropriate risk assessment scheme is available to address indirect exposure of birds to spores, vegetative cells and CryP by ingestion of insects (e.g., lepidoptera larvae) that might have ingested *Btk* ABTS-351 following application of Foray® 76B. In addition, neither levels of CryP, spores and vegetative cells of *Btk* ABTS-351 in infected insects that may be foraged by birds are known, nor are effect studies available characterizing potential adverse effects of vegetative cells of *Btk* ABTS-351 in birds.

Nevertheless, the risk posed by indirect exposure of birds to CryP, spores and vegetative cells of *Btk* ABTS-351 through consumption of infected insects can be considered low for the following reasons:

* Available data provide no evidence that *Btk* ABTS-351 may be toxic, pathogenic or infective in birds.
* *Btk* ABTS-351 is an ubiquitous soil microbe that has been used for decades in agricultural settings and forests across Europe, while no negative effects of *Btk* ABTS-351 on wildlife have been reported.
* *Btk* ABTS-351 levels are not expected to multiply in environmental compartments following application of Foray® 76B.
* Two field trials have been carried out in Canada and USA (Buckner *et al.*, 1974; Sopuck *et al.*, 2002), neither of which indicated any effect on birds after aerial application of *B. thuringiensis* subsp. *kurstaki*.
* It is very unlikely that birds will establish a reliance of the target organisms as a main source of diet, while application of Foray® 76B will control the population of the target insects and maintains non-target insects for birds to feed on. In addition, it is unknown whether or not birds will feed on infected or dead and decaying larvae containing vegetative cells of *B. thuringiensis* subsp. *kurstaki*.

Therefore, the risk to birds through dietary uptake of *Btk* ABTS-351 or associated toxins is considered low for all proposed uses of Foray® 76B.

*Exposure via drinking water*

In a conservative approach, the risk from *Btk* ABTS-351 to birds through uptake of contaminated drinking water can be assessed based for the worst-case scenario for exposure *via* drinking water according to EFSA/2009/1438, i.e., the small granivorous bird with a body weight of 15.3 g and a drinking rate of 7.0 mL/day (equivalent to 0.46 L/kg bw/d). The risk assessment below is based on the maximum predicted environmental density in surface water (i.e., PEDSW), which was calculated for application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) using the yearly total dose application as one single application without degradation of *Btk* ABTS-351 (worst-case approach; details in Part B Section 5).

**Table 10.1-3: Risk from *Btk* ABTS-351 to birds following exposure *via* drinking water**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Application**  **scenario** | **Appl. rate** | **PEDSW** | **Drinking rate a** | **Daily**  **dose** | **Relevant LD50** | **LD50 higher than daily dose?** | |
| **[L f.p./ha]** | **[CFU/L]** | **[L/kg bw/d]** | **[CFU/kg bw/d]** | **[CFU/kg bw/d]** | **(Trigger: < 1)** | |
| Pine trees, deciduous/coniferous forest, shrubs, ornamental trees and plants | 4 × 2.5 | 1.87 × 107 | 0.46 | 8.60 × 106 | > 5.7 × 1010 | 6626 | Yes |

Appl. rate = Maximum single application rate; f.p. = formulated product; PEDSW = Predicted spore density in surface water calculated based on the yearly total dose application as one single application assuming no degradation of *Btk* ABTS-351; **bold** values indicate risk quotients < 1.0, i.e., the LD50 of > 5.7 × 1010 CFU/kg bw/d is lower than the calculated daily dose

a Based on the worst-case scenario (EFSA/2009/1438) small granivorous bird with a body weight of 15.3 g and a drinking rate of 7.0 mL/day.

The drinking water assessment for the risk envelope application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) shows that the daily dose for birds through consumption *via* drinking water (i.e., 8.60 x 106 CFU/kg bw/d) is clearly lower than the relevant avian endpoint. Therefore, the risk to birds from contaminated drinking water is considered acceptable for all proposed uses.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 in birds**

No laboratory studies are available which specifically characterized the toxicity of relevant CryP to birds. However, the avian digestive tract is incompatible with the mode of action of *Btk* ABTS-351, since the pH in the avian digestive tract is slightly acidic, no specific cadherin receptors binding sites are present, and proteinaceous insecticidal δ-endotoxins would be inactivated by protease enzymes (refer to EFSA Journal 2021;19(10):6879 and RAR, 2020 Vol. 3 B.9). Therefore, oral exposure of birds to preformed CryP is not relevant. Optimum growth conditions for *Bacillus thuringiensis* subsp. *kurstaki* are 28 - 30°C and pH 6.8 - 7.2 (EFSA Journal 2021;19(10):6879), while the body temperature of birds usually ranges from 39 - 43°C, and pH in the avian gastrointestinal tract ranges from 2.5 – 5.5 in crop and gizzard and 5.0 - 8.0 in different segments of the intestine (documented for chicken in Journal of Applied Poultry Research 22.3 (2013): 628-636). Therefore, the avian gastrointestinal tract does not provide optimum growth conditions for *Btk* ABTS-351. In addition, (1) *B. thuringiensis* subsp. *kurstaki* has been used for decades for control of Lepidopteran pests in agricultural settings and forests in European countries, while no negative effects on birds have been reported, (2) *B. thuringiensis* is naturally occurring in the environment and thus birds are continuously exposed to low levels of *B. thuringiensis*, (3) *B. thuringiensis* is not persistent on foliage and its insecticidal activity declines rapidly within few days due to solar radiation, (4) and *B. thuringiensis* subsp. *kurstaki* is not expected to multiply in soil and water following application of Foray® 76B (details in Part B Section 5). In conclusion, the risk from CryP to birds is considered low following the proposed uses of Foray® 76B.

**Effects on mammals**

The effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on mammals were evaluated during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on studies with the MPCA *Btk* ABTS-351. Details on the available studies are available in EFSA Journal 2021;19(10):6879 and related documents (RAR, 2020). In addition, an acute oral toxicity study with the MPCP Foray® 76B is available (refer to IIIM 7.1.1/01). The relevant endpoints used for the present risk assessment are shown below.

**Table 10.1-4: Available data on adverse effects of *Btk* ABTS-351 to mammals**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test substance** | **Exposure system** | **Endpoint** | **Reference** |
| Rat | *Btk* ABTS-351 a | Acute, oral,  14-d observation period | LD50 > 5050 mg MPCA/kg bw  (> 2 × 1011 CFU/kg/bw) b | EFSA Journal 2021;19(10):6879 |
| Acute, oral,  29-d observation period | LD50 > 426 mg MPCA/kg bw  (> 2.75 × 109 CFU/rat) c | EFSA Journal 2021;19(10):6879 |
| Rat | Foray® 76B | Acute, oral,  14-d observation period | LD50 > 5050 mg MPCA/kg bw | Kuhn, 1991a  IIIM 7.1.1/01 |

CFU= Colony forming units

a Studies performed with DiPel® technical material, i.e., the source of *Btk* ABTS-351 in Foray® 76B.

b Study considered to provide only supporting information in RAR, 2020 Vol. 3 B.9.

c Test organisms were dosed once orally with 1 mL of test solution containing 2.75 × 1012 CFU/L.

Three acute oral toxicity studies on rats are available, i.e., two studies with the MPCA *Btk* ABTS-351 and one study with the MPCP Foray® 76B. In all of these three studies, no treatment-related adverse effects on survival, body weight, gross necropsy, or other clinical signs were observed over the observation periods of 14 and 29 days. Due to several shortcomings, the 14-d study with *Btk* ABTS-351 was considered to provide only supportive information (RAR, 2020 Vol. 3 B.6). The 29-d study resulted in an LD50 > 2.75 × 109 CFU/kg/bw (limit endpoint), while no evidence of pathogenicity or infectivity of *Btk* ABTS-351 in rats was observed in this study.

**Infectivity and pathogenicity of *Btk* ABTS-351 in mammals**

No signs of pathogenicity and infectivity were observed in the available 29-d oral acute toxicity study with the MPCA *Btk* ABTS-351. No treatment-related effects were apparent during necropsy and clearance of *Btk* ABTS-351 in rats was documented.

**Risk assessment for mammals**

Main exposure of mammals to the MPCA/MPCP occurs *via* consumption of residues on contaminated food such as vegetation, insects and earthworms, and *via* uptake of contaminated drinking water. Risk assessments for both exposure scenarios are shown below.

*Dietary exposure*

The risk from dietary uptake to mammals is quantitatively assessed based on similar same principles as described for birds considering (1) direct exposure to the MPCP Foray® 76B (containing spores of *Btk* ABTS-351) by ingestion of freshly sprayed plant parts and/or insects shortly after the application, or (2) indirect exposure to spores, vegetative cells and CryP by ingestion of insects (e.g., lepidoptera larvae) that may have ingested *Btk* ABTS-351 following application of Foray® 76B.

Note that the formulated product Foray® 76B contains spores and associated toxins but no vegetative cells. Since it is not known to what extent *Btk* ABTS-351 spores will produce vegetative cells following application of Foray® 76B, the risk from vegetative cells of *Btk* ABTS-351 to mammals cannot be quantitatively assessed. In addition, a quantitative risk assessment for oral exposure of mammals to CryP is not considered necessary, since CryPs are hydrolised and inactivated in the mammalian gastrointestinal tract (see Section “Risk posed by toxins/metabolites from *Btk* ABTS-351 in mammals”).

To quantitatively relate the potential exposure of mammals to spores of *Btk* ABTS-351 directly after application of Foray® 76B to the endpoint of the available pathogenicity studies with rats, a short-term screening assessment according to EFSA/2009/1438 is shown below (for details refer to quantitative risk assessment for birds above). The mammalian pathogenicity studies showed that exposure of mammals to *Btk* ABTS-351 spores at 426 mg MPCA/kg bw/d administered in one single dose does not cause adverse effects in rats. The screening assessment according to EFSA/2009/1438 is shown for applications of 1 x 619 g MPCA/ha and 4 x 516 g MPCA/ha based on a 5 d interval between applications. For multiple applications, the default DT50 for chemicals in feed matrices according to EFSA/2009/1438 was used, while it is noted that the default DT50 was established for chemicals and may not be adequate to describe exposure levels for MPCAs over time. However, since *Btk* ABTS-351 has a short half-life on e.g., foliage (details in RAR, 2020 Vol. 3 B7 and Vol. 3 B8), and biological activity of spores is expected to decline within few hours to days, the default DT50 of 10 days is considered to reflect a conservative approach in this case.

**Table 10.1‑5: Risk from spores of *Btk* ABTS-351 to mammals through dietary exposure directly following spray application of Foray® 76B**.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Intended use** | **Pine trees, deciduous/coniferous forest, shrubs, ornamental plants** | | | | |
| **Active substance/product** | *Btk* ABTS-351 / Foray® 76B | | | | |
| **Application rate [g MPCA/ha]** | 1 x 619 g MPCA/ha in deciduous forest,  4 x 516 g MPCA/ha (5-d interval) in pine trees, deciduous/coniferous forest, shrubs, and ornamental plants | | | | |
| **Relevant endpoint [mg MPCA/kg bw/d]** | 29-d LD50 > 426 mg MPCA/kg bw/d | | | | |
| **Short-term screening assessment - 1 x 619 g MPCA/ha** | | | | | |
| **Crop scenario** | **Model species** | **SV90** | **MAF90** | **DDD90**  **[mg/kg bw/d]** | **MoS** |
| Bare soil | Small granivorous mammal | 14.4 | 1.00 | 8.91 | > 47.8 |
| Bush and cane fruit | Small herbivorous mammal | 81.9 | 1.00 | 50.68 | > 8.4 |
| Bulbs and onion like crops, cereals, oilseed rape, potatoes, root and stem vegetables, strawberries, sugar beet and sunflower | Small herbivorous mammal | 118.4 | 1.00 | 73.26 | > 5.8 |
| Cotton, fruiting vegetables, grassland, leafy vegetables, legume forage, maize, orchards, ornamentals/nursery, pulses and vineyard | Small herbivorous mammal | 136.4 | 1.00 | 84.40 | > 5.0 |
| **Short-term screening assessment - 4 x 516 g MPCA/ha (5-d interval)** | | | | | |
| **Crop scenario** | **Model species** | **SV90** | **MAF90** | **DDD90**  **[mg/kg bw/d]** | **MoS** |
| Bare soil | Small granivorous mammal | 14.4 | 2.00 | 14.88 | > 28.6 |
| Bush and cane fruit | Small herbivorous mammal | 81.9 | 2.00 | 84.61 | > 5.0 |
| Bulbs and onion like crops, cereals, oilseed rape, potatoes, root and stem vegetables, strawberries, sugar beet and sunflower | Small herbivorous mammal | 118.4 | 2.00 | 122.32 | > 3.5 |
| Cotton, fruiting vegetables, grassland, leafy vegetables, legume forage, maize, orchards, ornamentals/nursery, pulses and vineyard | Small herbivorous mammal | 136.4 | 2.00 | 140.92 | > 3.0 |

SV: shortcut value; MAF: multiple application factor; DDD: daily dietary dose; MoS: Margin of safety. Exposure estimates are rounded to 2 significant figures; MoS represent accurate values underlying the actual exposure estimates.

The screening assessment above indicates an acceptable risk to mammals through dietary uptake of spores of *Btk* ABTS-351 directly following exposure of Foray® 76B with margins of safety between > 3.0 and > 47.8 for all assessment scenarios presented in EFSA/2009/1438. In addition, actual exposure of mammals to spores of *Btk* ABTS-351 by ingestion of freshly sprayed plant parts and/or insects shortly after the application of Foray® 76B is likely to be lower than considered for the risk assessment above, since *Btk* ABTS-351 has a short half-life on foliage and its biological activity degrades within hours (DT50 of < 24 h on foliage according to EFSA Journal 2021;19(10):6879). Moreover, *Btk* ABTS-351 is not translocated in plants. The margins of safety calculated above provide supportive evidence indicating a low risk posed by *Btk* ABTS-351 to mammals.

No appropriate risk assessment scheme is available to address indirect exposure of mammals to spores, vegetative cells and CryP by ingestion of insects (e.g., lepidoptera larvae) that might have ingested *Btk* ABTS-351 following application of Foray® 76B. In addition, neither levels of CryP, spores and vegetative cells of *Btk* ABTS-351 in infected insects that may be foraged by mammals are known. Nevertheless, the risk posed by indirect exposure of mammals to CryP, spores and vegetative cells of *Btk* ABTS-351 through consumption of infected insects can be considered low for the following reasons:

* Available data provide no evidence that *Btk* ABTS-351 may be toxic, pathogenic or infective in mammals.
* Mammalian studies evaluated during the latest EU Renewal of *Btk* ABTS-351 indicate that vegetative cells of *B. thuringiensis* are likely to be destroyed under conditions in the mammalian gastrointestinal system, while rats dosed either with vegetative cells or spores *Bacillus thuringiensis* subsp. *kurstaki* did not show enterotoxin production or effects on the composition of the faecal biota in the intestinal tract (details in RAR, 2020 Vol. 3 B.6).
* *Btk* ABTS-351 is an ubiquitous soil microbe that has been used for decades in agricultural settings and forests across Europe, while no negative effects of *Btk* ABTS-351 on wildlife have been reported.
* *Btk* ABTS-351 levels are not expected to multiply in environmental compartments following application of Foray® 76B.
* It is very unlikely that mammals will establish a reliance of the target organisms as a main source of diet, while application of Foray® 76B will control the population of the target insects and maintains non-target insects for mammals to feed on. In addition, it is unknown whether or not mammals will feed on infected or dead and decaying larvae containing vegetative cells of *B. thuringiensis* subsp. *kurstaki*.

Therefore, the risk to mammals through dietary uptake of *Btk* ABTS-351 or associated toxins is considered low for all proposed uses of Foray® 76B.

*Exposure via drinking water*

In a conservative approach, the risk from *Btk* ABTS-351 to mammals through uptake of contaminated drinking water can be assessed based for the worst-case scenario for exposure *via* drinking water according to EFSA/2009/1438, i.e., small granivorous mammal with a body weight of 21.7 g and a drinking rate of 5.1 mL/day (equivalent to 0.24 L/kg bw/d). The risk assessment below is based on the maximum predicted environmental density in surface water (i.e., PEDSW), which was calculated for application of 4 x 2.5 L Foray® 76B/ on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) using the yearly total dose application as one single application without degradation of *Btk* ABTS-351 (worst-case approach; details in Part B Section 5).

**Table 10.1-6: Risk from *Btk* ABTS-351 to mammals following exposure *via* drinking water**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Application**  **scenario** | **Appl. rate** | **PEDSW** | **Drinking rate a** | **Daily**  **dose** | **Relevant LD50** | **LD50 higher than daily dose?** | |
| **[L f.p./ha]** | **[CFU/L]** | **[L/kg bw/d]** | **[CFU/kg bw/d]** | **[CFU/kg bw/d]** | **(Trigger: < 1)** | |
| Pine trees, deciduous/coniferous forest, shrubs, ornamental trees and plants | 4 × 2.5 | 1.87 × 107 | 0.24 | 4.49 × 106 | > 1.68 × 107 b | ≥ 3.74 | Yes |

Appl. rate = Maximum single application rate; f.p. = formulated product; PEDSW = Predicted spore density in surface water calculated based on the yearly total dose application as one single application assuming no degradation of *Btk* ABTS-351; **bold** values indicate risk quotients < 1.0, i.e., the relevant mammalian endpoint is lower than the calculated daily dose.

a Worst-case (EFSA/2009/1438), i.e., small granivorous mammal with a body weight of 21.7 g and a drinking rate of 5.1 mL/day

b LD50 of 1.68 × 107 CFU/kg bw/d was calculated as worst-case based on test item concentration of 2.75 × 1012 CFU/L and the minimum body weight of 163.4 g that was determined for the test organisms in the available 29-d study with rats.

The drinking water assessment for the risk envelope application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) shows that the daily dose for mammals through consumption *via* drinking water (i.e., 4.49 x 106 CFU/kg bw/d) is lower than the relevant mammalian endpoint. Therefore, the risk to mammals from contaminated drinking water is considered acceptable for all proposed uses.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to mammals**

No laboratory studies are available which specifically characterized the toxicity of relevant CryP to mammals. However, as described for birds in the respective section above, CryP do not pose a risk to mammals following oral exposure since CryP are inactivated at low pH in the mammalian digestive tract. In line with this, it was concluded in EFSA Journal 2021;19(10):6879 that only spores of *Btk* ABTS-351 are able to survive the stomach passage and to reach the intestinal tract. Optimum growth conditions for *Bacillus thuringiensis* subsp. *kurstaki* are 28 - 30°C and pH 6.8 - 7.2 (EFSA Journal 2021;19(10):6879), while the body temperature of mammals usually ranges from 36 - 40°C, and pH in the mammalian gastrointestinal tract ranges from 1.5 – 5.0 in stomach and 6.0 - 8.0 in different segments of the intestine (documented pig, rat and human in Journal of animal science 94. suppl 3 (2016): 441-452). Therefore, the mammalian gastrointestinal tract does not provide optimum growth conditions for *Btk* ABTS-351. In addition, (1) none of the available toxicology studies (incl. product studies over 3 and 5 months; details in RAR, 2020 Vol. 3 B.6) documented signs of toxicity or pathogenicity after oral administration of *Bacillus thuringiensis* subsp. *kurstaki*, (2) *B. thuringiensis* subsp. *kurstaki* has been used for decades for control of Lepidopteran pests in agricultural settings and forests in European countries, while no negative effects on mammals have been reported, (3) *B. thuringiensis* is naturally occurring in the environment and thus mammals are continuously exposed to low levels of *B. thuringiensis*, (4) *B. thuringiensis* is not persistent on foliage and its insecticidal activity declines rapidly within few days due to solar radiation, (5) and *B. thuringiensis* subsp. *kurstaki* is not expected to multiply in soil and water following application of Foray® 76B (details in Part B Section 5). Therefore, the risk from CryP to mammals is low following the proposed uses of Foray® 76B.

**Conclusion on the overall risk to terrestrial vertebrates**

The risk from *Btk* ABTS-351 and CryP to terrestrial vertebrates following all proposed uses of Foray® 76B is considered acceptable, since (1) relevant environmental compartments and the gastrointestinal tract of terrestrial vertebrates do not provide optimum growth conditions for *Btk* ABTS-351, (2) *Btk* ABTS-351 is rapidly degraded on foliage, (3) and there is no evidence that *Btk* ABTS-351 or CryP exhibit toxicity, pathogenicity or infectivity in terrestrial vertebrates. In addition, (4) *Btk* ABTS-351 is an ubiquitous soil microbe that has been used for decades in agricultural settings and forests across Europe, while no negative effects of *Btk* ABTS-351 on wildlife have been reported.

IIIM 10.2 Effects on aquatic organisms

|  |  |
| --- | --- |
| Evaluator  Comments: | The presented approach was accepted.  The submitted justification was accepted.  The risk for aquatic organisms is acceptable. |

The effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on aquatic organisms were evaluated during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on studies with the microbial pest control agent (MPCA) *Btk* ABTS-351. Studies with the representative formulation DiPel® DF were not considered necessary as the ingredients of the formulation are not expected to present any hazard to the environment. Additional studies on the adverse effects of Foray® 76B on aquatic organisms are not available nor considered necessary (see rationale in Section IIIM 10). Details on the available studies are available in EFSA Journal 2021;19(10):6879 and related documents (RAR, 2020). The relevant endpoints used for the present risk assessment are shown below.

**IIIM 10.2.1 Effects on fish**

During the previous EU Renewal for *Btk* ABTS-351 (EFSA Journal 2021;19(10):6879), the potential effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351on fish were evaluated based on long-term studies with the MPCA *Btk* ABTS-351 (details in EFSA Journal 2021;19(10):6879 and RAR, 2020). The relevant endpoints are presented below.

**Table 10.2-1: Relevant endpoints on effects of *Btk* ABTS-351 on fish**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test substance** | **Exposure system** | **Endpoint** | **Reference** |
| Rainbow trout  (*Oncorhynchus mykiss*) | *Btk*  ABTS-351 a | 32 d,  semi-static | LC50 > 143.5 mg MPCA/L nom b  LC50 > 2.87 x 109 CFU/L nom b | EFSA Journal 2021;19(10):6879 |
| Bluegill sunfish  (*Lepomis macrochirus*) | LC50 > 143.5 mg MPCA/L nom b  LC50 > 2.87 x 109 CFU/L nom b | EFSA Journal 2021;19(10):6879 |

CFU= Colony forming units; nom = nominal concentration

a Studies performed with DiPel® technical material, i.e., the source of *Btk* ABTS-351 in Foray® 76B

b Fish were exposed *via* test water and diet simultaneously at 2.87 x 109 CFU/L and 2.87 x 109 CFU/g food.

Two long-term studies over 32 days are available on the adverse effects of *Btk* ABTS-351 on fish, which were considered to be acceptable and reliable during the previous EU Renewal for *Btk* ABTS-351 (EFSA Journal 2021;19(10):6879). The studies resulted in an LC50 of > 2.87 x 109 CFU/L for rainbow trout and bluegill sunfish.

In the 32-d study with rainbow trout, cumulative mortality in the test item group at test end was 20% (limit test), while histopathological examination showed no signs of pathogenicity and infectivity. Since the test item was not completely soluble in the test item solution and a cloudy suspension was observed during the test, the slightly increased mortality in the test item group was considered to potentially result from food competition or stress due to turbidity during the previous EU Renewal (details in RAR, 2020 Vol. 3 B.9). In the 32-d study with bluegill sunfish, no mortality, sublethal effects, behavioural abnormalities, or signs of pathogenicity during histopathological examination were observed following exposure to *Btk* ABTS-351.

**Infectivity and pathogenicity of *Btk* ABTS-351 in fish**

The available effect studies with fish did not show any signs of pathogenicity and infectivity over 32 days in two fish species. Despite no parameters were assessed in the available studies that would allow for a conclusive assessment of the potential infectivity of *Btk* ABTS-351 in the test organisms, the studies are considered sufficient to demonstrate that *Btk* ABTS-351 is not toxic, pathogenic or infective in fish (in line with EFSA Journal 2021;19(10):6879 and RAR, 2020 Vol. 3 B.9). In addition, fish do not provide optimal growth conditions for *Btk* ABTS-351, as water temperatures in European surface waters are well below the optimal growth temperature of *Btk* ABTS-351 (i.e., 28 - 30°C).

**Risk assessment for fish**

As stated in Ctgb’s (2022) Evaluation Manual for the Authorisation of Biopesticides according to Reg. (EC) No 1107/2009 (Part I: Microorganisms), the risk assessment schemes available for chemical plant protection products are generally regarded as not suitable for the assessment of risks posed by microorganisms to fish, since these assessment schemes were developed based on assumptions that apply to chemicals but not to microorganisms. Nevertheless, the risk from *Btk* ABTS-351 to fish can be assessed in a worst-case approach by calculating the margin of safety (MoS) as ratio of the Median Lethal Concentration (LD50; in CFU/L) from available laboratory studies and the Predicted Environmental Density of the MPCA (PEDSW) and Predicted Environmental Concentration of secondary metabolites (PECSW) in surface water.

EFSA considered the available information submitted during the previous EU Renewal (EFSA Journal 2021;19(10):6879) on the persistence and multiplication of *Btk* strain ABTS-351 in surface water as insufficient to conclude on *Btk* ABTS-351 levels in surface water following its entry into water bodies after spray application. However, the RMS disagreed and regards *Btk* ABTS-351 as microorganism that is not a common inhabitant of surface waters and is likely not to proliferate in natural water bodies due to hostile environmental conditions. Likewise, the RMS stated that available data indicate that CryP do not persist or accumulate in water and are degraded rapidly (details in RAR, 2020 Vol. 3 B.8). Nevertheless, worst-case PEDSW and PECSW for bacterial spores and crystal proteins were calculated in Part B Section 5 using Rautmann Drift values and FOCUS Steps 1-2 modelling assuming entry *via* spray drift based on a series of worst-case assumptions (e.g., total seasonal dose was used as a single application assuming no degradation and 0% crop interception). Degradation of the MPCA and CryP due to sunlight, predation and competition with other microorganisms in waterbodies were not considered. The risk assessment below is presented for application of Foray® 76B on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) at 4 x 2.5 L product/ha, based on spray drift rate of 33.2% for multiple applications, no crop interception and a default distance to water body of 3 m (for details refer to Part B Section 5). The risk envelope approach covers the risk for all proposed uses of Foray® 76B.

**Table 10.2-2: Risk from *Btk* ABTS-351 to fish following application of Foray® 76B**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Crop**  **scenario** | **Application rate** | **PEDSW a** | **Time scale** | **LC50** | **MoS** |
| [L f.p./ha] | [CFU/L] | [CFU/L] |
| Pine trees, deciduous/ coniferous forest, shrubs, ornamental trees and plants | 4 x 2.5 | 1.87 × 107 | Long-term | > 2.87 x 109 | > 153 |

f.p. = formulated product; MoS = margin of safety; values shown **in bold** fall below the margin of safety of 1.0;

a Based on 0% crop interception, drift rate of 33.2%, and total seasonal dose without degradation (details in Part B Section 5).

Since the conservative risk assessment above based on endpoints of the 32-d studies with rainbow trout and bluegill sunfish and the worst-case PEDSW value results in a margin of safety of > 153, the risk from *Btk* ABTS-351 to fish following application of Foray® 76B is considered acceptable for all proposed uses.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to fish**

Maximum CryP concentrations in surface water were calculated with a PECSW of 66.97 µg CryP/L (FOCUS Step 1) for application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) based on a series of worst-case assumptions (details in Part B Section 5). However, it must be considered that this exposure estimate reflects extreme worst-case assumptions, since *Bacillus thuringiensis* is not expected to multiply in freshwater systems due to hostile conditions (i.e., vegetative cells and spores of *Btk* ABTS-351 will be subject to sunlight, predation and natural competition by diverse microbiota of natural waters; details in Section IIIM 10 and Part B Section 5), and sporulation and germination of *Btk* ABTS-35 is considered unlikely. Consequently, CryP will not be synthesized or released to a significant extent.

While no information on CryP levels are provided in the available studies on fish, it is not expected that these host-specific toxins will be active in the fish gastrointestinal tract, since (1) the specific mode of action of CryP involves specific receptors on the apical brush border of the midgut microvillae of susceptible insects that are not present in fish, (2) CryP will be inactivated by protease enzymes in the gastrointestinal tract of fish, and (3) CryP are considered to not persist or accumulate in water bodies. This is supported by open literature studies showing that gastric pH values in fish range between 2 – 7.5, with pH values of 2-4 during food digestion in the stomach and up to 7.5 in different parts of the intestine (details for various fish species [[1]](#footnote-2) in e.g., Yufera *et al.*, 2012; Bravo *et al.*, 2018; Ojeda and Caceres 1995; Solovyev *et al.*, 2015). On the one hand, the low pH values in the stomach of fish result in inactivation of CryP; on the other hand, this shows that alkaline conditions, which are required for CryP activation, are not present in the gastrointestinal tract of fish. In addition, it is possible to derive an LC50 of CryP of > 14.35 mg/L based on the relevant endpoints from the available 32-d fish studies and an approximate content of protoxin in DiPel® Technical Powder of 10% (details in Part C). If this LC50 is related to the calculated worst-case PECSW of 66.97 µg CryP/L, a margin of safety of > 215 is calculated. Therefore, the weight-of-evidence indicates that risk from CryP to fish following the proposed uses of Foray® 76B is low.

**IIIM 10.2.2 Effects on aquatic invertebrates**

During the previous EU Renewal for *Btk* ABTS-351 (EFSA Journal 2021;19(10):6879), the potential effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on aquatic invertebrates were evaluated based on studies with the MPCA *Btk* ABTS-351 (details in EFSA Journal 2021;19(10):6879 and RAR, 2020). The relevant endpoints are presented below.

**Table 10.2-3: Relevant endpoints on effects of *Btk* ABTS-351 on aquatic invertebrates**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test substance** | **Exposure system** | **Endpoint** | **Reference** |
| *Daphnia magna* | *Btk*  ABTS-351 a | 10 d, semi-static | EC50 < 100 mg MPCA/L  (< 1.9 × 109 CFU/L nom) | EFSA Journal 2021;19(10):6879 |
| *Daphnia magna* | 21 d, semi-static | EC50 < 50 mg/L (1 x 109 CFU/L) | EFSA Journal 2021;19(10):6879 |
| *Daphnia magna* | 21 d, semi-static | Based on mortality/immobility:  EC50 = 13 mg/L (3.8 x 108 CFU/L nom)  Based on reproduction:  EC50 = 7.8 mg/L (2.3 x 108 CFU/L nom) | EFSA Journal 2021;19(10):6879 |
| Harpacticoid copepod  *(**Amphiascus minutus)* | 10 d, static | LC50 sed > 500 mg/kg sed nom  (> 1.00 × 1010 CFU/kg sed nom) | EFSA Journal 2021;19(10):6879 |
| Grass shrimp  *(**Palaemonetes vulgaris)* | 30 d, semi-static | EC50 > 1.7 x 107 CFU/L mm + 2.87 x 109 CFU/g food b, c | EFSA Journal 2021;19(10):6879 |

CFU= Colony forming units; mm = mean measured concentration; nom = nominal concentration

a Studies performed with DiPel® technical material, i.e., the source of *Btk* ABTS-351 in Foray® 76B

b Exposure *via* test water and diet

c Study considered to provide supportive information in RAR, 2020 Vol. 3 B.9.

Five studies are available on the adverse effects of *Btk* ABTS-351 on aquatic invertebrates that were considered acceptable and reliable during the previous EU review of *Btk* ABTS-351 (EFSA Journal 2021;19(10):6879).

In the available 10-day study with *Daphnia magna*, 100% mortality was observed after 10 days following exposure to *Btk* ABTS-351 at the measured concentration of 1.9 × 109 CFU/L. Likewise, effects on survival, reproduction and growth of *Daphnia magna* were observed following 21-d exposure to *Btk* ABTS-351 in the available reproduction studies. However, the effects in these studies were attributed to the high concentration of solids in the test media and not considered to reflect the biological activity of *Btk* ABTS-351 in aquatic invertebrates during the previous EU Renewal (EFSA Journal 2021;19(10):6879). This was corroborated with a series of open literature studies showing that natural concentrations and particle sizes of suspended sediments reduce the fecundity, survivorship and fitness of cladocerans (details in RAR, 2020 Vol. 3 B.9). In addition, Ctgb states in its Evaluation Manual for Biopesticides (Version 2.0; Dec 2022)[[2]](#footnote-3) that turbidity (in aqueous media linked to a high concentration of suspended particles and/or microorganisms) “can cause oxygen depletion in the test system and physical effects on the test organisms. These effects are unrelated to the infectivity and pathogenicity of the microorganism”. Moreover, the 21-day *Daphnia magna* reproduction test (OECD 211) is recognised as unsuitable for testing with microbials due to problems associated with turbidity (details in Series on Pesticides No. 76. ENV/JM/MONO (2014) and RAR (2020)). Therefore, it was acknowledged that the solids in the test solutions of the available laboratory studies with *D. magna* interfered with the filtering system of the daphnids and thereby reduced their growth, reproduction and survival. However, the lowest EC50 determined in these studies was an EC50 of 2.3 x 108 CFU/L based on effects on reproduction in *Daphnia magna* following 21 days of exposure. While this endpoint was considered suitable for risk assessment purposes in EFSA Journal 2021;19(10):6879 (and RAR, 2020 Vol. 3 B.9), it was accepted that this endpoint reflects physical effects of the solids and does not indicate pathogenicity or inherent toxicity of *Btk* ABTS-351 to aquatic invertebrates.

In the 10-d study with harpacticoid copepod, *Btk* ABTS-351 showed no adverse effects on survival and reproduction of the test organisms, while higher clutch size and naupliar production was observed in the test item group compared to the control. This beneficial biological effect was considered to be a result of the test organisms using *Btk* ABTS-351 as nutrition source.

In the 30-d study with grass shrimp, *Btk* ABTS-351 showed no adverse effects on survival or growth, and no signs of infectivity, pathogenicity, tumours, necrosis or abnormal growth attributable to the test item exposure were observed during the study.

Besides the laboratory studies described above, a series of non-GLP studies from the open literature were evaluated in the course of the previous EFSA Renewal (details in RAR, 2020 Vol. 3 B.9). In summary, studies with *Crassostra viginica*, *Hydatophylax argus*, larvae of *Simuliidae*, *Chironomidae*, *Trichoptera*, *Melagoptera*, and nymphs of *Ephemeroptera* and *Plectoptera* showed that *Bacillus thuringiensis* subsp. *kurstaki* and its β-toxins have no adverse effects on freshwater invertebrates at concentrations (or higher) that would be expected from common application rates of *Bacillus thuringiensis* subsp. *kurstaki* (e.g., Eidt 1985, Kreutzweiser 1992, Kreutzweiser 1994, Kreutzweiser 1996). This has also been shown for marine invertebrate species and freshwater invertebrate communities (e.g., Melin and Cozzi 1990, Richardson and Perrin 1994). Moreover, a study on the infectivity of *Bacillus thuringiensis* subsp. *kurstaki* in the oyster *Crassostrea virginica* showed that *Bacillus thuringiensis* subsp. *kurstaki* was quickly cleared from the blood stream of the oysters following intercardinal injection, while rapid ingestion of the vegetative cells of *Bacillus thuringiensis* subsp. *kurstaki* by leucocytes and phagocytosis was observed, suggesting that *Bacillus thuringiensis* subsp. *kurstaki* does not exhibit infectivity in oyster. During the previous EU Renewal (EFSA Journal 2021;19(10):6879), the weight of evidence was considered to sufficiently demonstrate that *Btk* ABTS-351 is not toxic, pathogenic or infectious in aquatic invertebrates. Moreover, taking into consideration the available data and the fact that the effects on *D. magna* that have been observed in the available laboratory studies resulted from turbidity rather than biological activity of *Btk* ABTS-351, an EC50 of > 1.0 x 109 CFU/L was established as the relevant endpoint for aquatic invertebrates in EFSA Journal 2021;19(10):6879.

**Infectivity and pathogenicity of *Btk* ABTS-351 in aquatic invertebrates**

No signs of infectivity and pathogenicity were observed in the laboratory studies with *D. magna,* Harpacticoid copepod and Grass shrimp, nor in the available open literature studies submitted in the course of the previous EU Renewal. In addition, the abovementioned study on the infectivity of *Bacillus thuringiensis* subsp. *kurstaki* in oyster (*Crassostrea virginica*) showed no infectivity and no other effects of *Bacillus thuringiensis* subsp. *kurstaki* on the test organisms following intercardinal injection, and it was concluded that vegetative bacteria of *Bacillus thuringiensis* subsp. *kurstaki* were rendered non-viable in oysters *via* phagocytosis. Therefore, in line with the previous EU Renewal, *Btk* ABTS-351 is not expected to exhibit infectivity and pathogenicity in aquatic invertebrates. This is corroborated by the fact that *Btk* ABTS-351 acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on other non-target species of other orders.

**Risk assessment for aquatic invertebrates**

The risk assessment below is presented for application of Foray® 76B on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) at 4 x 2.5 L product/ha and is based on identical exposure estimates and worst-case assumptions as described for fish (refer to Section IIIM 10.2.1). The risk envelope approach covers the risk for all proposed uses of Foray® 76B.

**Table 10.2-4: Risk from *Btk* ABTS-351 to aquatic invertebrates following application of Foray® 76B**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Crop**  **scenario** | **Application rate** | **PEDSW a** | **Species** | **Time scale** | **EC50** | **MoS** |
| [L f.p./ha] | [CFU/L] | [CFU/L] |
| Pine trees, deciduous/ coniferous forest, shrubs, ornamental trees and plants | 4 x 2.5 | 1.87 × 107 | *Daphnia magna* | Long-term | 2.3 x 108 | 12 |
| > 1.0 x 109 | > 53 |

f.p. = formulated product; MoS = margin of safety; values shown **in bold** fall below the margin of safety of 1.0;

a Based on 0% crop interception, drift rate of 33.2%, and total seasonal dose without degradation (details in Part B Section 5).

The conservative risk assessment above results in margins of safety of 12 and > 53 based on the lowest EC50 of the available laboratory studies with aquatic invertebrates and the EU agreed EC50 for aquatic invertebrates (EFSA Journal 2021;19(10):6879), respectively, and relating to the calculated worst-case PEDSW. Therefore, the risk from *Btk* ABTS-351 to aquatic invertebrates following application of Foray® 76B is considered acceptable for all proposed uses.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to aquatic invertebrates**

Maximum CryP concentrations in surface water were calculated with a PECSW of 66.97 µg CryP/L (FOCUS Step 1) for application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) based on a series of worst-case assumptions (details in Part B Section 5). However, it must be considered that this exposure estimate reflects extreme worst-case assumptions, since *Bacillus thuringiensis* is not expected to multiply in freshwater systems due to hostile conditions (i.e., vegetative cells and spores of *Btk* ABTS-351 will be subject to sunlight, predation and natural competition by diverse microbiota of natural waters; details in Section IIIM 10 and Part B Section 5), and sporulation and germination of *Btk* ABTS-35 is considered unlikely. Consequently, CryP will not be synthesized or released to a significant extent.

While toxicity of CryP to aquatic invertebrates was not particularly investigated in the available laboratory and open literature studies, the weight of evidence shows that CryP produced by *Btk* ABTS-351 pose no risk to aquatic invertebrates because no adverse effects on invertebrate species or communities were evident in the available studies that could be attributed to biological activity of *Btk* ABTS-351 (and hence to potential toxicity of CryP). This is in line with the conclusion of the RMS during the previous EU Renewal (EFSA Journal 2021;19(10):6879 and related documents). In addition, CryP are considered to not persist or accumulate in water bodies (see Section B Part 5). Moreover, it is possible to derive an EC50 of CryP of > 0.78 mg/L based on the worst-case EC50 from the laboratory studies with *Daphnia magna* and an approximate content of protoxin in DiPel® Technical Powder of 10% (details in Part C). If this EC50 is related to the calculated worst-case PECSW of 66.97 µg CryP/L, a margin of safety of approx. 12 is calculated. Taken together with the absence of toxicity and pathogenicity of *Btk* ABTS-351 in the available laboratory and literature studies, this shows that the risk from CryP to aquatic invertebrates following the proposed uses of Foray® 76B is low.

**IIIM 10.2.3 Effects on algal growth**

During the previous EU Renewal for *Btk* ABTS-351 (EFSA Journal 2021;19(10):6879), the potential effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on algae were evaluated based on a study with the formulated product DiPel® DF (MPCA is *Btk* ABTS-351). Details can be found in EFSA Journal 2021;19(10):6879 and RAR, 2020, and the relevant endpoint is presented below.

**Table 10.2-5: Relevant endpoints on effects of *Btk* ABTS-351 on algae**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test substance** | **Exposure system** | **Endpoint** | **Reference** |
| *Pseudokirchneriella subcapitata* | DiPel® DF | 72 h, static | EC50 = 50.8 mg Dipel® DF/L  (5.94 × 108 CFU/L nom) | EFSA Journal 2021;19(10):6879 |

CFU= Colony forming units; nom = nominal concentration

A 72-h study on the effects of *Btk* ABTS-351 (tested as DiPel® DF) on algal growth is available that has been considered acceptable and reliable during the previous EU Renewal (RAR, 2020 Vol. 3 B.9). Inhibition of algal growth was observed in the two highest test item concentrations (i.e., 50.16 and 99.98 mg/L), and the EU agreed endpoint of 5.94 × 108 CFU/L was derived. Since pronounced inhibition of algal growth was also observed in the inactivated control, the effects observed in the test item groups were not considered to reflect biological activity of *Btk* ABTS-351, and it was concluded by the RMS that *Btk* ABTS-351 does not exhibit toxicity to algae (details in RAR, 2020 Vol. 3 B.9).

In addition, an open literature study by Koskella and Stotzky (2002) is available on the microbicidal activity of larvicidal toxins from *Bacillus thuringiensis* (subspp. *kurstaki*, *morrisoni*, and *israelensis*) towards selected bacteria, fungi, and algae *in vitro* (details in RAR, 2020 Vol. 3 B.9). The study showed that larvicidal toxins from *B. thuringiensis* subspp. *kurstaki*, *morrisoni*, and *israelensis* have no microbicidal activity on a variety of bacteria (8 Gram-negative, 5 Gram-positive and a cyanobacterium), fungi (2 Zygomycetes, 1 Actinomycete, 2 Deuteromycetes, and 2 yeasts), and algae (primarily green and diatoms) in pure and mixed culture. Since algae cultures were exposed to *Btk* toxins but not *Bacillus thuringiensis* itself, the study was considered to indicate that *Btk* toxins have no adverse effects on algae. No details were provided in this study on *Btk* strains and type and concentrations of *Btk* toxins.

**Infectivity and pathogenicity of *Btk* ABTS-351 in algae**

The pathogenicity and infectivity of *Btk* ABTS-351 have not been evaluated in the available algal study. However, since no biological activity of *Btk* ABTS-351 was observed in the available study and *Btk* ABTS-351 acts *via* a highly specific insecticidal mode of action, *Btk* ABTS-351 is not considered to exhibit pathogenicity or infectivity in algae (in line with RMS in RAR, 2020 Vol. 3 B.9).

**Risk assessment for algae**

The quantitative risk assessment below is presented for application of Foray® 76B on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) at 4 x 2.5 L product/ha and is based on identical exposure estimates and worst-case assumptions as described for fish (refer to Section IIIM 10.2.1). The risk envelope approach covers the risk for all proposed uses of Foray® 76B.

**Table 10.2-6: Risk from *Btk* ABTS-351 to algae following application of Foray® 76B**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Crop**  **scenario** | **Application rate** | **PEDSW a** | **Species** | **EC50** | **MoS** |
| [L f.p./ha] | [CFU/L] | [CFU/L] |
| Pine trees, deciduous/ coniferous forest, shrubs, ornamental trees and plants | 4 x 2.5 | 1.87 × 107 | *Pseudokirchneriella subcapitata* | 5.94 x 108 | 32 |

f.p. = formulated product; MoS = margin of safety; values shown **in bold** fall below the margin of safety of 1.0

a Based on 0% crop interception, drift rate of 33.2%, and total seasonal dose without degradation (details in Part B Section 5).

The conservative risk assessment above results in a margin of safety of 32 based on the EU agreed endpoint for algae and the calculated worst-case PEDSW. Therefore, the risk from *Btk* ABTS-351 to algae following application of Foray® 76B is considered acceptable for all proposed uses.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to algae**

Maximum CryP concentrations in surface water were calculated with a PECSW of 66.97 µg CryP/L (FOCUS Step 1) for application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) based on a series of worst-case assumptions (details in Part B Section 5). However, it must be considered that this exposure estimate reflects extreme worst-case assumptions, since *Bacillus thuringiensis* is not expected to multiply in freshwater systems due to hostile conditions (i.e., vegetative cells and spores of *Btk* ABTS-351 will be subject to sunlight, predation and natural competition by diverse microbiota of natural waters; details in Section IIIM 10 and Part B Section 5), and sporulation and germination of *Btk* ABTS-35 is considered unlikely. Consequently, CryP will not be synthesized or released to a significant extent.

While no suitable endpoint for effects of CryP on algae is available, the risk from CryP to algae can be considered low due to the highly specific insecticidal mode of action of *Btk* ABTS-351. This is corroborated by the open literature study by Koskella and Stotzky (2002) on the microbicidal activity of CryP from *Bacillus thuringiensis* towards selected bacteria, fungi, and algae. In addition, CryP are considered to not persist or accumulate in water bodies (see Section B Part 5). Moreover, it is possible to derive an EC50 of CryP of 5.08 mg/L based on the 72-h EC50 from the laboratory studies with *Pseudokirchneriella subcapitata* and an approximate content of protoxin in DiPel® Technical Powder of 10% (details in Part C). If this EC50 is related to the calculated worst-case PECSW of 66.97 µg CryP/L, a margin of safety of approx. 76 is calculated. In conclusion, the risk from CryP to algae following the proposed uses of Foray® 76B is low.

**IIIM 10.2.4 Effects on plants other than algae**

No studies are available on the effects of *Btk* ABTS-351 or Foray® 76B on aquatic plants other than algae. Nevertheless, the risk from *Btk* ABTS-351 or CryP to aquatic plants other than algae following the proposed uses of Foray® 76B can be considered low, since (1) *Btk* ABTS-351 acts *via* a highly specific insecticidal mode of action, (2) adverse effects of *Btk* ABTS-351 on plants are unlikely since *B. thuringiensis* subsp. *kurstaki* is a common organism in the phyllosphere of plants (see RAR, 2020 Vol 3 B.7) and no adverse effects have been reported during decades of use of *Btk* ABTS-351 in agricultural settings or forests, (3) and *Btk* ABTS-351 is not expected to multiply or persist in surface water.

**Conclusion on the overall risk to aquatic organisms**

In line with the conclusions for DiPel® DF in EFSA Journal 2021;19(10):6879, a low risk from *Btk* ABTS-351 and CryP to aquatic organisms can be concluded for the proposed uses of Foray® 76B, since (1) *Btk* ABTS-351 showed only low toxicity and no signs of pathogenicity or infectivity in the available studies with aquatic organisms, (2) the quantitative risk assessment resulted in margins of safety from 12 - 153 based on worst-case exposure estimates for *Btk* ABTS-351 following the proposed uses of Foray® 76B, and (3) *Btk* ABTS-351 and CryP are not expected to persist in surface water at high level.

IIIM 10.3 Effects on bees

|  |  |
| --- | --- |
| Evaluator  Comments: | The presented approach was accepted.  The submitted justification was accepted.  The risk for bees is acceptable. |

The effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on bees were assessed during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on studies with the MPCA *Btk* ABTS-351, the EU representative formulation DiPel® DF, and the MPCP Foray® 48B. Details on the available studies are available in EFSA Journal 2021;19(10):6879 and related documents (RAR, 2020). In addition, two short-term studies on the adverse effects of Foray® 76B on adult honeybees are available (details in Appendix 2). The relevant endpoints used for the present risk assessment are shown below.

**Table 10.3-1:**  **Relevant endpoints for effects of *Btk* ABTS-351 and Foray® 76B on bees**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test substance** | **Exposure system** | **Endpoint** | **Reference** |
| Honey bee  (*Apis mellifera*) | DiPel® DF | 48 h, oral | LD50 > 222.4 µg f.p./bee  (2.60 × 106 CFU/bee) | EFSA Journal 2021;19(10):6879 |
| 48 h, contact | LD50 > 185.0 μg f.p./bee  (2.16 × 106 CFU/bee) |
| Honey bee  (*Apis mellifera*) | Foray® 48B | 48 h, oral | LD50 > 578 μg f.p./bee  (> 6127 IU/bee) | EFSA Journal 2021;19(10):6879 b |
| 48 h, contact | LD50 > 555 μg f.p./bee  (> 5883 IU/bee) |
| Honey bee  (*Apis mellifera*) | Foray® 48B | 48 h, oral | LD50 > 542 μg f.p./bee  (> 5745 IU/bee) | EFSA Journal 2021;19(10):6879 b |
| 48 h, contact | LD50 > 555 μg f.p./bee  (> 5883 IU/bee) |
| Honeybee  (*Apis mellifera*) | Foray® 76B | 48 h, oral | LD50 > 629.07 μg f.p./bee | Vergé, 2010a,  IIIM 10.3/01 |
| 48 h, contact | LD50 > 100 μg f.p./bee | Vergé, 2010b,  IIIM 10.3/02 |
| Honey bee  (*Apis mellifera*) | *Btk*  ABTS-351 a | 14 d, oral | LD50 > 4042 μg MPCA/bee  (>3.6 × 105 IU/bee, > 8.1 × 107 CFU/bee)  Pathogenicity not observed | EFSA Journal 2021;19(10):6879 |
| Honey bee larva  (*Apis mellifera*) | *Btk*  ABTS-351 a | 17 d, single exposure | LD50 > 100 μg MPCA/larva  (> 5513 IU/larva)  Pathogenicity not observed | EFSA Journal 2021;19(10):6879 |

f.p. = formulated product

a Studies performed with DiPel technical material, i.e., the source of *Btk* ABTS-351 in Foray® 76B.

b Details on studies in RAR 2020 Vol. 3 MA B.9.

Four laboratory studies are available on the acute toxicity to bees following oral and contact exposure over 48 h to formulated products containing *Btk* ABTS-351 (details in RAR 2020 Vol. 3 B.9). The studies showed low mortality (i.e., ≤ 20%) after oral and contact exposure to the test items, and no behavioural or morphological abnormalities were observed. Infectivity and pathogenicity were not assessed in these studies.

A laboratory study is available on adverse effects of *Btk* ABTS-351 on larval and pupal development of honey bee larvae following single exposure to the MPCA. Over the 17-d observation period, *Btk* ABTS-351 showed no adverse effects on development and emergence of the test organisms, and no behavioural or morphological abnormalities up to the test dose of 100 μg MPCA/larva (i.e., 5513 IU/larva). No signs of pathogenicity were apparent, while infectivity was not assessed in this study.

A laboratory study is available on the adverse effects of *Btk* ABTS-351 on adult honey bees during chronic exposure over 14 days up to test doses of 4042 μg MPCA/bee (i.e., 3.6 × 105 IU/bee). No effects of *Btk* ABTS-351 on survival of the test organisms and no signs of pathogenicity were observed during the study. Infectivity was not assessed.

In addition to the above-mentioned laboratory studies, open literature studies are available from the previous EU Renewal demonstrating that *Btk* ABTS-351 has no adverse effects on bees (details in RAR 2020 Vol. 3 B.9). In the study of Buckner *et al.* (1974), adverse effects of *Btk* ABTS-351 on honey bee populations were investigated in a field trial over one week. Following aerial application of different formulations containing *Btk* ABTS-351, no adverse effects of *Btk* ABTS-351 on mortality, pollen collection, hive activity, hive weight, and brood development were detected. In the study of Malone *et al.* (1999), adult honey bees were orally exposed over 7 days to pollen-based diet containing either crystalline δ-endotoxin or *Btk* HD-1. Crystalline δ-endotoxin and *Btk* strain HD-1 were tested at concentrations up to 1.0% in the pollen-based diet which was considered to reflect an unrealistically high proportion of test item in pollen. Bee survival was monitored for up to 70 days until all test bees were dead. No adverse effects on survival and food consumption were observed in the test item groups compared to the control, with the exception that bees treated with *Btk* HD-1 consumed significantly more food compared to the control. In addition, a non-GLP long-term study with bumble bees over 11 weeks is available from the open literature (Mommaerts *et al*., 2009). The study was considered acceptable by the RMS in the course of the latest EU Renewal for *Btk* ABTS-351 (see RAR 2020 Vol. 3 MA B.9), while details on the study are provided in RAR 2020 Vol. 3 MP B.9 for *Bacillus thuringiensis* ssp. *aizawai* strain ABTS-1857. In the study, bumble bees were exposed once to aqueous test item solution *via* topical application to the dorsal thorax. In addition, the test organisms were continuously supplied with sugar water and pollen treated with DiPel (i.e. *Btk* ABTS-351) over 11 weeks. Results showed that *Btk* ABTS-351 has no effects on mortality, reproduction, foraging behaviour and nest performance of bumble bees following oral and contact exposure of bumble bees to 0.1% Dipel (i.e. 1.6 × 104 IU/mL). The RMS considered the results of this study as strain specific evidence that *Btk* ABTS-351 has no toxic or pathogenic effects on bumble bees, since no signs of pathogenicity were observed following oral and contact exposure to 1.6 × 104 IU/mL over 11 weeks, which presents a highly unrealistic worst-case scenario.

In addition, a field study of Leza *et al.* (2014; IIIM 10.3/03) is available, which investigated the adverse effects of aerial application of *Bacillus thuringiensis* subsp. *kurstaki* ABTS-351 (applied as Foray® 48B) on colony performance (i.e., queen health and brood development breeding rates) of *Apis mellifera* over a period of four months. The study included two groups each of hives in two areas, i.e., one group treated with Foray® 48B (i.e. *Btk* ABTS-351) at 3.5 L product/ha and one control group without treatment. No differences in colony performance were detected between the treated and untreated groups of colonies over the observation period of four months. Results were considered to indicate that *Bacillus thuringiensis* subsp. *kurstaki* has no adverse effects on brood development of honey bees under natural conditions.

**Infectivity and pathogenicity of *Btk* ABTS-351 in bees**

Available studies have not purposefully investigated the potential infectivity of *Btk* ABTS-351 in bees. However, the weight of evidence is considered sufficient (as in RAR 2020 Vol. 3 B.9) to demonstrate the absence of pathogenicity and infectivity *Btk* ABTS-351 in bees, since (1) no adverse effects were observed in the available laboratory and open literature studies, (2) no signs of pathogenicity were observed in the 14-d and 17-d studies with adult and larval honey bees, and (3) *Btk* ABTS-351 does not cause adverse effects on mortality, reproduction, foraging behaviour and nest performance of bumble bees under very worse-case exposure conditions (Mommaerts *et al*., 2009). In addition, (4) *Btk* ABTS-351 acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on beneficials and other non-target species of other insect orders.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to bees**

No laboratory study is available which specifically characterized and quantified the toxicity of relevant CryP to bees. However, since *Btk* ABTS-351 showed no adverse effects in the available laboratory and open literature studies, and the content of total protoxin in DiPel Technical Powder (i.e., source of *Btk* ABTS-351) is approximately 10% (w/w; details in Part C), the risk from CryP to adult bees and bee larvae can be considered low. This is underpinned by multiple open literature studies showing that CryP have no adverse effects on bees. For instance, a study of Malone *et al.* (1999) showed that crystalline δ-endotoxins showed no adverse effects on survival of adult honey bees during 7-d exposure *via* treated pollen-based diet (RAR 2020 Vol. 3 B.9). In another study (Rose *et al*., 2007; IIIM 10.3/04), *Bt* corn had no adverse effects on colony health and worker foraging activity of honey bees during a 4-week test period where honey bees were foraging on pollen of transgenic *Bt* corn. This is supported by a review of Malone (2004; IIIM 10.3/05), which summarises a series of studies demonstrating that the *Bt* toxins Cry1Ab and Cry1Ac have no effects on survival of adult and larval honey bees. This is supported by the fact that to date nearly all commercial *Bt* maize and *Bt* cotton varieties express the two Cry toxins Cry1Ab and Cry1Ac, while adverse effects on bees have not been documented during decades of extensive use of such plants (O’Callaghan *et al*., 2005; IIIM 10.3/06). In addition, the risk from CryP to bees can be considered low, since CryP activation needs an alkaline environment, while the midgut of bees is characterised by acidic or neutral pH.

Bee larvae in particular, are only indirectly exposed to CryP when adult bees transfer *Btk* ABTS-351 or CryP-containing paraspores from crops to the hive. However, this indirect exposure can be expected to be low, as the activity of *B. thuringiensis* and CryP decreases rapidly (< 24 hours) when exposed to sunlight (details in RAR 2020 Vol. 3 B.8). In addition, the available 17-d study on adverse effects of *Btk* ABTS-351 on larval and pupal development of honey bee larvae showed no effects that could indicate toxicity or pathogenicity of *Btk* ABTS-351 on bee brood. In addition, bee larvae can be expected to be exposed to *B. thuringiensis* independent of the application of Foray® 76B, since *Bacillus thuringiensis* is ubiquitous and occurs at low levels in the environment.

In conclusion, the risk from CryP to adult bees and bee brood following application of Foray® 76B is low.

**Risk assessment for bees**

The risk assessment schemes available for chemical plant protection products (e.g., hazard quotient based on ratio of application rate and LD50) are generally regarded as not suitable for the assessment of risks posed by microorganisms to bees, as relevant assessment parameters (e.g., shortcut values and twa values) were developed based on assumptions that apply to chemicals but not to microorganisms. This is additionally supported by the revised guidance on the risk assessment of plant protection products on bees (EFSA/2023/7989) stating that “*The guidance document does not cover the risk assessment for microorganism active substances for which specific considerations are needed […]*”. In addition, current EU Regulations (i.e. Commission Regulations (EU) 2022/1439 and 2022/1440) and Ctgb’s “Evaluation Manual for the Authorisation of Biopesticides according to Reg. (EC) No 1107/2009 Part I: Microorganisms” (v2.0, Dec 2022) state that the risk from microorganisms to non-target organisms shall be assessed considering (1) its mode of action, (2) other biological properties, (3) studies on toxicity, pathogenicity and infectivity, and (4) survival and dispersal of the active microorganism in the environment. Therefore, the risk from *Btk* ABTS-351 to bees following application of Foray® 76B is assessed qualitatively in a weight-of-evidence approach. A low risk from *Btk* ABTS-351 and associated CryP following application of Foray® 76B can be concluded for the following reasons:

1. *Bacillus thuringiensis* occurs naturally and ubiquitously in the environment.
2. No signs of toxicity were observed in the available short-term studies on oral and contact toxicity of DiPel® DF, Foray® 48B and Foray® 76B to adult honey bees.
3. No signs of pathogenicity were observed in the available 14- and 17-d studies with honey bee larvae and adult honey bees.
4. No signs of pathogenicity were observed in the available open literature study by Mommaerts *et al*. (2009) following oral and contact exposure to DiPel (i.e. *Btk* ABTS-351) over 11 weeks under unrealistic worst-case exposure conditions.
5. No adverse effects on queen health and brood development of honey bee colonies were observed over four months following aerial application of *Btk* ABTS-351 (applied as Foray® 48B; Leza *et al*., 2014).
6. Concentrations tested in the available studies reflect worst-case exposures (oral exposure considered as most relevant route of exposure for bees according to the MoA of *Btk* ABTS-351) compared to realistic exposures of bees in the environment. The test organisms in the available studies were exposed to high and stable concentrations of *Btk* ABTS-351 over several days or weeks, while *Btk* ABTS-351 and CryP are not expected to persist over a longer period following spray application of Foray® 76B, since spores and CryP of *Btk* ABTS-351 are rapidly degraded under environmental conditions. *Btk* ABTS-351 has a half-life of less than 24 h on foliage (RAR 2020, Vol.3 B7), and δ-endotoxins and endospores are rapidly degraded and inactivated when exposed to UV radiation following spray application (EFSA Journal 2021;19(10):6879). In addition, the realistic exposure of honey bee larvae following application of Foray® 76B is significantly lower than simulated in the available 17-d laboratory study, since honey bee larvae do not come into direct contact with the spray solution and are only indirectly exposed to *Btk* ABTS-351 and CryP via collected pollen and nectar.
7. Available data demonstrate low toxicity of CryP associated with *Btk* ABTS-351 to adult bees and bee brood.
8. *Btk* ABTS-351 acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on beneficials and other non-target species of other insect orders.

Therefore, the weight-of-evidence sufficiently demonstrates an acceptable risk posed by *Btk* ABTS-351 and associated CryPs to bees following the proposed uses of Foray® 76B**.**

**Conclusion on the overall risk to bees**

In line with conclusions for DiPel® DF in EFSA Journal 2021;19(10):6879, the risk from *Btk* ABTS-351 and CryP to bees following the proposed uses of Foray® 76B is low. Adequate studies are available with adult and larval honey bees and adult bumble bees showing that *Btk* ABTS-351 is only of low toxicity to bees and does not exhibit pathogenicity in bees under worst-case exposure scenarios.

IIIM 10.4 Effects on arthropods other than bees

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| --- | --- |
| Evaluator  Comments: | The presented approach was accepted.  The submitted justification was accepted.  In accordance with EFSA Journal 2021;19(10):6879: *In the two-glass plate limit test studies on Aphidius rhopalosiphi and Typhlodromus pyri with strain ABTS-351, no adverse effects were observed on reproduction. The study designs did not take the relevant oral route into account and no assessment of infectivity and pathogenicity was performed.*  The risk for arthropods other than bees is acceptable. |

The effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on non-target arthropods other than bees were assessed during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on studies with the MPCA *Btk* ABTS-351. Additional studies on the adverse effects of Foray® 76B on non-target arthropods are not available nor considered necessary (see rationale in Section IIIM 10). Details on the available studies are available in EFSA Journal 2021;19(10):6879 and related documents (RAR, 2020). The relevant endpoints used for the present risk assessment are shown below.

**Table 10.4-1: Available data on effects of *Btk* ABTS-351 on non-target arthropods other than bees**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test item** | **Exposure system** | **Endpoint** | **Reference** |
| *Aphidius rhopalosiphi* | *Btk*  ABTS-351 a | 48 h mortality, 12 d reproduction,  laboratory test | ER50 > 3.9 kg MPCA/ha  (> 2.38 x 1011 IU/ha) | EFSA Journal 2021;19(10):6879 |
| *Typhlodromus pyri* | 7 d mortality, 14 d reproduction, laboratory test | ER50 > 3.9 kg MPCA/ha  (> 2.38 x 1011 IU/ha) | EFSA Journal 2021;19(10):6879 |
| Predatory mite  (*Metaseiulus occidentalis*) | Approx. 8 d, extended laboratory test | EC50 > 62 g MPCA/L b | EFSA Journal 2021;19(10):6879 |
| Two-spotted spider mite *(Tetranychus*  *urticae*) | EC50 < 6.2 g MPCA/L b |
| Green lacewing  (*Chysoperla*  *carnea*) | Approx. 27 d, laboratory test | No reliable endpoint can be derived due to high control mortality. | ~~EFSA Journal 2021;19(10):6879~~  RAR Volume 3MA B.9.4 |
| Parasitic wasp (*Trichogramma*  *pretiosum*) | 6 d mortality, 11 d reproduction | No reliable endpoint can be derived due to high control mortality. | ~~EFSA Journal 2021;19(10):6879~~  RAR Volume 3MA B.9.4 |

a Studies performed with DiPel® technical material, i.e., the source of *Btk* ABTS-351 in Foray® 76B

b Expression in potency of spore density not possible since no details provided on content of *Btk* ABTS-351 in test item.

One glass plate study each is available on the adverse effects of *Btk* ABTS-351 on *Typhlodromus pyri* and *Aphidius rhopalosiphi*, which were considered acceptable during the latest EU Renewal (EFSA Journal 2021;19(10):6879). Both studies resulted in an ER50 of > 2.38 x 1011 IU/ha, since no adverse effects of *Btk* ABTS-351 on survival and reproduction of *Aphidius rhopalosiphi* were observed, and only a slight effect on survival (i.e., 14.1% corrected mortality) and no effect on reproduction of *Typhlodromus pyri* were apparent.

A laboratory test on the adverse effects of *Btk* ABTS-351 to the spider mite *Tetranychus urticae* and predatory mite *Metaseiulus occidentalis* is available, which was considered acceptable and suitable for risk assessment during the latest EU Renewal (EFSA Journal 2021;19(10):6879). Exposure of *T. urticae* to 62 g MPCA/L resulted in 31.94% mortality of adult gravid host prey mites, while no mortality of protonymphs or effects on larva hatching were observed. Exposure of *M. occidentalis* to 6.2 and 62 g MPCA/L resulted in 10.29 - 12.3% mortality. No effects on number of eggs laid were observed compared to the control. Hatching rate of *M. occidentalis* was reduced in all treatment groups (i.e., 0.62 - 62 g MPCA/L) compared to the control. However, since the observed effects on hatching are not consistent with the mode of action of *Btk* ABTS-351 and did not follow a concentration-response relationship, reduced hatching likely reflects physical effects of the test item application. Insufficient information is available to allow conversion of test concentrations to test rates for this study. In addition, it must be noted that the EU agreed endpoint for *T. urticae* (i.e., EC50 < 6.2 g MPCA/L) is likely not correct, since effects > 50% were not observed for *T. urticae* in the respective study.

A laboratory test on survival and reproduction of *Chysoperla carnea* is available, which does not provide a reliable endpoint for risk assessment purposes due to high mortalities in all test groups including the control and large variations between replicates. In this study, no statistically significant differences between the control and test item groups were found for larval mortality, time to pupation, pupation rate, pupal mortality, total mortality and egg production of *C. carnea*.

A study on survival and reproduction of the insect egg parasitoid *Trichogramma pretiosum* is available, which does not provide a reliable endpoint for risk assessment purposes due to high mortalities in all test groups including the control, and since fecundity decreased considerably in all test groups (incl. control) during the test period. Despite the high mortalities and decreasing fecundity in all test groups, survival (at 0.62, 6.2 and 62 g MPCA/L) and fecundity (at 6.2 and 62 g MPCA/L) were significantly lower in the test item groups compared to the control. No adverse effects were observed for hatching rate, sex ratio, emergence, physical appearance, and behaviour of the test organisms.

In addition to the abovementioned laboratory studies, a series of studies from the open literature providing supportive information has been evaluated during the previous EU Renewal (details in RAR, 2020 Vol. 3 B.9). In brief, Melin and Cozzi (1990) reviewed the safety to non-target invertebrates of Lepidopteran strains of *Bacillus thuringiensis* and their exotoxins. Reviewed studies showed that (1) exposure of invertebrates on leaves is short due to degradation of spores, cells and CryP *via* e.g., solar radiation, and that (2) *Bacillus thuringiensis* subsp. *kurstaki* has no detrimental effects on insects of the orders Orthoptera, Dermaptera, Heteroptera, Coleoptera, Diptera, and Hymenoptera following various routes of oral and contact exposure. Buckner *et al.* (1974) showed that *Bacillus thuringiensis* subsp. *kurstaki* has no adverse effects on non-target insect populations (incl. forest ground-dwelling to foliage-dwelling species) over 30 days following aerial application of a DiPel® formulation (i.e., *Btk* ABTS-351) for spruce budworm control. Hamed (1979) showed that oral exposure to a DiPel® formulation (i.e., *Btk* ABTS-351) at a spore density of 5 x 108 spores/mL over 6 days has no adverse effects on two tachnids (*Bessa fugax* and *Zenillia dolosa*), one ichneumonid (*Trichionotus* sp.) and the predatory shieldbug (*Picromerus bidens*). Adverse effects on mortality and reproduction as well as presence of vegetative cells in the bodies of dead parasites were observed for four test species of the order Hymenoptera (i.e., *Diadegma armillata*, *Pimpla turionella*, *Ageniaspis fuscicollis*, and *Tetrastichus evonymellae*). However, it was concluded that such effects would not be expected under realistic application regimes and environmental conditions, since *Bacillus thuringiensis* subsp. *kurstaki* and CryP are expected to be quickly degraded, and, consequently, insects in the field are not exposed to such high levels of vegetative cells of *Btk* ABTS-351 and CryP over long periods. Boulton (2004) showed that aerial spray application of Foray® 48B may result in adverse effects in non-target lepidopteran populations in forestry. Following application, lepidopteran species richness was decreased in sprayed areas, and species abundance of uncommon species groups varied significantly between treated and untreated areas. While these results suggests that application of Foray® 48B may reduce populations of non-target lepidopteran species in sprayed areas if these species are actively feeding on the treated vegetation, it was concluded that this potential impact will only be temporary and limited to larvae that are actively feeding, since *Btk* ABTS-351 has a relatively short half-life on e.g., foliage. In addition, most non-target lepidopteran species are expected to recover quickly due to multiple life-cycles per year. This is in line with the review of Joung and Coté (2000), where it was concluded that permanent changes in non-target arthropod populations are highly unlikely due to adverse effects of *Bacillus thuringiensis* subsp. *kurstaki*.

**Infectivity and pathogenicity of *Btk* ABTS-351 in non-target arthropods other than bees**

Five laboratory studies and a series of open literature studies are available on potential adverse effects of *Btk* ABTS-351 on non-target arthropods other than bees. Infectivity of *Btk* ABTS-351 in non-target arthropods was not purposefully investigated in the available studies. Adverse effects that could indicate potential pathogenicity or infectivity of *Btk* ABTS-351 in non-target arthropods were observed only in some studies with test species of the orders Hymenoptera and Lepidoptera. Adverse effects of *Btk* ABTS-351 on mortality and reproduction of *Trichogramma pretiosum* were described in a laboratory study, which should be interpreted with caution since high mortality and significantly decreasing fecundity were observed in the control group. In addition, adverse effects of *Btk* ABTS-351 on different test species of the order Hymenoptera were observed following 6-day exposure to *Btk* ABTS-351, but only following exposure to high levels that would not be expected under realistic application regimes and environmental conditions. Besides these findings for Hymenoptera, data from an open literature study suggest that *Btk* ABTS-351 may have adverse effects on non-target lepidopteran species. This is consistent with the highly specific mode of action of *Btk* ABTS-351 against insect species of the order Lepidoptera (details in RAR, 2020 Vol. 3 B2). However, since (1) exposure of lepidopteran species to *Btk* ABTS-351 is expected to occur only during active feeding on treated vegetation, while *Btk* ABTS-351 is rapidly degraded on foliage due to its short half-life, and (2) since most non-target species are expected to recover quickly due to multiple life cycles per year, the observed effects of *Btk* ABTS-351 on non-target lepidopteran species are expected to be of temporary and limited nature. Therefore, the weight of evidence confirms the absence of adverse effects (and hence pathogenicity) of *Btk* ABTS-351 on non-target arthropods following field application of Foray® 76B. This is in line with the RMS’ conclusions during the previous EU Renewal (RAR, 2020 Vol. 3 B.9), where *Btk* ABTS-351 was considered as not toxic, pathogenic or infective to arthropods other than target pests, albeit EFSA concluded that available information was not sufficient to address the potential infectivity and pathogenicity of *Btk* ABTS-351 in non-target arthropods.

**Risk assessment for non-target arthropods other than bees**

The risk assessment schemes available for chemical plant protection products (e.g., hazard quotient based on ratio of application rate and LD50) are generally regarded as not suitable for the assessment of risks posed by microorganisms to non-target arthropods, as these assessment schemes were developed based on assumptions that apply to chemicals but not to microorganisms. Nevertheless, the risk from *Btk* ABTS-351 to non-target arthropods can be quantitatively assessed in a worst-case approach by calculating the margin of safety (MoS) as ratio of the median effective concentrations/test rates of available effect studies and the worst-case field exposure, both expressed either in spore density or potency. MoS ratios were calculated using *Aphidius* and *Typhlodromus* endpoints because the dose level is given as an application rate, while for the other species insufficient information is given to allow a conversion of the relevant endpoints to application rates. This is in line with the RMS’ approach during the previous EU Renewal (RAR, 2020 Vol. 3 B.9). MoS were also calculated based on maximum single application rates of the intended uses of Foray® 76B, since it is considered very unlikely that the same population of non-target arthropods is exposed to each application throughout the year. Furthermore, *Btk* ABTS-351 (1) is recognised to be a poor infectious agent that rarely recycles, (2) is unlikely to multiply in bulk soil, (3) does not persist or multiply on edible plant commodities (fruiting vegetable and leafy crops), (4) declines rapidly (viable spores) due to environmental factors such as solar radiation, rainfall, plant growth and temperature, and (5) has a half-life of less than 24 h on foliage (for details and references refer to Section IIIM 10 in the present document).

The risk assessment is shown for application of 3.0 L Foray® 76B/ha (refer to Table 10-1 or Appendix 2) and assumes exposure of test species to undiluted spraying solutions (i.e., in-field exposure), whereas non-target arthropods in off-field areas will be exposed to significantly lower levels of Foray® 76B, since exposure of off-field areas *via* drift is much lower compared to direct spray application (in-field), and widespread contamination of off-crop areas with *Btk* ABTS-351 and/or CryP *via* other dispersion routes is unlikely due to the low persistence of *Btk* ABTS-351 and CryP under environmental conditions (e.g., solar radiation). Consequently, the risk assessment covers the potential risk from *Btk* ABTS-351 to non-target arthropods in both in-field and off-field areas.

**Table 10.4-2: Risk assessment for effects of *Btk* ABTS-351 and Foray® 76B** **on non-target arthropods.**

| **Crop**  **scenario** | **Maximum single AR** | | | **Scenario** | **Relevant endpoint** | **MoS b** |
| --- | --- | --- | --- | --- | --- | --- |
| [L f.p./ha] | [g MPCA/ha] | [IU/ha] a | EC50 [IU/ha or g MPCA/ha] |
| Deciduous forest | 3.0 | 619 | 6.59 × 1010 | *A. rhopalosiphi* and *T. pyri* | > 2.38 × 1011  or > 3900 | > 3.6  or > 6.3 |

AR = maximum single application; Cspray = worst-case density of *Btk* ABTS-351 in application solution expressed in potency; f.p. = formulated product; MoS = margin of safety; values shown **in bold** fall below the margin of safety of 1.0;

a Based on maximum single application rate (i.e., g MPCA/ha) and mean potency of 19600 IU/mg product as specified in Part C.

b MoS calculated based on maximum single application rate and relevant effect data expressed either in IU/ha or in g MPCA/ha.

When based on International Units/ha, the quantitative risk assessment above shows margins of safety (MoS) > 3.6 for *A. rhopalosiphi* and *T. pyri* for all proposed uses of Foray® 76B. When based on g MCPA/ha the MoS are > 6.3 for all proposed uses. Accordingly, the quantitative risk assessment indicates an acceptable risk from all proposed uses of Foray® 76B to non-target arthropods based on endpoints from studies with *A. rhopalosiphi* and *T. pyri* with high margins of safety. Data available from studies with additional species support this assessment, since corrected mortality of *C. carnea* was < 50% at all test concentrations, results for *T. cacoeciae* are not reliable due to the high control mortality, and effects on mortality and reproduction of *M. occidentalis* and *T. urticae* were < 50% at all test concentrations. The only pronounced effect that has been observed in the available effect studies was a reduced hatching rate of *M. occidentalis* following direct application of *Btk* ABTS-351 to eggs, which is not consistent with the mode of action of *Btk* ABTS-351 and thus likely reflects physical effects of the test item application.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to non-target arthropods other than bees**

No laboratory studies are available which specifically characterized or quantified the toxicity of relevant CryP to non-target arthropods. In the DAR (2008) and the latest EU Renewal of *Btk* ABTS-351 (details in RAR, 2020 Vol. 3 B2), it was concluded that *Btk* ABTS-351 acts highly specific against members of the insect order of Lepidoptera and has no or only very limited activity against members of the orders Coleoptera, Diptera and Orthoptera. In line with this, the available data package (laboratory studies and open literature) shows low toxicity of *Btk* ABTS-351 to insects of various insect orders such as Parasitiformes, Trombidiformes, Neuroptera, Orthoptera, Dermaptera, Hemiptera, Coleoptera, and Diptera. Adverse effects on arthropods of the order Hymenoptera were described in the open literature, but these were apparent only after oral exposure to very high concentrations of *Btk* ABTS-351 over a period of six days. Overall, available data are consistent with the highly specific biological activity of *Btk* ABTS-351 against insect species of the order Lepidoptera and confirm that *Btk* ABTS-351 has no detrimental effects on non-target arthropods of other insect orders under realistic application regimes and exposure scenarios. In addition, since most of the available studies were conducted with formulated products on the basis of DiPel® Technical Powder (i.e., *Btk* ABTS-351), which contains approximately 10% of protoxin, available data are considered to cover the toxicity of CryP to non-target arthropods.

This is further supported by available open literature studies that have been evaluated in the course of the previous EU review of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 (details in RAR, 2020; RMS was The Netherlands). In laboratory feeding experiments using rice plants containing a synthetic cry1Ab gene derived from *Bacillus thuringiensis* Berliner (Bai *et al.*, 2006; IIIM 10.4/01), no adverse effects were observed in *Propylea japonica* after they were fed on brown planthopper contaminated with Cry1Ab toxins. Further studies (Sisterson *et al.*, 2004 and Truter *et al.*, 2014; IIIM 10.4/02 & IIIM 10.4/03) demonstrated that the abundance and diversity of arthropods in crop fields with cotton and maize plants that have been genetically modified to produce *Bacillus thuringiensis* Berliner toxins for pest control (*Bt* cotton and *Bt* maize) is similar compared to fields with non-*Bt* cotton and non-*Bt* maize plants.

Toxicity of CryP to non-target lepidopteran species following the proposed uses of Foray® 76B cannot be excluded. However, since non-target lepidopteran populations are expected to recover rapidly (Boulton, 2004; Joung and Coté, 2000) and *Btk* ABTS-351 and CryP are not expected to persist or accumulate in the environment, the risk from CryP to non-target arthropods following the proposed uses of Foray® 76B is considered acceptable (as concluded by RMS in RAR, 2020 Vol. 3 B.9). In addition, when evaluating the risk from the proposed uses of Foray® 76B to non-target arthropods it should be considered that *Btk* ABTS-351 has very high specificity to target lepidopteran species and therefore poses only a low risk to other non-target organisms compared to available chemical insecticides.

**Risk to beneficial arthropods**

Since the intended applications of Foray® 76B may result in exposure of beneficial arthropods (e.g., natural enemies from integrated and non-integrated pest management), the potential risk posed by *Btk* ABTS-351 to beneficial arthropods must be assessed. However, none of the available effect studies with bees, *A. rhopalosiphi*, *T. pyri*, *M. occidentalis*, and *T. urticae* showed effects above 30% that could be attributed to the biological activity of *Btk* ABTS-351 or associated CryPs (the studies with *Chrysoperla* and *T. pretiosum* are excluded from this evaluation as their reliability is limited due to high control mortalities). This is owed to the highly specific MoA of *Btk* ABTS-351 against members of the insect order of Lepidoptera, which is supported by available specificity data (details in RAR, 2020 Vol. 3 B2) and open literature studies (discussed above) that show that *Btk* ABTS-351 has no or only very limited activity against members of other insect orders. Therefore, the risk posed by *Btk* ABTS-351 to pollinators and other beneficial arthropods following application of Foray® 76B is considered acceptable based on the same arguments that support the risk assessment for non-target arthropods.

**Conclusion on risk to non-target arthropods other than bees**

Since (1) *Btk* ABTS-351 and CryP are quickly degraded on foliage and are not expected to accumulate to high levels in the environment, (2) the quantitative risk assessment indicates an acceptable risk from all proposed uses of Foray® 76B to non-target arthropods based on endpoints for *A. rhopalosiphi* and *T. pyri* with high MoS, (3) no effects > 50% were observed in the available laboratory studies with *C. carnea*, *M. occidentalis* and *T. urticae*, (4) *Btk* ABTS-351 has a highly specific MoA against insect species of the order Lepidoptera, (5) open literature studies do not provide evidence that *Btk* ABTS-351 is pathogenic or infective in non-target arthropods (orders other than Lepidoptera) or that toxicity of CryP to arthropods other than the target pest is likely, and (6) non-target lepidopteran species are expected to recover quickly due to multiple life cycles per year, the risk from *Btk* ABTS-351 and CryP to non-target arthropods (incl. beneficial arthropods) is considered acceptable for all proposed uses of Foray® 76B.

IIIM 10.5 Effects on earthworms

|  |  |
| --- | --- |
| Evaluator  Comments: | The presented approach was accepted.  The submitted justification was accepted.  The risk for earthworms is acceptable. |

The effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on earthworms were assessed during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on a study with the MPCA *Btk* ABTS-351. Additional studies on the adverse effects of Foray® 76B on earthworms are not available nor considered necessary (see rationale in Section IIIM 10). Details on available studies are available in EFSA Journal 2021;19(10):6879 and related documents (RAR, 2020). The relevant endpoints used for the present risk assessment are shown below.

**Table 10.5-1: Available data on effects of *Btk* ABTS-351 on earthworms**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test item** | **Exposure system** | **Endpoint** | **Reference** |
| Earthworm  (*Eisenia fetida*) | *Btk*  ABTS-351 a | 30 days;  test item mixed into artificial soil | NOEC ≥ 1000 mg MPCA/kg dry soil  (NOEC ≥ 1.1 × 1010 CFU/kg dry soil) | EFSA Journal 2021;19(10):6879 |

a Studies performed with DiPel® technical material, i.e., the source of *Btk* ABTS-351 in Foray® 76B

One laboratory study on the adverse effects of *Btk* ABTS-351 on *Eisenia fetida* is available, which was considered acceptable during the latest EU Renewal of *Btk* ABTS-351 (details in RAR, 2020 Vol. 3 B.9). Since no adverse effects on mortality, body weight or other signs of toxicity were observed during the 30-day observation period, a NOEC of ≥ 1000 mg MPCA/kg dry soil was established.

In addition, two open literature studies are available from the latest EU Renewal of *Btk* ABTS-351 showing that applications of different DiPel® formulations (i.e., *Btk* ABTS-351) have no adverse effects on earthworms in forest soils (details in RAR, 2020 Vol. 3 B.9). The first study (Benz and Altweg, 1975) showed that *Btk* ABTS-351 has no adverse effects on earthworm populations in forest soils over 9 weeks following application of DiPel® up to application rates of 1.5 x 109 CFU/ha. The second study (Lolmes, 1996) found no adverse effects of *Btk* ABTS-351 on the forest earthworm *Dendrobaena octaedra* following application of two different DiPel® formulations in a soil-litter microcosm experiment at applications rates up to 1000-times of expected environmental concentrations.

**Infectivity and pathogenicity of *Btk* ABTS-351 in earthworms**

In the available 30-d laboratory study and available open literature studies on adverse effects of *Btk* ABTS-351 on earthworms, no signs of pathogenicity were observed. Therefore, *Btk* ABTS-351 is not expected to exhibit pathogenicity and infectivity in earthworms, although infectivity of *Btk* ABTS-351 was not specifically assessed in this study. This is corroborated by the fact that *Btk* ABTS-351 acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on other non-target species of other orders. In addition, earthworms are well adapted to ubiquitous soilborne bacteria such as *B. thuringiensis* and have developed adequate immune systems to cope with microorganisms without being infected or negatively affected (OECD 2012, Series on Pesticides No. 67). This was also investigated in a study of Çotuk and Dales (1984; IIIM 10.5/01) which indicates that coelomic fluids of earthworms provide protection from infections from microorganisms such as *B. thuringiensis*. Moreover, since the biological activity of *B. thuringiensis* declines rapidly when exposed to e.g., solar radiation (EFSA Journal 2021;19(10):6879), and *Btk* ABTS-351 is not expected to multiply in soil, *Btk* ABTS-351 is not expected to exhibit pathogenicity and infectivity in earthworms following application of Foray® 76B.

**Risk assessment for earthworms**

The risk assessment schemes available for chemical plant protection products are generally regarded as not suitable for the assessment of risks posed by microorganisms to earthworms, as these assessment schemes were developed based on assumptions that apply to chemicals but not to microorganisms. Nevertheless, the risk from *Btk* ABTS-351 to earthworms can be quantitatively assessed in a worst-case approach by calculating the margin of safety (MoS) as ratio of the No Observed Effect Concentration (NOEC; in CFU/kg dry soil) from available effect studies and the Predicted Environmental Density in soil (PEDSOIL).

Since the PEDSOIL is calculated based on the yearly total dose application as one single application and no decline was considered for *Btk* ABTS-351, the risk assessment below presents a worst-case scenario. In a risk envelope approach, the risk assessment below is shown for the risk envelope application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens). Since all other proposed uses result in lower PEDSOIL, the risk assessment below covers all proposed uses.

**Table 10.5-2: Risk from *Btk* ABTS-351 and Foray® 76B to earthworms**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Crop scenario** | **Yearly total dose** | | **PEDSOIL** | **NOEC** | **MoS** |
| [L f.p./ha] | [CFU/ha] a | [CFU/kg dry soil] | [CFU/kg dry soil] |
| Pine trees, deciduous/ coniferous forest, shrubs, ornamental trees and plants | 4 x 2.5 | 1.69 × 1014 | 2.26 × 108 | > 1.1 × 1010 | > 48.7 |

f.p. = formulated product; MOS = margin of safety; values shown **in bold** fall below the margin of safety of 1.0

a Calculated based on max. spore density 1.69 × 1013 CFU/L f.p., yearly total dose application as one single application, and assuming no decline of *Btk* ABTS-351 as worst-case scenario in line with Part B Section 5 (Environmental Fate).

Since the quantitative risk assessment above shows a margin of safety of > 48.7, the risk from *Btk* ABTS-351 to earthworms following the application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) (i.e., risk envelope) is indicated to be acceptable. Since PEDSOIL values for all other proposed uses of Foray® 76B are lower compared to the risk envelope, the risk assessment covers all uses presented in the GAP (refer to Part A).

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to earthworms**

No laboratory studies are available which specifically characterized the toxicity of relevant CryP to earthworms. However, the results of the available 30-d laboratory study with *E. fetida,* the open literature studies by Benz and Altweg (1975) and Lolmes (1996), and the high margin of safety (i.e., > 48.7) calculated in the risk assessment above show that no adverse effects or unacceptable risk result from exposure of earthworms to CryP following the proposed uses of Foray® 76B. To quantitatively support this conclusion, it is possible to derive an NOEC for CryP based on the relevant endpoint from the available 30-d study on adverse effects of *Btk* ABTS-351 to *E. fetida* and an approximate content of protoxin in DiPel ®Technical Powder of 10% (details in Part C). If this NOEC (i.e., 100 mg CryP/kg dry soil) is related to the calculated worst-case PECSOIL of 350 µg CryP/kg dry soil (calculated in Part B Section 5), a margin of safety of > 285.7 is calculated.

Further evidence is provided by two open literature studies that have been considered relevant and reliable during the latest EU Renewal of *Bta* ABTS-1857 (EFSA Journal 2020;18(10):6294 and RAR, 2020). In the study by Shu *et al.* (2011; IIIM 10.5/02), earthworms were exposed to CryP (Cry1Ab) derived from *Bt* toxin producing corn plants. Over the test period of 30 to 60 days, no adverse effects of CryP on survival, growth and reproduction of *E. fetida* were observed. Concentrations of CryP in the test substrate were verified in this study and ranged between 0.74 ± 0.07 to 19.91 ± 4.16 ng/g Cry1Ab protein (mean ± SD). However, as these CryP levels are lower than exposure estimates calculated for CryP levels in soil following application of Foray® 76B (refer to Part B Section 5), these findings cannot be used to quantitatively assess the potential risk of CryP to earthworms following the proposed uses of Foray® 76B. Results of a further study (Zeilinger *et al.*, 2010; IIIM 10.5/03) indicate that exposure of earthworms (i.e., *Aporrectodea caliginosa*, *Aporrectodea trapezoides*, *Aporrectodea tuberculate*, and *Lumbricus terrestris*) to CryP (i.e., Cry1Ab and Cry3Bb1) produced from maize plants over four years has no effects on biomass of juveniles and adults in the soil. However, although this study suggests that earthworms ingest CryP from root exudates of plants, clay particles in soil, and/or crop litter, results of this study have to be considered only as supportive information, since approximate CryP levels in the soil were not reported or verified in this study.

In conclusion, the risk from CryP to earthworms following the proposed uses of Foray® 76B is low, since available data indicate low toxicity of CryP to earthworms, and *Btk* ABTS-351 and CryP are not expected to persist or multiply to high levels in soil (details in Part B Section 5).

**Conclusion on risk to earthworms**

In line with conclusions for DiPel® DF in EFSA Journal 2021;19(10):6879, a low risk from *Btk* ABTS-351 and CryP to earthworms can be concluded for the proposed uses of Foray® 76B, since (1) *Btk* ABTS-351 showed no signs of toxicity, pathogenicity or infectivity in the available studies with earthworms, (2) the quantitative risk assessment resulted in a margin of safety ≥ 48.7 based on worst-case exposure estimates for *Btk* ABTS-351 in soil, and (3) *Btk* ABTS-351 and CryP are not expected to multiply or accumulate to high levels in soil.

IIIM 10.6 Effects on soil microorganisms

|  |  |
| --- | --- |
| Evaluator  Comments: | The presented approach was accepted.  The submitted justification considering the risk was accepted.  The risk for soil micro-organisms is acceptable. |

The effects of *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 on soil microorganisms were assessed during the latest EU Renewal (EFSA Journal 2021;19(10):6879) based on an open literature study with a DiPel® formulation containing *Btk* ABTS-351. Details on available studies are available in EFSA Journal 2021;19(10):6879 and related documents (RAR, 2020). The relevant endpoint used for the present risk assessment are shown below.

**Table 10.6-1: Available data on the effects of *Btk* ABTS-351 to soil microflora**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test species** | **Test substance** | **Exposure system** | **Endpoint** | **Reference** |
| Carbon and nitrogen mineralisation | DiPel® 176  (*Btk* ABTS-351) | 8-week soil microcosm | NOEL = 0.226 µL product/10 g soil  (approx. 1.42 x 108 CFU/kg dry soil) | EFSA Journal 2021;19(10):6879 |

NOEL: No Observed Effect Level

An open literature study is available from the latest EU Renewal of *Btk* ABTS-351 (Visser *et al.,* 1994; details in RAR, 2020 Vol. 3 B.9), which investigated the adverse effects of *Btk* ABTS-351 on carbon and nitrogen mineralisation of soil microflora in soil microcosms. Soil samples were treated with two test item concentrations (i.e., 0.226 µL/10 g soil and 226 µL/10 g soil; potency of test item: 1.69 x 1010 IU/L), and soil respiration and nitrification were measured over 8 weeks. No significant differences in nitrification were observed between the control and the test item groups after 8 weeks. Soil respiration was statistically significantly higher in the high-test item group compared to control after 8 weeks, while no significant differences were detected between the low-test item group and the control. Therefore, the NOEL of this study was established as 0.226 µL product/10 g soil (approx. 1.42 x 108 CFU/kg dry soil).

**Risk assessment for soil microorganisms**

The risk from *Btk* ABTS-351 to microorganisms can be assessed in a worst-case approach by calculating the margin of safety (MoS) as ratio of the No Observed Effect Level (NOEL; in CFU/kg dry soil) from available effect studies and the Predicted Environmental Density in soil (PEDSOIL).

Since the PEDSOIL is calculated based on the yearly total dose application as one single application and no decline was considered for *Btk* ABTS-351, the risk assessment below presents a worst-case scenario. The risk assessment below is shown for application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens). Since all other proposed uses result in lower PEDSOIL, the risk assessment below covers all proposed uses.

**Table 10.6-2: Risk from *Btk* ABTS-351 to soil microorganisms following application of Foray® 76B**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Crop**  **scenario** | **Yearly total dose** | | **PEDSOIL** | **NOEL** | **MoS** |
| [L f.p./ha] | [CFU/ha] a | [CFU/kg dry soil] | [CFU/kg dry soil] |
| Pine trees, deciduous/ coniferous forest, shrubs, ornamental trees and plants | 4 x 2.5 | 1.69 × 1014 | 2.26 × 108 | 1.42 × 108 | **0.63** |

f.p. = formulated product; MoS = margin of safety; values shown **in bold** fall below the margin of safety of 1.0

a Calculated based on max. spore density 1.69 × 1013 CFU/L f.p., yearly total dose application as one single application, and assuming no decline of *Btk* ABTS-351 as worst-case scenario in line with Part B Section 5 (Environmental Fate).

The quantitative risk assessment above results in a risk quotient below 1.0 for application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens). Currently, no appropriate guidance is available on the interpretation of ratios between relevant effect data and exposure estimates for MPCAs. However, as the very conservative risk assessment is based on worst-case exposure estimates (e.g., PEDSOIL was based on yearly total dose application as one single application assuming no decline of *Btk* ABTS-351 in soil), the calculated risk quotient overestimates the potential risk from *Btk* ABTS-351 to soil microorganisms. In addition, tests on adverse effects of MPCA on soil microflora activity are acknowledged to have limited relevance for risk assessment purposes, since (1) result interpretation often is ambiguous as microorganisms may be affected by various stressors, and (2) natural microbial communities in soil are well adapted to their habitat, show good resilience, and are able to recover even upon extreme decimation (details in RAR, 2020 Vol. 3 B.9). Furthermore, *Btk* ABTS-351 is considered an ubiquitous soilborne microorganism that is not expected to accumulate to high levels in soil following application of Foray® 76B (details in Part B Section 5). Therefore, the risk from *Btk* ABTS-351 to microorganisms following application of Foray® 76B can be considered low, can be considered low despite the risk quotient being below 1.0.

**Infectivity and pathogenicity of *Btk* ABTS-351 in soil microorganisms**

The concept of infectivity and pathogenicity of microorganisms in non-target organisms is not applicable to non-target microorganisms. For this reason, adverse effects of MPCAs on soil microflora do not need to be further considered according to new data requirements set out in Commission Regulations (EU) 2022/1439 and 2022/1440.

**Risk posed by toxins/metabolites from *Btk* ABTS-351 to soil microorganisms**

No laboratory studies are available which specifically characterized the toxicity of relevant CryP to microorganisms. However, *Btk* ABTS-351 is an ubiquitous soilborne microorganism that is not expected to accumulate to high levels in soil following application of Foray® 76B (details in Part B Section 5). Likewise, CryP are unlikely to persist or accumulate in soil (details in Part B Section 5). In addition, a study is available from Koskella and Stotzky (2002; details in RAR, 2020 Vol. 3 B.9) that showed that larvicidal toxins from *B. thuringiensis* subspp. *kurstaki*, *morrisoni*, and *israelensis* have no microbicidal activity on a variety of bacteria (8 Gram-negative, 5 Gram-positive and a cyanobacterium), fungi (2 Zygomycetes, 1 Actinomycete, 2 Deuteromycetes, and 2 yeasts), and algae (primarily green and diatoms) in pure and mixed culture. Moreover, information from open literature studies indicate that other native soil microorganisms utilize *Bacillus thuringiensis* subspp. *kurstaki* as a source of nutrition, and thus prevent growth of *Bacillus thuringiensis* subspp. *kurstaki* in soil (details in RAR, 2020 Vol. 3 B.9). Therefore, the risk from CryP to soil microorganisms following the proposed uses of Foray® 76B is considered low.

**Conclusion on risk to soil microorganisms**

In line with conclusions for DiPel® DF in EFSA Journal 2021;19(10):6879, the risk from *Btk* ABTS-351 and CryP to soil microorganisms following the proposed uses of Foray® 76B is low, since (1) *Btk* ABTS-351 is an ubiquitous soilborne microorganism that is not expected to multiply to high levels in soil, (2) CryP are unlikely to persist or accumulate in soil, (3) microbial communities in soil are well adapted to their habitat and show good resilience and recovery towards stressors, (4) open literature studies indicate that *Bacillus thuringiensis* subspp. *kurstaki* has no microbial activity, while some native soil microorganisms may utilize *Bacillus thuringiensis* subspp. *kurstaki* as source of nutrition and thus prevent its growth in soil.

IIIM 10.7 Additional studies

This section includes summaries of studies that have not been evaluated during the 1st Annex Inclusion or latest EU Renewal of *Btk* ABTS-351. However, the presented studies may have been evaluated during the latest EU Renewal of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 (RAR, 2020). For details on studies evaluated during the latest EU Renewal of *Btk* ABTS-351, refer to EFSA Journal 2021;19(10):6879 and related documents (i.e., RAR, 2020).

**IIIM 10.7.1 Effects terrestrial vertebrates**

No additional studies available.

**IIIM 10.7.2 Effects on aquatic organisms**

No additional studies available.

**IIIM 10.7.3 Effects on bees**

**Study 1: Acute Oral Toxicity to the Honeybee *Apis mellifera* L.**

|  |  |
| --- | --- |
| Comments of zRMS: | The oral toxicity study to bees is acceptable.  The validity criteria were met.  The study was conducted according to OECD guideline 213.  No mortality was observed.  Oral LD50 at 24 and 48h > 685.86 μg formulation/bee  The toxicity was recalculated for active substance: LD50 > 108.95 μg a.s./bee.  The endpoints were used in the risk assessment.  . |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.3/01 |
| Author(s) (year) | Vergé, E., (2010a) |
| Title | Foray 76B Acute Oral Toxicity to the Honeybee *Apis mellifera* L. in the Laboratory |
| Report number | S09-03398 |
| Test facility | Eurofins Agroscience Services GmbH, Germany |
| Published | No |
| Test guideline | OECD Guideline No. 213 (1998) |
| Deviations | None |
| GLP | Yes |
| Acceptability | Acceptable |

**Materials and Methods:**

The study was conducted with Foray® 76B (Batch No. 185-87-BJ; nominal purity: *Bacillus thuringiensis* subsp. *kurstaki* active ingredient 18.44% (w/w)) on the honeybee in an oral toxicity limit test.

The nominal dose was 100 µg a.s./bee, equivalent to 629.07 µg of formulated product / bee. The actual calculated intake was 108.95 µg a.s./bee, corresponding to 685.37 µg of formulated product / bee. The control group received a 50% (w/v) aqueous sugar solution. Perfekthion (a.s. dimethoate was used as a reference item, with nominal test concentrations of 0.08, 0.11, 0.14 and 0.18 µg a.s./bee. The bees were exposed to the test item during feeding.

The bees were kept in cages made of stainless steel (width 8.2 cm; depth: 4 cm; height: 6 cm). The front side of the cages was equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated steel, which guaranteed sufficient air supply for the test insects. The test cages were lined with filter paper. Each concentration and treatment consisted of five replicate groups of ten honeybees per cage, with the exception of one cage in the treatment group and one cage in the 0.18 µg dimethoate/bee group that contained 11 bees.

In the oral toxicity test the test item and reference item was dissolved in tap water. Doses were then diluted into 50% (w/v) aqueous sucrose solution in order to obtain the intended nominal dose per bee in 20 µL, even though 25µL were provided. The control bee received 50% (w/v) aqueous sucrose solution. The amount of test item in the feeding solutions were set 25% higher than required with 250 µL of solution offered per cage in order to compensate for potential decrease in food uptake as often seen in such tests.

The bees were starved for 2 hours prior to dosing. 250 µL of appropriate solution was offered to each cage of bees for 6 hours. The amount of solution consumed per bee was determined by weighing the feeders before and after feeding. After the exposure period, 50% (w/v) aqueous sucrose solution was provided to each cage *ad libitum.*

During the experimental phase the test organisms were kept under constant darkness, with the feeding and observations made under neon light. The temperature during the test was 23.5 to 25°C and the relative humidity was 53% to 65%. The number of dead bees in the individual test cages was recorded after 4 h, 24 h and 48 h. In case of symptoms of poisoning the behavioural differences between the bees of the control group and those of the test item treatment were noted at each observation interval.

**Findings:**

In the oral toxicity test the nominal concentration of 100 µg a.s./bee was determined to be an actual intake of 108.95 µg a.s./bee. No mortality was observed at this dose after 48 hours. The 48-hour LD50 is therefore >108.95 µg a.s./bee. There was no mortality in the control group during the test period. Mortality occurred in the reference item treatment group, with between 18.0% and 92.2% mortality observed at 0.08 and 0.18 µg a.s./bee respectively after 48 hours. One affected bee was observed 4 hours after start of feeding. No more behavioural effects were observed at the following assessments 24 and 48 hours after test start.

**Table 10.7.3-1: Mortality in the oral toxicity test of Foray 76B**

| **Treatment** | **Intake of test or reference item** | **Mortality (%)** | |
| --- | --- | --- | --- |
| **24 h** | **48 h** |
| Control (50% w/v aqueous sucrose solution) | - | 0.0 | 0.0 |
| Foray® 76B (µg a.s./bee) | | | |
| 100 | 108.95 | 0.0 | 0.0 |
| Perfekthion (dimethoate, µg a.s./bee) | | | |
| 0.08 | 0.09 | 12.0 | 18.0 |
| 0.11 | 0.12 | 62.0 | 68.0 |
| 0.14 | 0.15 | 92.0 | 92.0 |
| 0.18 | 0.18 | 86.3 | 92.2 |

**Conclusion:**

The results of this study show that the oral LD50 is >108.95 µg a.s./bee for Foray® 76B.

**Study 2: Acute Contact Toxicity to the Honeybee *Apis mellifera* L.**

|  |  |
| --- | --- |
| Comments of zRMS: | The contact toxicity study to bees is acceptable.  The validity criteria were met.  The study was conducted according to OECD guideline 214.  No mortality was observed.  The contact LD50 at 24 h and 48h > 100 μg formulation/bee  The toxicity was recalculated for active substance: LD50 > 15.88 μg a.s./bee.  The endpoints were used in the risk assessment.  . |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.3/02 |
| Author(s) (year) | Vergé, E., (2010b) |
| Title | Foray 76B – Acute Contact Toxicity to the Honeybee *Apis mellifera* L. in the Laboratory |
| Report number | S10-02724 |
| Test facility | Eurofins Agroscience Services GmbH, Germany |
| Published | No |
| Test guideline | OECD Guideline No. 214 (1998) |
| Deviations | None |
| GLP | Yes |
| Acceptability | Acceptable |

**Materials and Methods:**

The study was conducted with Foray® 76B (Batch No. 185-087-BJ; nominal purity: *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 active ingredient 18.44% (w/w)) on the honeybee in a contact toxicity limit test.

The nominal doses tested were 20, 40, 60, 80, and 100 µg Foray® 76B/bee. The control group received mineral tap only. Dimethoate (BAS 152 11 I 414.8 g/L) was used as a reference item, with nominal test concentrations of 0.10, 0.15, 0.23 and 0.34 µg a.s./bee.

The bees were kept in cages made of high-grade steel (width 10 cm; depth: 5.5 cm; height: 8.5 cm). The front side of the cages was equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test insects. The test cages were lined with filter paper. Each concentration and treatment consisted of five replicate groups of ten honeybees per cage.

In the contact toxicity test the test item and reference item was diluted with tap water. The bees were anaesthetised with carbon dioxide and treated individually by the topical application of a 2 µL drop of appropriate solution dispensed from a micro applicator to the ventral thorax of each bee. The needle of the micro applicator was cleaned between each application using water and water-wetting agent in order to ensure the dispersion of the treatment solutions after application. After dose administration, the bees were returned to their cages and fed with a 50% (w/v) aqueous sucrose solution in excess.

During the experimental phase the test organisms were kept under constant darkness, with the observations made under neon light. The temperature during the test was 25°C and the relative humidity was 60% to 66%. The number of dead bees in the individual test cages was recorded after 4 h, 24 h and 48 h. In case of symptoms of poisoning the behavioural differences between the bees of the control group and those of the test item treatment were noted at each observation interval.

**Findings:**

In the contact toxicity test the nominal concentrations were 20, 40, 60, 80, and 100 µg Foray® 76B/bee, corresponding to 3.18, 6.35, 9.53, 12.71, and 15.89 µg a.s./bee. Mortality of 8% occurred in the dose level tested of 80% product/bee over 48-hour observation period. Mortality of 4% occurred in the dose level tested of 100% product/bee over 48-hour observation period which indicates that the mortality is not dose related. The 48-hour LD50 is therefore >100 µg product/bee. There was no mortality in the control group during the test period. The 24-hour contact LD50 for the reference item was 0.24 µg dimethoate/bee. Apathetic and affected bees were observed 4 hours after application in all treatment groups. At the assessment 24 hours after treatment one apathetic bee was recorded in the 80 µg product/bee group. No more behavioural abnormalities were observed at the following assessment after 48 hours after treatment.

**Table 10.7.3-2: Mortality in the contact toxicity test of Foray 76B.**

| **Treatment** | **Mortality (%)** | |
| --- | --- | --- |
| **24 h** | **48 h** |
| Control (water) | 0.0 | 0.0 |
| Foray® 76B (µg product/bee) | | |
| 20 | 4.0 | 6.0 |
| 40 | 2.0 | 4.0 |
| 60 | 0.0 | 0.0 |
| 80 | 6.0 | 8.0 |
| 100 | 4.0 | 4.0 |
| Dimethoate (Perfekthion µg a.s./bee | | |
| 0.10 | 2.0 | 6.0 |
| 0.15 | 8.0 | 14.0 |
| 0.23 | 60.0 | 66.0 |
| 0.34 | 56.0 | 56.0 |

**Conclusion:**

The results of this study show that the contact LD50 is >100 µg product/bee for Foray® 76B.

**Study 3: Adverse effects on colony performance of *Apis mellifera* L.**

|  |  |
| --- | --- |
| Comments of zRMS: | The study was evaluated during active substance evaluation. Please refer to Volume 3 B.9 (MPCA), 2020. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.3/03 |
| Author(s) (year) | Leza, M.M., Llado, G., Petro, A.B., and Alemany, A. (2014) |
| Title | First field assessment of *Bacillus thuringiensis* subsp. *kurstaki* aerial application on the colony performance of *Apis mellifera* L. (Hymenoptera: Apidae) |
| Report number | Spanish Journal of Agricultural Research 2014 12(2): 405-408 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

**Executive Summary**

Honeybee populations around the world are experiencing a decrease in colony numbers probably due to a combination of different causes, such as diseases, poor nutrition and frequent applications of insecticides to control pests. The aim of this study was to analyse the effect of field aerial applications of *Btk* on bee colony performance, specifically on the brood cell percentage evolution, which can be used as an indicator of queen health and brood development breeding rates. To achieve it, the brood cell surface was photographed in every sampling, and data were analysed using a method based on image treatment software. A total of 480 pictures were examined from two groups of four nucleus hives in two areas, one receiving aerial spraying with *Btk* and the other without treatment. A mixed factorial design was realized to analyse the data showing no differences in colony performance between the two groups of colonies either before the treatment, during and at the end of the assay. Furthermore, the brood surface ratio of *Btk* treated/ untreated increased along the experiment.

The results of the present study suggest that *Btk* aerial applications did not affect the brood development of honeybees under natural conditions. Nevertheless, further field studies are required to ascertain a safe use of *Btk* in forest pest management.

**I. MATERIALS AND METHODS**

**A. MATERIALS**

**Test item**

Foray® 48B, Kenogard S.A., Spain (*B. thuringiensis* subsp. *kurstaki*, 11.8% p/v (11.8 x 106 of IU g–1);a suspension concentrate (SC); 3.5 L ha–1 ultra-low volume application, drop diameter: 100-125 microns)*.*

**Hives and testing locations**

Eight Langstroth nucleus hives were located in two pine forests (four nuclei per field) of Ibiza (572.56 km2), West Mediterranean Island. One of the forests is located in a zone treated with *Btk* (UTM: 31S 79032 m E 4322751 m N)*,* while the second one is in a treatment-free protected area and was considered as control (UTM: 31S 367720 m E 4321474 m N). The two zones are separated by about 10 km, an insurmountable distance for a bee flight, and far enough to be free of the spray wind drift. To make sure those bee populations of all nucleus hives were as homogeneous as possible, sister queens from the same breeding line were reared by using Doolittle method (Flores *et al.*,1998[[3]](#footnote-4)). The frames of all colonies were made from organic wax and bees did not receive any chemical treatment.

**B. STUDY DESIGN AND METHODS**

BACI (Before-After Control-Impact) design (Green, 1979) was conducted in this study. The first measurements were taken on August 25, 2009, two months before the treatment applied, which was realized by helicopter on October 20, 2009. The product applied was Foray® 48 B, Kenogard S.A., Spain (*B. thuringiensis* subsp. *kurstaki*, 11.8% p/v (11.8 x 106 of IU g–1). It was a suspension concentrate (SC); 3.5 L ha–1 ultra-low volume application, drop diameter: 100-125 microns)*.* By knowing the initial state, the environmental heterogeneity was controlled. First’s five samplings were taken fortnightly, except the last sampling, which were taken one month later on December 16, 2009. Both faces of every frame were photographed in every sampling. A total of 480 pictures were taken and examined. In each sampling, 80 pictures were taken (10 pictures per hive). Each digital photograph was processed with the Image Analysis Software SIG ArcGIS (ESRI), in order to calculate the percentage of cells occupied with brood (open brood as well as capped brood) in relation to the total surface of the frame, as an effective measurement of the bee’s brooding efficiency (Dai *et al.*, 2012).

**C. STATISTICAL ANALYSES**

Data were analysed by a 2 × (6 × 4) mixed factorial design with one between-factor (control/treated groups) and one within-factor (six temporal points) in SPSS v. 20.0 (SPSS Inc., Chicago, IL, USA). A level of *p* < 0.05 was accepted as significant. Means and standard deviations were computed for variable.

**II.** **RESULTS AND DISCUSSION**

The percentage of brood of both groups of hives showed a strong parallelism throughout the experiment. No significant differences between groups were found (F = 2.59, *p* = 0.159).

A graph of a graph with lines and numbers

Description automatically generated

During the first three samplings the brood were increased. In the third sampling (just after the *Btk* treatment) the brood area was triplicated in both groups (3.8-fold in *Btk* colonies and 3.2-fold in control hives). In the fourth sampling three colonies of treated site (*Btk1, Btk2* and *Btk4)* and all colonies of control site began to decrease. In the fifth sampling, the brood surface was practically non-existent in six nucleus hives, three of the treated colonies (*Btk1, Btk2 and Btk4*) and three in the control forest zone (control 1, control 2 and control 4). Actually, in both groups there were significant differences between fourth and fifth sampling (t = 4.573, *p* = 0.020 in control group; and t = 3.472, *p* = 0.040 in treated group).

A screenshot of a graph

Description automatically generated

In addition to this drastic brood decrement, new queen cells in all of these nucleus hives were observed, as well as the new honeybee swarms in the nearby trees. All of these symptoms suggested that the nucleus hives had lost their queens because of a natural swarming process.

Although in control 4 there was a low brood percentage, it was observed that all of them remained in a pupal stage without any young or old larvae, which indicates that there was no recent queen laying. Interestingly, in the last sampling, four of these six colonies were recovered (*Btk1, Btk4,* control 1 and control 4) and a similar brood percentage was observed in both hive groups. On the other hand, *Btk* 3 and control 3 were maintained with normal growth (Table 1). At the end of the assay no significant differences between groups of hives were found. None of the nucleus hives showed any disease during the assay, being the reserves of honey and pollen enough to the development of the colonies. Furthermore, if the brood percentage of both groups are compared through the ratio efficiency *Btk*/control, it can be observed that, even though the *Btk* hives had an initial brood surface smaller than those of the control group, the brood mean ratio increased throughout the experiment: from an initial value of 0.66 (12.56/19.03) to a final 0.79 (3.65/4.59) (Table 1). So, the treated group breeding had increased comparatively to the control group breeding.

**III.** **CONCLUSIONS**

The evolution between the groups of colonies was very similar (Fig. 1), without any significant difference along the experiment between groups, and the breeding mean ratio efficiency *Btk*/control increased throughout the experiment. So, the results of the effect of *Btk* aerial treatment (which is applied against processionary caterpillar in pine forest in Ibiza) on *A. mellifera* suggest that the *Btk* do not affect the brood development of honeybees.

**Study 4: Toxicity of crystalline proteins to bees**

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| Comments of zRMS: | The submitted study report was accepted as a supportive/additional data. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.3/04 |
| Author(s) (year) | Rose, R., Dively, G. P., and Pettis, J. (2007) |
| Title | Effects of Bt corn pollen on honey bees: Emphasis on protocol development |
| Report number | Apidologie, 38(4), 368-377 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

1. **Abstract**

Laboratory feeding studies showed no effects on the weight and survival of honey bees feeding on Cry1Ab-expressing sweet corn pollen for 35 days. In field studies, colonies foraging in sweet corn plots and fed *Bt* pollen cakes for 28 days showed no adverse effects on bee weight, foraging activity, and colony performance. Brood development was not affected by exposure to *Bt* pollen but significantly reduced by the positive insecticide control. The number of foragers returning with pollen loads, pollen load weight, and forager weight were the most consistent endpoints as indicators of foraging activity. Using variances of measured endpoints, experimental designs required to detect a range of effect sizes at 80% statistical power were determined. Discussed are methods to ensure exposure to pollen, duration of exposure, positive controls, and appropriate endpoints to consider in planning laboratory and field studies to evaluate the nontarget effects of transgenic pollen.

1. **MATERIALS AND METHODS**

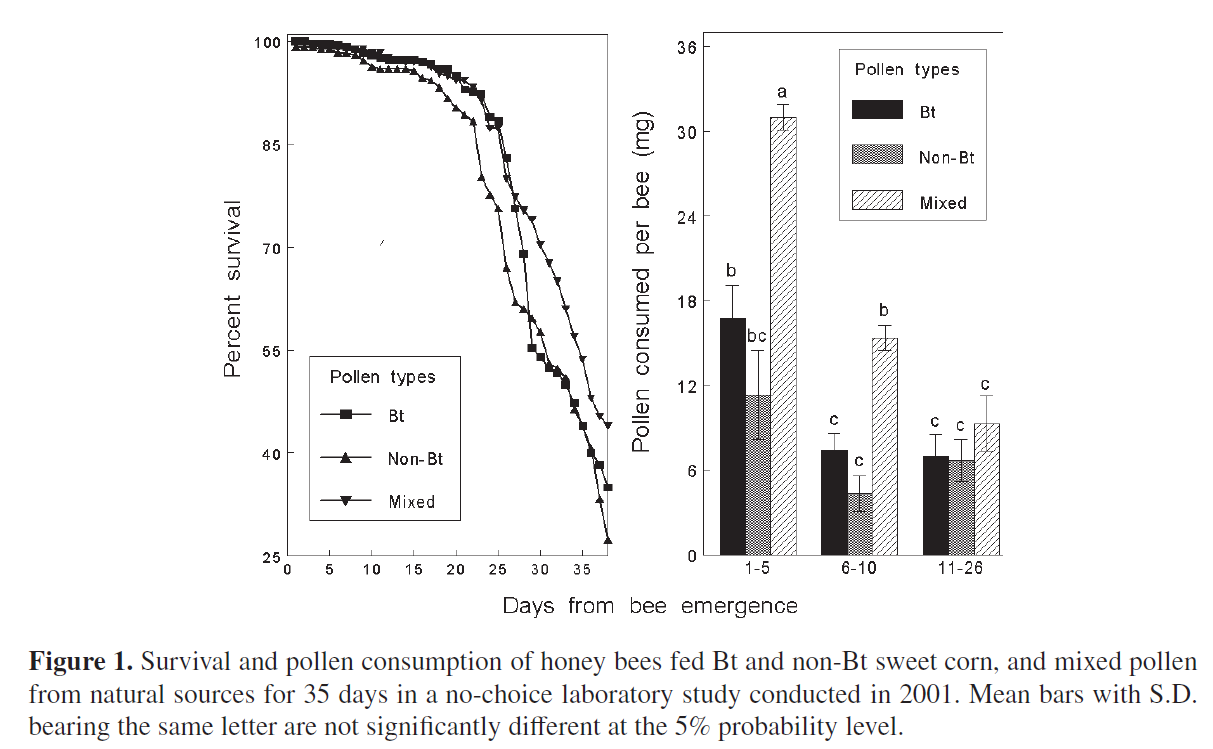
**Laboratory studies on adult survival and pollen consumption**

No-choice dietary feeding studies were conducted to measure effects of corn pollen on development and survival of adult honey bees. Cohorts of newly-emerged bees were fed a diet of *Bt* pollen from sweet corn hybrid Attribute GSS0966 (Syngenta Seeds, Golden Valley, MN; event *Bt*11) and non-*Bt* pollen from an isoline (Syngenta Seeds; Prime Plus). Corn pollen was collected from plant tassels in the field and stored at –80°C until initiation of the study. Bee cohorts were provided access to pollen, sugar syrup and water sources in small wooden cages (11 cm × 9 cm× 7 cm) with a sliding glass front, which allowed daily counting and removal of dead bees. Cages were kept in an incubator at 34°C, 50% RH, and 24 h of dark. Bee mortality was recorded daily and the body weights of ten randomly selected bees were recorded on day 10. In 2001, *Bt*, non-*Bt*, and mixed pollen (from pollen traps) diets were fed to newly emerged bees (<24 h old). Six replicate cages were assigned to each diet treatment. Each cage contained 60 bees randomly selected from one of six source colonies. Two grams of powdered pollen in an artificial plastic comb were placed in each cage on days 0, 5 and 10. Small plastic lures with queen pheromone were placed in cages to simulate the presence of a queen. The amount of pollen consumed was determined on days 5, 10 and 26 for each treatment cage by weighing pollen before and after introduction. In 2002, cohorts of 50 newly emerged bees from ten source colonies were evenly allocated to three sets of 10 replicate cages. Treatments of *Bt* pollen, non-*Bt* pollen, or non-*Bt* pollen treated imidacloprid were assigned to each replicate set. The pollen types were mixed with honey to increase consumption and were provided on days 0, 7, 14 as 5 g cakes (80% pollen and 20% honey w/w). Imidacloprid was added to the non-*Bt* pollen at the rate of 200 μg per kg of cake. The difference in weights of pollen cakes before and after introduction was determined on days 7, 14 and 21.

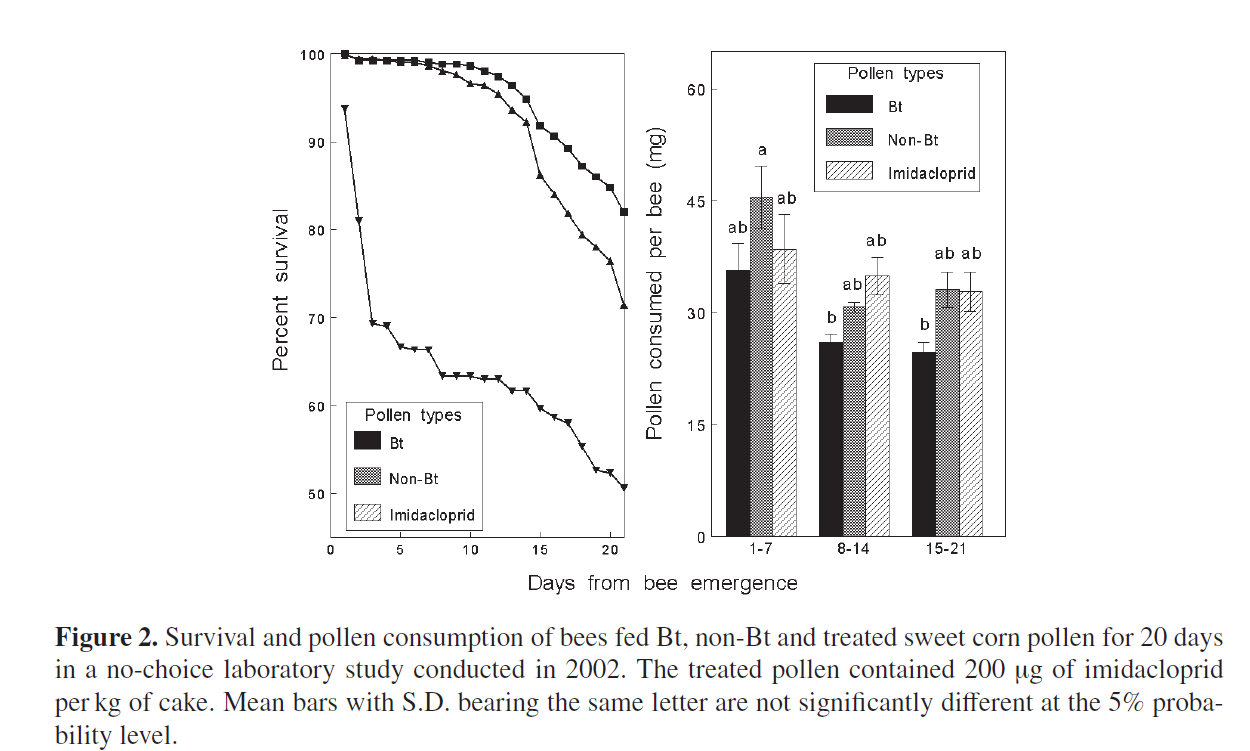
**Field studies on colony activity and development**

In 2002 four 65 m × 65 m (0.4 ha) replicate plots of *Bt* hybrid Attribute GSS0966 (Syngenta Seeds) and its non-*Bt* isoline Prime Plus were planted at least 300 m apart from each other at University of Maryland Research facilities. Two plots of non-*Bt* sweet corn were also planted for positive control colonies exposed to imidacloprid. Packages of 900 g of bees (approximately 10 000) were obtained from a commercial supplier (Wilbanks Apiaries; Claxton, GA, USA) and used to establish colonies for 5–6 weeks prior to initiation of the field study. Sister queens from the same breeding line were used in these colonies which were established in nucleus hives with four brood frames and one foundation frame to allow for expansion. Two weeks prior to anthesis, hives were opened to visually assess and equalize bee and brood densities. Seven days prior to anthesis, three hives were randomly selected and placed on wooded pallets over a 3 m × 4 m tarp in a cleared area at the center of each plot. During the evening of the same day, each hive was opened and sides of each comb with bees were photographed with a digital camera. Bees were then dislodged into the hive box and each comb photographed a second time to obtain pre-exposure images of brood, pollen, and honey cells. Colonies were allowed to forage for 4 weeks, which overlapped the period of anthesis. No pollen loads were trapped to avoid interference with the functioning of the nucleus hive. Each colony was provisioned with pollen cakes to increase the level of exposure. Pollen was collected from plants within each respective plot and processed into 50 g cakes consisting of 2 parts pollen, 2 parts soy flour, 2 parts honey, and 1 part sugar by weight. Pollen cakes containing either 10, 100 or 1000 μg of imidacloprid per kg of cake were assigned to one of the three hives in each positive control plot. Concentrations

were based on studies conducted to assess the impact of imidacloprid on honey bee behaviour in France (Schmuck, 1999). Four cakes were placed directly on the top of the frames in each hive once or twice weekly to allow bees ad libitum access. Records were kept on the weight of pollen cakes consumed by each colony. Hives were observed three times weekly during the exposure period to record the number of foraging bees returning to the hive, with and without pollen pellets, over a 5-min. period between 0900 and 1100 h. Weights of foraging bees and their pollen loads were measured once weekly from a sample of 10 bees returning to hives. At the end of the exposure period, combs of each hive were again photographed to obtain post-exposure images of bee strength, brood development, and stores of food. The percentage of bees covering each comb, and the percentage of capped brood, pollen and honey cells were visually estimated to the nearest 10% from matching pre- and post-exposure images displayed on a computer monitor with a grid overlay. An actual count of bees was also recorded on representative grid sections showing 10 to 100% of the area covered with bees.

1. **Results**

**Figure 10.3-02/01**. Survival and pollen consumption of honey bees fed *Bt* and non-*Bt* sweet corn, and mixed pollen from natural sources for 35 days in a no-choice laboratory study conducted in 2001. Mean bars with S.D. bearing the same letter are not significantly different at the 5% probability level.



**Figure 10.3-02/02**. Survival and pollen consumption of bees fed *Bt*, non-*Bt* and treated sweet corn pollen for 20 days in a no-choice laboratory study conducted in 2002. The treated pollen contained 200 μg of imidacloprid per kg of cake. Mean bars with S.D. bearing the same letter are not significantly different at the 5% probability level.

1. **Conclusion**

In field studies, colonies foraging in sweet corn plots and fed *Bt* pollen cakes for 28 days showed no adverse effects on bee weight, foraging activity, and colony performance. Brood development was not affected by exposure to *Bt* pollen but significantly reduced by the positive insecticide control.

**Study 5: Toxicity of crystalline proteins to bees**

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| Comments of zRMS: | The submitted report was accepted as a supportive/additional data. |

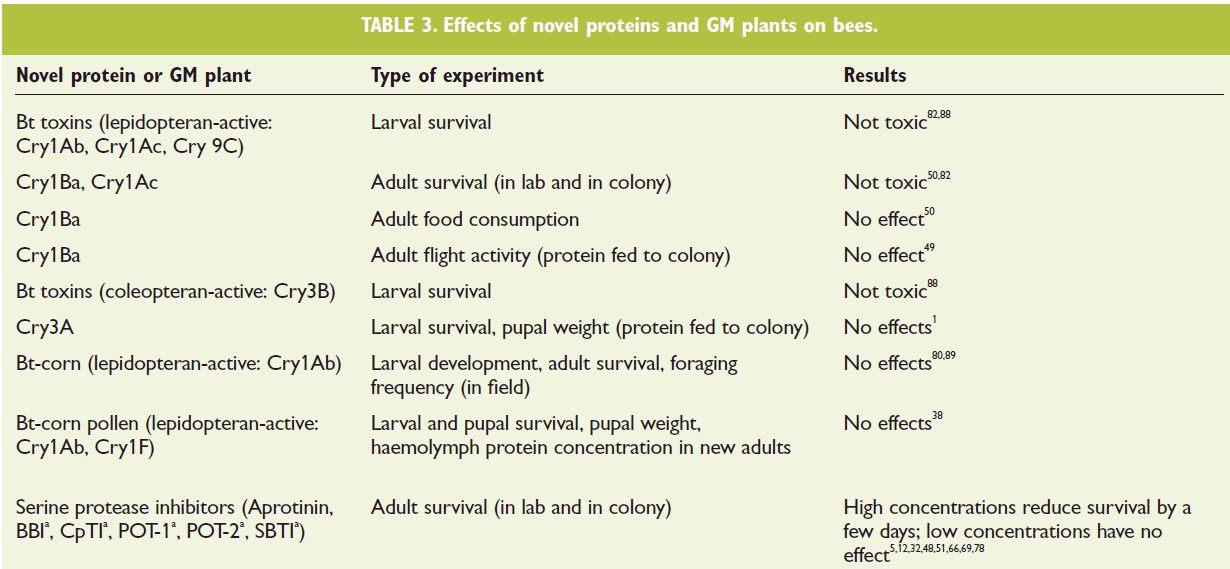
|  |  |
| --- | --- |
| Data point addressed | IIIM 10.3/05 |
| Author(s) (year) | Malone, L. (2004) |
| Title | Potential effects of GM crops on honey bee health |
| Report number | Bee World, 85(2), 29-36. |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

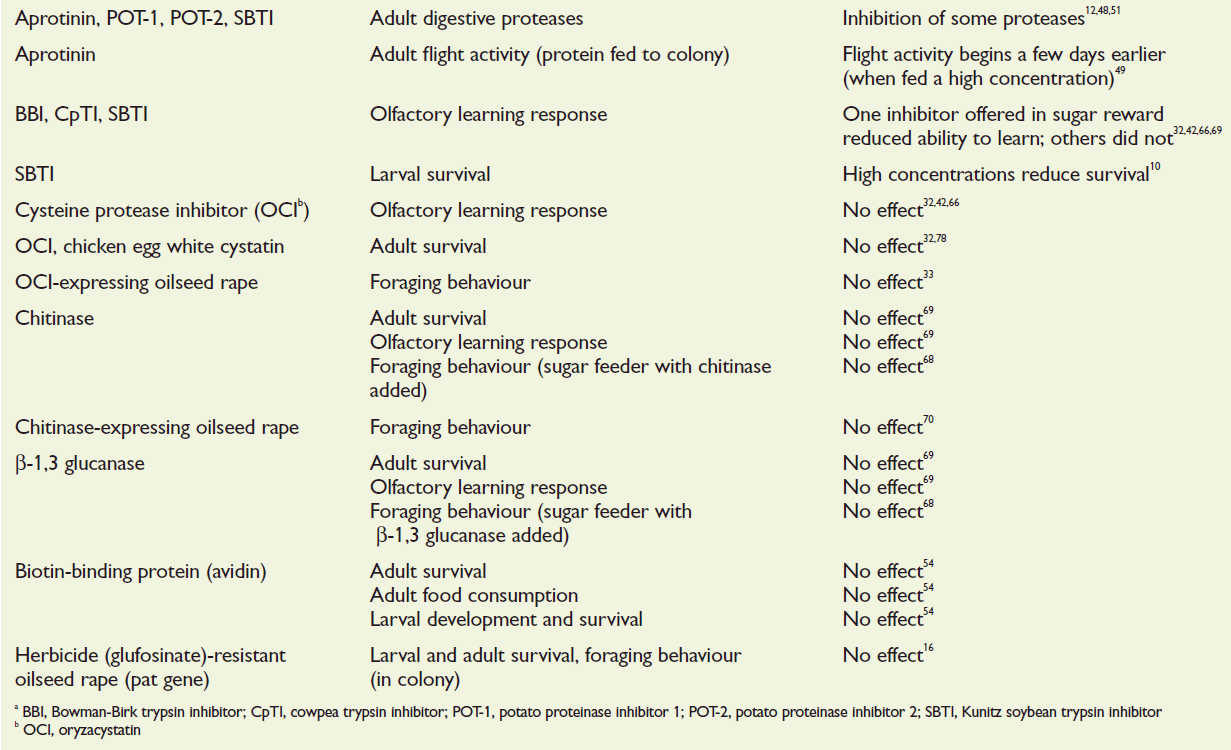
1. **Abstract**

Recent increases in the global area planted in GM crops have been accompanied by rising public awareness of agricultural practices and concern about the environment. Honey bees are widely recognized as important beneficial insects. In most countries, regulators assessing potential risks and benefits from GM plants list the honey bee among the ‘non-target’ species that need to be considered before these plants are released. Research on honey bees and GM crops has focused on the presence of GM material in honey, the roles that bees may play in the flow of genes from GM crops, and the potential impacts of GM plants on bee health. This article summarizes research on bee health and GM. There is now a considerable body of knowledge on this topic and some well-established techniques for assessing risks to bees before new plants are released.

1. **Results**

Many experiments have been conducted in which bees were fed with purified novel proteins at concentrations estimated to approximate or to exceed likely pollen expression levels (figs 2 and 3). Novel proteins with insecticidal properties aimed at making GM plants pest resistant have been the most thoroughly tested. There have also been trials conducted with small colonies of bees and potted flowering GM plants in glasshouses or under mesh in the field. Parameters measured include food consumption by adult bees, adult bee survival, olfactory learning and foraging behaviour in adult bees, larval bee development and survival. Results to date are briefly summarized in table 3.

**Table 10.3-03/01**. Effects of novel proteins and GM plants on bees.



1. **Conclusion**

Evidence available so far shows that none of the GM plants currently commercially available have significant impacts on honey bee health. There is a rapidly-growing body of scientific knowledge on the potential effects of GM plants with a variety of traits on these beneficial insects.

**Study 6: Toxicity of crystalline proteins to bees**

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| Comments of zRMS: | The submitted report was accepted as a supportive/additional data.. |

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| --- | --- |
| Data point addressed | IIIM 10.3/06 |
| Author(s) (year) | O'Callaghan, M., Glare, T. R., Burgess, E. P., and Malone, L. A. (2005) |
| Title | Effects of plants genetically modified for insect resistance on nontarget organisms |
| Report number | Annu. Rev. Entomol., 50, 271-292 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

1. **Abstract**

Insect resistance, based on *Bacillus thuringiensis* *(Bt*) endotoxins, is the second most widely used trait (after herbicide resistance) in commercial genetically modified (GM) crops. Other modifications for insect resistance, such as proteinase inhibitors and lectins, are also being used in many experimental crops. The extensive testing on nontarget plant-feeding insects and beneficial species that has accompanied the long-term and wide-scale use of *Bt* plants has not detected significant adverse effects. GM plants expressing other insect-resistant proteins that have a broader spectrum of activity have been tested on only a limited number of nontarget species. Little is known about the persistence of transgene-derived proteins in soil, with the exception of *Bt* endotoxins, which can persist in soil for several months. *Bt* plants appear to have little impact on soil biota such as earthworms, collembolans, and general soil microflora. Further research is required on the effects of GM plants on soil processes such as decomposition. Assessment of nontarget impacts is an essential part of the risk assessment process for insect-resistant GM plants.

**IIIM 10.7.4 Effects on arthropods other than bees**

**Study 1: Toxicity of crystalline proteins to *Propylea japonica***

|  |  |
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| Comments of zRMS: | The submitted study report was accepted as a supportive/additional data.. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.4/01 |
| Author(s) (year) | Bai, Y. Y., Jiang, M. X., Cheng, J. A., and Wang, D. (2006) |
| Title | Effects of Cry1Ab Toxin on *Propylea japonica* (Thunberg) (Coleoptera: Coccinellidae) Through Its Prey, *Nilaparvata lugens* Stål (Homoptera: Delphacidae), Feeding on Transgenic *Bt* Rice |
| Report number | Environmental Entomology, 35(4), 1130-1136 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

**Summary**

Laboratory feeding experiments using rice plants containing a synthetic cry1Ab gene derived *from Bacillus thuringiensis* Berliner (*Bt*) were carried out to study the effects of *Bt* rice-fed prey on the predator *Propylea japonica* (Thunberg). Plants were obtained from two homozygous transgenic *Bt*-cry1Ab expressing rice lines, Kemingdao 1(KMD1) and Kemingdao 2(KMD2), and their untransformed parental variety Xiushui 11 (XS11). The herbivorous prey species tested was the brown planthopper, *Nilaparvata lugens* Stål, one of most serious insect pests of rice and not targeted by KMD1 or KMD2. The concentrations of Cry1Ab toxin expressed in KMD1 and KMD2 plants and that of the toxin transferred to *N. lugens* feeding on these plants were determined by enzyme immunosorbent assay technique. Development parameters of *P. japonica* reared on KMD1- or KMD2-fed *N. lugens* were assessed in the laboratory. The results showed that the concentration of Cry1Ab in rice leaves and stems significantly increased from the booting to grain spilling stage and subsequently decreased as the plants matured. Cry1Ab could be detected in nymphs and adults of *N. lugens* feeding on the *Bt* rice plants. Development time, pupation, adult eclosion, pupal and adult weight, and male-adult locomotive activity of *P. japonica* that had preyed on KMD1- or KMD2-fed *N. lugens* nymphs as larvae were not significantly different from those that preyed on XS11-fed nymphs. In short, our results indicate that the nontarget insect *N. lugens* and its predator *P. japonica* are exposed to Cry1Ab toxin from transgenic cry1Ab rice, but development of this predator was not affected by the toxin through tritrophic interactions.

**RMS Comment** (RAR, 2020 for *Bta* ABTS-1857): The current research paper demonstrates that there are no tritrophic interaction on the predator Coleoptera *P. japonica*, feeding on the brown planthopper after exposure on Cry1Ab toxin from transgenic cry1Ab rice. Although the plants are not sprayed, the exposure to the Cry toxin is ensured due to the use of transgenic rice. The concentration of toxins in plants were also demonstrated in the laboratory. In the field, the predators such as lady beetles are exposed to the toxins from pray by ingestion of the gut of the prey by chewing. The results can be used in the risk assessment in a weight of evidence.

**Study 1: Arthropod Abundance and Diversity in *Bt* and Non-*Bt* Cotton Fields**

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| Comments of zRMS: | The submitted study report was accepted as a supportive/additional data. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.4/02 |
| Author(s) (year) | Sisterson, M. S., Biggs, R. W., Olson, C., Carrière, Y., Dennehy, T. J., and Tabashnik, B. E. (2004) |
| Title | Arthropod Abundance and Diversity in *Bt* and Non-*Bt* Cotton Fields |
| Report number | Environmental Entomology, 33(4), 921-929 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

**Summary**

The widespread planting of crops genetically modified to produce *Bacillus thuringiensis* Berliner (*Bt*) toxins for pest control may affect nontarget arthropods. To address this issue, we compared the abundance and diversity of arthropods on plants in field plots of *Bt* cotton, non-*Bt* cotton, and a row mixture of 75% *Bt* cotton and 25% non-*Bt* cotton at two sites in Arizona. Over three sampling dates during 2 year, we recorded all of the arthropods found on 120 cotton plants per treatment. This yielded 3,309 individual arthropods from 69 families. Excluding pink bollworm, *Pectinophora gossypiella* Saunders, the pest targeted by *Bt* cotton, we compared the abundance and diversity of all arthropods, chewing herbivores, sucking herbivores, rasping-sucking arthropods, and natural enemies. Arthropod abundance was significantly affected by site, plant height, and cotton type. More arthropods were collected from row mixture plots than *Bt* plots, but arthropod abundance did not differ significantly between *Bt* plots and non-*Bt* plots. The number of families collected was 57 for row mixture plots, 55 for non-*Bt* plots, and 47 for *Bt* plots. The number of families increased as arthropod abundance increased, suggesting that the differences in diversity among treatments were caused by differences in abundance. Within row mixture plots, arthropod abundance and diversity did not differ significantly between *Bt* plants and non-*Bt* plants. We conclude that the differences between *Bt* and non-*Bt* cultivars had relatively minor effects on the arthropod community on cotton plants.

**Comment RMS** (RAR, 2020 for *Bta* ABTS-1857): The current research paper demonstrated that there is no effect on abundance and diversity of arthropods between the *Bt* crops and non *Bt* cotton crops. The abundance of arthropods was affected by site, plant height and cotton type. The conclusion of this study can be used as supplementary information in the risk assessment.

**Study 3: Effects of crystalline proteins on arthropod diversity**

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| Comments of zRMS: | The submitted study report was accepted as a supportive/additional data. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.4/03 |
| Author(s) (year) | Truter, J., Van Hamburg, H., and Van Den Berg, J. (2014) |
| Title | Comparative Diversity of Arthropods on *Bt* Maize and Non-*Bt* Maize in two Different Cropping Systems in South Africa |
| Report number | Environ. Entomol. 43(1): 197-208 (2014) |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

**Abstract**

The biodiversity of an agroecosystem is not only important for its intrinsic value but also because it influences ecological functions that are vital for crop production in sustainable agricultural systems and the surrounding environment. A concern about genetically modified (GM) crops is the potential negative impact that such crops could have on diversity and abundance of nontarget organisms, and subsequently on ecosystem functions. Therefore, it is essential to assess the potential environmental risk of the release of a GM crop and to study its effect on species assemblages within that ecosystem. Assessment of the impact of *Bt* maize on the environment is hampered by the lack of basic checklists of species present in maize agroecosystems. The aims of the study were to compile a checklist of arthropods that occur on maize in South Africa and to compare the diversity and abundance of arthropods and functional groups on *Bt* maize and non-*Bt* maize. Collections of arthropods were carried out during two growing seasons on *Bt* maize and non-*Bt* maize plants at two localities. Three maize fields were sampled per locality during each season. Twenty plants, each of *Bt* maize and non-*Bt* maize, were randomly selected from the fields at each site. The arthropods collected during this study were classified to morphospecies level and grouped into the following functional groups: detritivores, herbivores, predators, and parasitoids. Based on feeding strategy, herbivores and predators were further divided into sucking herbivores or predators (piercing-sucking mouthparts) and chewing herbivores or predators (chewing mouthparts). A total of 8,771 arthropod individuals, comprising 288 morphospecies and presenting 20 orders, were collected. Results from this short-term study indicated that abundance and diversity of arthropods in maize and the different functional guilds were not significantly affected by *Bt* maize, either in terms of diversity or abundance.

**Comment RMS** (RAR, 2020 for *Bta* ABTS-1857): The current research paper demonstrated that there is no effect on abundance and diversity of arthropods between the *Bt* crops and non *Bt* maize crops. The conclusion of this study can be used as supplementary information in the risk assessment.

**IIIM 10.7.5 Effects on earthworms**

**Study 1: Effect of the coelomic fluid of the earthworm *Eisenia foetida***

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| --- | --- |
| Comments of zRMS: | The submitted report was accepted as a supportive/additional data. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.5/01 |
| Author(s) (year) | Çotuk, A. and Dales, R.P. (1984) |
| Title | The effect of the coelomic fluid of the earthworm *Eisenia foetida* Sav. on certain bacteria and the role of the coelomycetes in internal defence |
| Report number | Comp. Biochem. Physiol. 78A (2): 271 275 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

**Executive Summary**

1. Bacteria were isolated from the coelomic fluid and medium of *Eisenia foetida* and the effect of the coelomic fluid on them assayed.

2 While a normal complement of coelomycetes appeared essential in preventing growth of bacteria, cell-free fluid inhibited some of the strains isolated, but most were not affected.

3 Injection of various strains into the coelomic cavity demonstrated that large numbers of *A. hydrophila* and *S. marcescens* led to septicaemia and death, but that low numbers (3 × 106) were not pathogenic providing coelomycetes were present.

4. *B. thuringiensis* (Heimpel EN‑1) do not appear to be pathogenic to *E. foetida*.

5. Injection of vaccines of formalized *A. hydrophila* and *B. thuringiensis* failed to induce lytic activity in the fluid.

**I MATERIALS AND METHODS**

**A MATERIALS**

|  |  |
| --- | --- |
| **1 Test Material:** | *Bacillus thuringiensis* var. *thuringiensis* Berliner (Heimpel EN‑1). |
| **Source:** | Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für biologische Schädlingsbekämpfung, Darmstadt, Germany. |
|  |  |
| **2 Test Animals -** | |
| **Species:** | *Eisenia foetida unicolor*. |
| **Age/growth stage:** | Not reported. |
| **Source:** | Stock cultures established at London University, originally derived from animals sourced from a single locality in Sussex, UK and from the College grounds in London. |
|  |  |

**B STUDY DESIGN**

**1 In-life dates:** Unknown, prior to September 1983.

**2 Treatments**Bacteria were isolated from the coelomic fluid and from the original medium of each strain of Eisenia. *B. thuringiensis* (Heimpel EN‑1) was obtained from the source indicated above. Other bacteria isolated both from worms and from the medium were identified by standard microscopical and staining methods and by use of API 20E reagents (Analytab) together with Bergey’s Manual. Samples of body fluid were obtained aseptically by swabbing the body wall with 70% alcohol following anaesthetization by immersion in 5% alcohol in earthworm saline (Hofftreter) and using sterile microcapillaries. Bacteria from body fluid and from the medium collected by means of sterile swabs were initially grown on yeast extract peptone or McConkey and isolated according to standard microbiological practice. Numbers of bacteria were estimated either by dilution plating or by dilution and counting on a haemocytometer slide after fixation in 5% formalin and using phase contrast optics.

Formalised bacteria were prepared from overnight (log phase) broths washed with saline and suspended in 3% formalin-saline. Vaccines were prepared by resuspension in sterile saline following repeated washing and centrifugation and numbers injected were estimated by nephelometry. Freeze-dried preparations of bacteria for use as substrates in tests for bacteriolytic action of the body fluid were prepared in earthworm saline and the optical density measured spectrophotometrically after 20 min. The growth of living bacteria in coelomic fluid was measured similarly. Growth of bacteria was also followed by addition of bacteria enumerated by direct counting as above, to 200 µL volumes of sterile, cell-free body fluid in Cooke U‑well microtitre plates.

Worms were depleted of cells and coelomic fluid by repeated stimulation (5 V) using a Grass Stimulator (Roch *et al.*, 1975). Cell-free fluid was obtained by centrifugation. Cell extracts were made by sonication. Sterile fluid was obtained by first centrifuging at 3000 rpm at room temperature followed by 20,000 g at 0°C for 15 min and/or filtration through membrane filters (0.2 µm pore). The effect of body fluid on different bacteria was also tested by the staining method of Speece (1964). The effect of coelomic fluid was assayed either by placing sterile 5 mm filter paper discs saturated with fluid or by addition of drops of fluid directly onto bacterial lawns grown on agar.

**3 Statistical analysis**  
None applied.

**II RESULTS AND DISCUSSION**

*Assay for bacteriolytic action of coelomic fluid*

Sterile filter paper discs saturated with coelomic fluid did not inhibit bacterial growth when incubated overnight on a bacterial lawn of *B. thuringiensis*.

The effect of coelomic fluid on different bacteria was also tested by direct application of single drops of centrifuged fluid onto bacterial lawns or microscope slides which were then stained after 1, 2 and 24 h incubation with alcian blue/basic fuchsin. The effect of the coelomycetes resuspended in sterile earthworm saline and sonicated cell extracts in saline were also tested against saline and hen egg white lysozyme (HEL; 250 i.u.) controls. Lysis of *Micrococcus lysodeikticus* NCTC 2665 by HEL provided a control for the method. *B. thuringiensis* was unaffected by coelomic fluid and was also not lysed by HEL. Outcomes are presented in the table below, with results for other, non-*B. thuringiensis* species included to show the responsiveness of the method.

**Table 10.7.5-1: Inhibition of various bacteria by coelomic fluid of *Eisenia foetida***

|  |  |  |  |
| --- | --- | --- | --- |
| **Bacterium** | **No of worms tested** | **No showing inhibition** | **mean diameter of inhibition zone (mm)** |
| *B. thuringiensis* | 12 | 0 | 0 |
| *B. megaterium* | 12 | 0 | 0 |
| *E. coli* | 99 | 31 | 7.6 |
| *Ps. aeruginosa* | 89 | 60 | 7.8 |
| *M. lysodeikticus* | 73 | 4 | 6.8 |
| *Ps. maltophilia* | 29 | 5 | 8.0 |
| *Kl. pneumoniae* | 32 | 0 | 0 |
| *Fl. odoratum* | 25 | 25 | 7.8 |
| *S. marcescens* | 25 | 25 | 8.5 |
| *Ac. calcoaceticus* | 25 | 25 | 8.7 |

**Table 10.7.5-2: Cumulative mortality (%) of normal and depleted *Eisenia foetida* following injection with various doses of *B. thuringiensis***

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **No of bacteria injected** | **Normal worms** | | **‘Depleted’ worms** | |
| **No of worms injected** | **Mortality (%) after 72 h** | **No of worms injected** | **Mortality (%) after 72 h** |
| 6 × 105 | 8 | 0 | - | - |
| 3 × 106 | 8 | 0 | - | - |
| 6 × 106 | 8 | 0 | 6 | 0 |
| 9 × 106 | 18 | 0 | 10 | 0 |

*Growth of bacteria in coelomic fluid in vivo*

Normal worms were injected with 10 µL of either earthworm saline or with coelomic fluid containing a known number of bacteria estimated by dilution/plating. Worms depleted of coelomic fluid and cells injected with 100 µL of body fluid containing a similar number of bacteria or with earthworm saline as controls to test mortality of initially normal worms compared to those depleted of their coelomycetes. Numbers of bacteria at 4, 24 and 48 h post-injected were estimated by dilution/plating. *B. thuringiensis* had no lethal effect even on depleted worms 72‑h post-injection.

*Effect of injection of formalized vaccines*

Formalized vaccines prepared from log‑phase *B. thuringiensis* were injected into normal worms. Fluid was pooled from 5 worms 24‑h post-injection and assayed for lytic activity using suspensions of formalized and lyophilized bacteria following the same method as for the lysozyme assay. No lytic activity was detected.

**III CONCLUSIONS**

The authors concluded that the coelomic phagocytes of earthworms constitute an important line of defence to bacteria which gain entry to the coelom. Although the lytic or static action of the coelomic fluid is weak it may nevertheless assist the worm to maintain the balance with the bacteria present in the coelom by promoting phagocytosis.

**RMS comment** (RAR, 2020 for *Bta* ABTS-1857): The paper by Çotuk and Dales (1984) indicates that coelomic fluids of earthworms protect them from infections from microorganisms such as *B. thuringiensis*. The study is considered reliable and relevant with restrictions because it concerns a different strain than the one being evaluated. The information from this study can be used as supporting information in the risk assessment.

**Study 2: Toxicity of crystalline toxins to *Eisenia fetida***

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted report was accepted as a supportive/additional data. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.5/02 |
| Author(s) (year) | Shu, Y., Ma, H., Du, Y., Li, Z., Feng, Y., and Wang, J. (2011) |
| Title | The presence of *Bacillus thuringiensis* *(Bt*) protein in earthworms *Eisenia fetida* has no deleterious effects on their growth and reproduction |
| Report number | Chemosphere 85: 1648-1656 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

**Executive Summary**

Earthworms *Eisenia fetida*, bred in substances with stover of two genetically engineered *Bacillus thuringiensis* (*Bt*) corn varieties (5422*Bt*1 (Event *Bt*11) and 5422CBCL (MON810)) expressing Cry1Ab and their near-isogenic non-*Bt* corn (5422) were used to investigate the non-target effects of *Bt*‑corn on soil-dwelling organisms. Cry1Ab concentrations in substances, casts and guts of *E. fetida* were also investigated by Enzyme-Linked Immunosorbent Assay (ELISA). More than 90% individuals of *E. fetida* survived over a period of 30 d, irrespective of whether they received *Bt* corn or non-*Bt* corn. Compared to 5422 treatments, significantly higher relative growth rate and a greater number of new offspring and cocoons of *E. fetida* were found in 5422*Bt*1 and 5422CBCL treatments. These results were unlikely to be directly caused byCry1Ab released from *Bt* corns but rather by differences in other factors of plants such as plant components (soluble sugar, total organic carbon, total protein and available phosphorus of *Bt* corns were more than 52422). ELISA results indicated immunoreactive Cry1Ab was detectable in substances under the corresponding experimental conditions. With the increase of treated time, a strong decline was observed in Cry1Ab from substances and casts of *E. fetida*, whereas Cry1Ab in guts of *E. fetida* from 5422*Bt*1 treatments gradually increased and that from 5422CBCL treatments increased between 14 and 30 d. Therefore, the presence of Cry1Ab in *E. fetida* had no deleterious effects on their growth and reproduction.

**I MATERIALS AND METHODS**

**A corn varieties**

|  |  |
| --- | --- |
| **1 *Bt* variety:** | Cry1Ab [5422Bt1 (Event Bt11)]. |
| **Source:** | Beck’s Hybrids, USA. |
|  |  |
| **2 *Bt* variety:** | Cry1Ab [5422CBCL (MON810)]. |
| **Source:** | Beck’s Hybrids, USA. |
|  |  |
| **3 Non-*Bt* variety:** | 5422 |
|  |  |
| **Cultivation and stover preparation** | Corn plants were cultivated at 40 cm spacing in three 3.0 × 3.4 m plots in a greenhouse. During the growth phase, plants were watered at 3 d intervals and were fertilised on five occasions with 20 g compound fertiliser by top spray application. Three weeks after pollen was shed, the stover, including leaves and stalks was cut into pieces (ca. 2‑4 cm), freeze-dried, ground and sieved through a 1 mm mesh. The plant material was stored at ‑20°C until use. |
| **B test SOIL** | |
| **Source:** | Natural soil collected from the upper 5‑25 cm in a field used for conventional sweet corn cultivation at the test facility (23°08’N, 113°15’E). |
| **Preparation:** | Air-dried at room temperature and sieved sequentially through 15 mm and 1 mm mesh. |
| **Classification:** | Clay loam. |
| **Characterisation:** | 18.20 g/kg organic matter, 0.31 g/kg total N, 0.87 g/kg total P, 32.28 g/kg total K, pH: 5.9. |
|  |  |
| **C test Organism** | |
| **Species, strain:** | *Eisenia fetida* Daping No. 2 |
| **Source:** | Breeding stock maintained at the test facility, established with animals obtained from Hollen Ecological Agricultural Company, Guangzhou, China. The earthworms were kept in a controlled climate chamber (25°C, 65% relative humidity, 24 h darkness) in the same soil as that used for the experiments and fed powdered stover from conventional field-grown corn. |
| **Age at test start:** | Worms selected for use in the test were ca 2-months old, with a clitellum and an average weight of 200 mg (range 180 – 220 mg). |
| **Preparation:** | Rinsed with distilled water and blotted dry on filter paper before allocation to test vessels. |

**D STUDY DESIGN**

**1 In-life dates:** Not reported. Tests were performed sequentially.

**2 Survival and growth assay**

Plastic cups (8.5 cm diameter, 9.5 cm deep) were filled with 200 g total substrate weight, comprising air-dried soil amended by incorporation with 5 g (2.5% of total weight), 10 g (5% of total weight) or 15 g (7.5% of total weight) of powdered stover from each corn variety. The water content of the treated soils was maintained at 30% of water holding capacity with distilled water. After introducing a single worm to each container, the vessels were covered with gauze to permit gas exchange. There were 30 replicates per concentration per corn variety.

Numbers of surviving earthworms were recorded on days 7, 14 and 30 by emptying the test vessel contents onto white plates and gently probing the earthworms to stimulate movement. Missing worms were assumed to have died and undergone autolysis. Survivors were rinsed to clean them, blotted dry and weighed, then returned together with the test substrate to their respective containers. The mortality counts and bodyweight data were used to determine survival and relative growth rates (RGR), respectively.

**3 Reproduction assay**

Plastic cups filled as described above each received a pair of earthworms at the start of the test, with 30 replicates per treatment and corn variety. At 30 d intervals the numbers of cocoons and juveniles were counted, and the juveniles removed before returning the live adults and cocoons to their respective vessels, refilled with freshly prepared substrate containing the appropriate concentration of ground corn stover.

**4 ELISA assay for Cry1Ab**

Worms were added singly to 30 replicate vessels per treatment and corn variety, as for the survival and growth assay described above. On days 7, 14 and 30 five worms per treatment were removed, washed with distilled water to remove external substrate, then dissected in phosphate-buffered saline (PBS). Guts were removed, washed three times in PBS and collected. Earthworm casts and ca. 2.0 g of the substrate contents of the vessels that had housed the sacrificed worms were also collected. All samples were frozen immediately in liquid nitrogen and stored at ‑80°C prior to Cry1Ab extraction. All Cry1Ab concentrations were expressed as ng Cry1Ab/g dry weight.

**5 Analysis of stover composition**

Triplicate samples of the stover of each corn variety were analysed to determine soluble sugar (enthrone-sulphuric acid colorimetric assay), total protein (trichloroacetic acid-acetone extraction), total organic C (rapid titration), total N (micro Kjeldahl), available P (Bray and Krutz method) and total K (flame emission).

**6 Statistical analysis**

Statistical analysis was performed using SPSS software (Version 13.0, SPSS, USA). A 5% significance level was applied in all tests. Survival rates, RGRs, Cry1Ab in test substrates, casts and guts of earthworms were analysed by a repeated measure of generalised linear model. One-way ANOVA was performed to test for significances among three corn varieties in relation to the following parameters: chemical composition, Cry1Ab content of corn stover, earthworm survival rate and reproduction. Significances of RGR and Cry1Ab concentrations in the test substrates and the casts and guts of earthworms at three timepoints during the experiment were also analysed by one-way ANOVA, and ANOVA was used to determine the differences in RGR and Cry1Ab concentrations in substrates, casts and guts of earthworms among treatments (stover of three corn varieties applied at different concentrations at the same time point). Survival and RGR data were arcsine square-root transformed before analysis. Other data were log-transformed to maintain homogeneous variances.

**II RESULTS AND DISCUSSION**

**A Survival**

No significant differences were found in the survival rate of *E. fetida* between the *Bt* and non‑*Bt* corn treatments and survival after 30 days exposure exceeded 90% regardless of *Bt* exposure. One-way ANOVA showed no significant difference between the three stover incorporation rates (5, 10 and 15 g/kg soil) of each corn hybrid and the same incorporation rates were therefore suitable for subsequent tests.

**B relative growth rate (rgr)**

RGR declined over the course of the experiment, in the non‑*Bt* as well as the *Bt*‑corn treatments. The RGRs from all *Bt* treatments were significantly higher than that of the corresponding non‑*Bt* corn treatments at contemporary time points. The RGR of earthworms of the non‑*Bt* treatments increased significantly in response to the stover incorporation rate at contemporary time points. The differences in RGR between the three stover treatment rates were generally significant. Over the duration of the experiment there were no significant differences between the 5422*Bt*1 and 5422CBCL treatments at the same stover concentrations and time points.

**C reproduction**

The production of juveniles and cocoons in the substrates treated with *Bt*‑corn stover was significantly higher than in the corresponding non‑*Bt* 5422 substrates in both 30‑day phases of the reproduction assay. The results of the reproduction assay indicated a consistent trend for the three corn varieties, whereby the numbers of juveniles and cocoons increased significantly in response to the rate of stover incorporation into the test soil. The differences between the *Bt* and non‑*Bt* treatments were attributed to differences in the chemical composition of the various sources of corn stover, rather than exposure to Cry1Ab.

**d cry1ab in the test substrates**

The concentration of Cry1Ab was higher in the *Bt*‑corn treated substrates than in the corresponding non‑*Bt* treatments. Cry1Ab concentrations in both *Bt*‑corn variety treatments gradually declined over time. The Cry1Ab in the 5422CBCL treatments was lower than in the equivalent 5422*Bt*1-amended substrates at corresponding timepoints.

**e cry1ab in *e. fetida* casts**

Earthworm casts of the 5422 treatments registered extremely low levels of immunoreactive Cry1Ab and higher levels were detected in casts of earthworms of the *Bt*-corn treatments. Levels generally declined over time in the *Bt*‑corn treatments, apart from the lowest treatments rate. Earthworms were able to excrete some ingested Cry1Ab via casts and Cry1Ab in casts was closely related to the stover incorporation rate as well as transformation events as a function of exposure time.

**f cry1ab in *e. fetida* guts**

ELISA results showed that *E. fetida* could store Cr1Ab in the gut. Cry1Ab gut concentrations were generally lower in the 5422CBCL treatments than in the corresponding 5422*Bt*1 treatments (corresponding to the relative levels in the test substrates) and whereas gut concentrations increased gradually over time in the worms exposed to 5422*Bt*1, the increase was limited to the period between days 14 and 30 in the 5422CBCL-exposed groups. These findings suggested that Cry1Ab may have the potential to accumulate in the guts of earthworms.

**G Transfer of cry1ab from *bt* corn to *e. fetida***

Earthworms in substrates emended with incorporated *Bt*-corn were able to absorb Cry1Ab by feeding and excreted Cry1Ab via casts. Concentrations of Cry1Ab in earthworm guts and casts were consistently lower than in the amended soil substrates.

**h chemical composition of corn stover**

There were no significant differences between the three corn varieties in terms of concentrations of soluble sugar, total organic C, and available P, although the concentrations of these were higher in the two *Bt* varieties than in the non‑*Bt* 5422. The total protein content of the two *Bt*‑corn varieties was significantly higher than in 5422. Total N and P concentrations were both significantly higher in 5422 than in the two *Bt*‑varieties. The Cry1Ab content of 5422*Bt*1 (Event *Bt*11) and 5422CBCL (MON810) determined by ELISA were 269.0 ± 4.0 ng Cry1Ab/g dw and 209.0 ± 6.0 ng Cry1Ab/g dw, respectively, and did not differ significantly.

**III CONCLUSIONS**

In this laboratory study, *E. fetida* were exposed in a natural field soil to stover meal sourced from two varieties of transgenic *Bt* corn (5422*B*t1 (Event *Bt*11) and 5422CBCL (MON810)) expressing Cry1Ab and the near-isogenic non‑*Bt* 5422, all incorporated at concentrations of 5, 10 and 15 g/kg soil. Assays were undertaken to determine effects on survival, growth and reproduction, as well as to determine the fate of Cry1Ab in the test substrate and earthworm casts.

No significant differences were found in the survival rate of *E. fetida* in a field soil amended with incorporated *Bt* corn meal (two varieties) and corresponding non‑*Bt* corn treatments. To some extent earthworm growth and reproduction in the soils amended with *Bt*‑corn meal exceeded those recorded in the analogous non‑*Bt* treatments, however there were also differences in earthworm life history parameters between the 5422CBCL (MON810) and 5422*Bt*1 (Event *Bt*11) *Bt* corn varieties. These differences are unlikely to have been caused directly by exposure to Cry1Ab, but rather by variations in other factors such as the composition of the plant material: soluble sugar, total organic C, total protein and available P were all present at higher levels in *Bt* corn than in the non‑*Bt* 5422.

A significant decline occurred over time in the concentration of Cry1Ab present in the amended soil test substrates and in the *E. fetida* casts, whereas Cry1Ab concentrations increased in the earthworm gut throughout the test duration in the case of 5422*Bt*1 and between days 14 and 30 for 5422CBCL.

**Study 3: Toxicity of crystalline proteins to earthworms**

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted report was accepted as a supportive/additional data. |

|  |  |
| --- | --- |
| Data point addressed | IIIM 10.5/03 |
| Author(s) (year) | Zeilinger, A.R., Andow, D.A., Zwahlen, C., and Stotzky, G. (2010) |
| Title | Earthworm populations in a northern U.S. Cornbelt soil are not affected by long-term cultivation of *Bt* maize expressing Cry1Ab and Cry3Bb1 proteins |
| Report number | Soil Biology & Biochemistry 42: 1284-1292 |
| Test facility | Not applicable |
| Published | Yes |
| Test guideline | Not applicable |
| Deviations | Not applicable |
| GLP | No |
| Acceptability | Acceptable |

**Executive Summary**

Earthworms, which play a key role in biogeochemical processes in soil ecosystems, could be negatively affected by the cultivation of transgenic *Bt* crops. Studies to date have found few effects of *Bt* maize on earthworm species. If adverse effects occur, they are likely to be chronic or sub-lethal and expressed over large spatial and temporal scales. The present multiple-year field study sought to investigate potential effects on earthworm populations in soil cultivated with *Bt* maize. Earthworm populations were surveyed in 0.16 ha experimental field plots of two varieties of Cry1Ab *Bt* maize, one variety of Cry3Bb1 *Bt* maize, and three non-transgenic control varieties cultivated for four years. Four earthworm species were found: *Aporrectodea caliginosa*, *Aporrectodea trapezoides*, *Aporrectodea tuberculata* (collectively the *Aporrectodea* species complex), and *Lumbricus terrestris*. No significant differences were found in the biomass of juveniles and adults for all four species between *Bt* and non-*Bt* maize varieties. From this and previous studies it was concluded that the effects of Cry1Ab and Cry3Bb1 *Bt* maize on the *A. caliginosa* species complex and *L. terrestris* are small.

**I MATERIALS AND METHODS**

**A test Maize varieties**

|  |  |
| --- | --- |
| **1 *Bt* variety:** | Cry1Ab Novartis N45‑A6 (*Bt*11). |
| **Target organism:** | European corn borer, *Ostrinia nubilalis* Hübner [Lepidoptera: Crambidae]. |
| **Seed treatment:** | None. |
|  |  |
| **2 *Bt* variety:** | Cry1Ab Pioneer 38A25 (MON810). |
| **Target organism:** | European corn borer, *Ostrinia nubilalis* Hübner [Lepidoptera: Crambidae]. |
| **Seed treatment:** | None. |
|  |  |
| **3 *Bt* variety:** | Cry3Bb1 DeKalb DKC46‑24 (MON863) (Year 1);  Cry3Bb1 DeKalb DKC46‑23 (Years 2‑4). |
| **Target organism:** | 3 root worm species, *Diabrotica* spp. [Coleoptera: Chrysomellidae]. |
| **Seed treatment:** | Poncho 250® (clothianidin a.s.) and Gaucho ® (imidacloprid a.s.) insecticide coatings for DKC46‑24 and DKC46‑23, respectively. |
|  |  |
| **4 Non-*Bt* variety:** | Novartis N45‑T6 (*Bt*11) |
| **Seed treatment:** | None. |
|  |  |
| **5 Non-*Bt* variety:** | Pioneer 38A24 |
| **Seed treatment:** | None. |
| **6 Non-*Bt* variety:** | DeKalb DKC46‑28 (Year 1) DeKalb DKC46‑26 (Years 2-4) |
| **Seed treatment:** | Poncho 250® (clothianidin a.s.) and Gaucho ® (imidacloprid a.s.) insecticide coatings for DKC46‑24 and DKC46‑23, respectively. |
|  |  |

**B STUDY DESIGN**

**1 In-life dates:** Four consecutive years: 2003 to 2006

**2 Test design**

The purpose of the study was to investigate potential changes in populations of naturally occurring earthworm species in soil cultivated with two Cry1Ab *Bt* maize varieties (*Bt*11 and MON810) and one Cry3Bb1 *Bt* maize variety (MON863). The authors hypothesized that adverse effects on earthworms resulting from the cultivation of *Bt* maize would reduce earthworm population biomass by one or more direct or indirect sub-lethal effect pathways, and that the influence of such effects should be easier to detect in long-term field studies than in laboratory tests.

The study was conducted in an experimental field in Rosemount, Minnesota, USA (44°43’12” N, 93°06’54” W). Six maize varieties were sown in 40 m × 40 m plots in a complete randomized block design with four replicates, i.e., a total of 24 plots. Plots were separated by a 20 m non‑*Bt* maize buffer. The same varieties were sown in the same plots for four growing seasons, except that the Cry3Bb1 maize variety sown in 2003 became commercially unavailable and was substituted, together with the corresponding near-isogenic non-*Bt* control in each of the three following years. The maize was sown using standard reduced tillage agricultural practices and only the kernels were harvested, leaving large quantities of crop stover on the soil after harvest.

**3 Earthworm sampling**

Sampling was conducted prior to harvest over 2 days in late October in both 2005 and 2006. Sampling used the mustard extraction technique: 10 g mustard powder dispersed in 3.8 L water was poured over a 0.13 m2 area of cleared soil enclosed within a 7.6 cm tall aluminium frame inserted 2.5 cm into the soil. The mustard dispersion was applied one third at a time, allowing 5 min intervals between applications to allow time to infiltrate the soil and to retrieve emerged earthworms. Collected worms were killed and stabilised for long-term preservation. Biomass is considered a more accurate measure of earthworm population size than abundance or density. Based on literature reports, the mustard extraction procedure gave higher or similar estimates of biomass than digging and hand-sorting for anecic earthworm species and comparable estimates for endogeic worms. Overall, it was considered to correlate well with biomass determined by digging and hand-sorting and to be suitable for the needs of this study.

4 Statistical analysis

The data were analysed for differences between maize varieties, sample location (within or between maize rows) and year in a randomised complete block fractional split-split plot design. The whole plots were the six maize varieties, the split plots were the location of the sample within plots (within or between rows) and the split-split plots were the years within location. A fractional design was used because between-row samples were not taken in 2006 and consequently the year by location interaction term could not be estimated. The maize variety, variety by location interaction and variety by year interaction effects were further investigated with planned orthogonal contrasts. These contrasts compared Cry1Ab varieties against their isolines, Cry3Bb1 variety against its isoline and the two Cry1Ab-isoline pairs against the Cry3Bb1-isoline pair. the contrasts of the interaction terms, variety by location and variety by year, facilitated the isolation of interactions of biologically relevant combinations of varieties. In total 81 contrasts were analysed for the variety main effect, variety by location interaction and variety by year interaction for all nine response variables; of these five were significant (6.2% experiment-wise error rate).

Differences in nine response (dependent) variables were tested. Because adult earthworms were identified to species whereas juveniles were identified only to genus, biomass was analysed separately for adults and juveniles. Six variables were chosen to examine adult biomass: *A. caliginosa* biomass, *A. trapeziodes* biomass, *A. tuberculata* biomass, *Aporrectoidea* spp. total adult biomass, *L. terrestris* biomass and total adult biomass. Three variables were used for juveniles: *Aporrectoidea* juvenile biomass, *Lumbricus* spp. juvenile biomass and total juvenile biomass. All earthworm biomass data were analysed as calculated from the allometric equation. To meet the assumptions of normality and constant error variance for ANOVA the inverse transformation was applied to all the adult biomass variables and natural log transformation was used for all juvenile biomass variables.

A multivariate analysis using six of the response variables (*A. caliginosa* biomass, *A. trapeziodes* biomass, *A. tuberculata* biomass, *L. terrestris* biomass, *Lumbricus* spp. juvenile biomass and *Aporrectoidea* juvenile biomass) was used to test for relationships between the dependent variables, *i.e.,* between earthworm species and genera in the samples. No correlations were found between response variables. All data were analysed using the gLM procedure from the SAS 9.1 statistical package (SAS, 2004).

**II RESULTS AND DISCUSSION**

**A earthworm community**

A total of 1276 earthworms (17.04 individuals/m2) was collected over all sampling dates. Adult earthworms comprised 51.3% of the total earthworm biomass, with juveniles comprising the remainder. Four species were found: the anecic *L. terrestris* (85.8% of total adult biomass) and the endogeic *A. caliginosa* (6.4%), *A. tuberculata* (5.6%) and *A. trapezoides* (2.2%). *Lumbricus* spp. juveniles comprised 57.2% of the total juvenile biomass collected and *Aporrectodea* comprised the remainder. The collections made in 2005 and 2006 contributed 46.4% and 53.6% of the total earthworm biomass, respectively.

**B Biomass of adult earthworms**

No significant differences in adult biomass were found for any of the six response variables among maize varieties across years and sample locations. No significant differences were found in any of the planned comparisons: Cry1Ab varieties versus their isolines, Cry3Bb1 variety versus its isoline and the two CryAb1-isoline pairs against the Cry3Bb1-isoline pair. The result of the third contrast implies that no effect from the neonicotinoid seed treatment was detected.

Biomass of adult earthworms was generally higher in samples taken inside maize rows than between maize rows for all adult response variables. There were no significant differences between the sample locations in adult response variables, except for total adult biomass.

**C biomass of juvenile earthworms**

No significant differences were found in the three juvenile response variables for the maize variety main effect or for any of the associated comparisons. As for adult earthworms, the lack of statistical significance of the third contrast implies that no effect of the neonicotinoid seed treatment was detected.

There was significantly more biomass of *Aporrectoidea* spp. juveniles, *Lumbricus* spp. juveniles and total juveniles within maize rows than between maize rows.

None of the juvenile biomass response variables was significantly different for the maize variety by simple location interaction, but some significant interaction contrasts were found. First, inside the maize rows, juvenile biomass of *Aporrectoidea* spp. was greater in Cry3Bb1 maize relative to its non-*Bt* isoline. In contrast, between rows, biomass was lower in the Cry3Bb1 maize plots than in the non‑*Bt* isoline. Second, within rows, juvenile biomass of *Lumbricus* spp. was greater in the Cry3Bb1-isoline pairs than in the Cry1Ab-isoline pairs, whereas between rows it was lower in the Cry3Bb1-isoline plots.

**III CONCLUSIONS**

The purpose of the study was to investigate potential changes in populations of naturally occurring earthworm species in soil cultivated with two Cry1Ab *Bt* maize varieties (*Bt*11 and MON810) and one Cry3Bb1 *Bt* maize variety (MON863). No differences were found in adult or juvenile earthworm biomass between *Bt* maize and the non‑*Bt* isolines for any of the four earthworm species detected. Five comparisons of the interactions of maize variety by location and maize variety by year were statistically significant, but no biologically significant patterns could be linked to them. Significantly higher population densities were recorded within rows than between rows for total adult biomass and all three juvenile biomass responses. Higher earthworm populations within rows may have resulted from lower soil bulk density, higher biological activity or a combination of both, associated with the maize rhizosphere and the absence of compaction that occurred in the wheel tracks between rows. The lack of statistically significant differences in adult biomass may have been due to greater mobility, lower population density and/or greater tolerance of higher soil bulk density compared with juveniles. The results obtained support the findings of a majority of previous studies of the impacts of *Bt* maize on *L. terrestris* and the *A. caliginosa* species complex, which found no effect from *Bt* maize varieties. A prospective power analysis of the data collected in 2005 indicated that the sample size during the following year should have been sufficient to detect a significant difference in earthworm population due to maize variety.

Based on the geographic distribution ranges of the earthworm species encountered in these trials it was concluded that the risks to *L. terrestris* and the A*. caliginosa* complex from exposure to Cry1Ab and CryBb1 *Bt* maize in temperate North America and Europe are small.

**IIIM 10.7.6 Effects on microorganisms**

No additional studies available.

IIIM 11 Summary and evaluation of environmental impact

IIIM 11.1 Distribution and fate of the MPCA

For details on distribution and fate of *Bacillus thuringiensis* subsp. *kurstaki* strainABTS-351in relevant environmental compartments refer to Part B Section 5 (i.e., Environmental Fate).

**IIIM 11.2 Identification of non-target species at risk and extent of their exposure**

According to the presented risk assessment, the use of Foray® 76B at the proposed label rates according to good agricultural practice poses no risk to any of the terrestrial or aquatic non-target species.

**Terrestrial vertebrates**

*Bacillus thuringiensis* subsp*. kurstaki* strain ABTS-351 showed no treatment-related mortalities or signs of pathogenicity in birds over 30 days following 5-d oral exposure to 2857 mg MPCA/kg bw/d (equivalent to approx. 5.7 × 1010 CFU/kg bw/d), and no evidence is available indicating that *Btk* ABTS-351 is infective in birds. Likewise, *Btk* ABTS-351 showed no treatment-related adverse effects, pathogenicity or infectivity in rats following oral exposure to the MPCA. The highest limit endpoint in the studies on rats was established as LD50 > 426 mg MPCA/kg bw. The risk from *Btk* ABTS-351 and CryP to terrestrial vertebrates following the proposed uses of Foray® 76B is considered acceptable, since (1) the quantitative risk assessment based on worst-case assumptions resulted in margins of safety between > 3.0 and > 6626 for the risk through dietary uptake and drinking water, (2) the gastrointestinal tract of terrestrial vertebrates does not provide optimum growth conditions for *Btk* ABTS-351, (3) *Btk* ABTS-351 is rapidly degraded on foliage, and (4) there is no evidence that *Btk* ABTS-351 or CryP exhibit toxicity, pathogenicity or infectivity in terrestrial vertebrates.

**Aquatic organisms**

The risk from *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 to aquatic organisms following application of Foray® 76B was evaluated based on relevant endpoints from available laboratory studies and the Predicted Environmental Densities in surface water (i.e., PEDSW). PEDSW were calculated based on a series of worst-case assumptions (i.e., total seasonal dose was used as a single application assuming no degradation and 0% crop interception), Rautmann spray drift values, and a default distance to water bodies of 3 m. The risk from *Btk* ABTS-351 to aquatic organisms was assessed for application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) (i.e., PEDSW = 1.87 × 107 CFU/L).

**Fish**

The risk from *Btk* ABTS-351 to fish was quantitatively assessed based on a 32-d NOEC value of > 2.87 × 109 CFU/L for *Oncorhynchus mykiss* and *Lepomis macrochirus*. A margin of safety (MoS) of ≥ 153 was calculated indicating an acceptable risk to fish from all proposed uses of Foray® 76B. Available studies show that *Btk* ABTS-351 does not exhibit pathogenicity and infectivity in fish and that toxicity of CryP to fish is unlikely. Therefore, the risk posed by *Btk* ABTS-351 and CryP to fish is low for all proposed uses of Foray® 76B.

**Aquatic invertebrates**

The risk from *Btk* ABTS-351 to aquatic invertebrates was quantitatively assessed based on the lowest EC50 for available laboratory studies (i.e., EC50 of 2.3 x 108 CFU/L for *D. magna*) and the EU agreed endpoint for aquatic invertebrates (i.e., > 1.0 x 109 CFU/L). MoS were calculated as 12 and > 53 indicating an acceptable risk to aquatic invertebrates for all proposed uses of Foray® 76B. No adverse effects attributable to the biological activity of *Btk* ABTS-351 and no signs of pathogenicity of infectivity were observed in the available laboratory studies with *D. manga*, *A. minutus* and *P. vulgaris*. A series of open literature studies with freshwater and marine invertebrates corroborated these findings, and, in addition, provided no evidence for potential toxicity of CryP to aquatic invertebrates. Therefore, the risk posed by *Btk* ABTS-351 and CryP to aquatic invertebrates is low for all proposed uses of Foray® 76B.

**Algae and aquatic plants**

The risk from *Btk* ABTS-351 to algae was quantitatively assessed based on the 72 h EC50 of 5.94 × 108 CFU/L from a laboratory study. An MoS of 32 was calculated indicating an acceptable risk for all proposed uses of Foray® 76B. In addition, *Btk* ABTS-351 is unlikely to exhibit pathogenicity or infectivity in algae and other aquatic plants due to its highly specific insecticidal mode of action. Likewise, CryP are unlikely to have adverse effects on algae or aquatic plants, which was also confirmed in an open literature study on the microbial activity of *Bacillus thuringiensis* subspp. *kurstaki* on bacteria, fungi and algae. Therefore, the risk from *Btk* ABTS-351 and CryP to algae and other aquatic plants is low for all proposed uses of Foray® 76B.

**Bees**

Laboratory studies are available showing that *Btk* ABTS-351 has no adverse effects on mortality or behaviour of adult honey bees and honey bee larvae following oral and contact exposure. In addition, open literature studies are available providing evidence that *Btk* ABTS-351 and CryP have no adverse effects on bees. In particular, an open literature study is available (Mommaerts *et al*., 2009) which shows no signs of pathogenicity and toxicity of *Btk* ABTS-351 and CryP in bumble bees following oral and contact exposure to DiPel over 11 weeks under unrealistic worst-case exposure conditions. Therefore, the weight of evidence indicates a low risk from *Btk* ABTS-351 and associated CryPs to bees following the proposed uses of Foray® 76B, based on the absence of toxicity and pathogenicity in the available studies, the highly specific MoA of *Btk* ABTS-351 against insect species of the order Lepidoptera, and rapid degradation of *Btk* ABTS-351 and associated CryPs under environmental conditions following spray application of Foray® 76B.

**Other non-target arthropods**

The in-field risk (covers also off-field risk) from *Btk* ABTS-351 to non-target arthropods other than bees was quantitatively assessed for all proposed uses of Foray® 76B by calculating the MoS based on maximum single application rates and effect data (i.e., EC50) from the available laboratory studies. An MoS of > 3.61 was calculated for all proposed uses of Foray® 76B based on endpoints available for *A. rhopalosiphi* and *T. pyri*. Risk quotients for *M. occidentalis* and *T. urticae* were calculated to be < 1.0 for undiluted uses Foray® 76B and MoS above 1.0 were calculated for all applications of diluted Foray® 76B based on a minimum water volume of 60 L/ha. However, since all endpoints from the available effect studies with *A. rhopalosiphi*, *T. pyri*, *M. occidentalis*, and *T. urticae* were limit endpoints, calculated risk quotients generally overestimate the risk from *Btk* ABTS-351 to non-target arthropods. Available laboratory and literature studies provide evidence that *Btk* ABTS-351 does not exhibit pathogenicity or infectivity in non-target arthropods of various insect orders. Likewise, the available data package suggests low toxicity of CryP to insects of various insect orders such as Parasitiformes, Trombidiformes, Neuroptera, Orthoptera, Dermaptera, Hemiptera, Coleoptera, and Diptera. Adverse effects have been described only for test species of the orders Hymenoptera and Lepidoptera. However, the observed adverse effects on Hymenoptera resulted from exposure to high levels of *Btk* ABTS-351 that would not be expected under realistic conditions. In addition, exposure of lepidopteran species to *Btk* ABTS-351 following the proposed uses of Foray® 76B is expected to occur only during active feeding on treated vegetation, and lepidopteran species are expected to recover quickly due to multiple life cycles per year.

Since (1) *Btk* ABTS-351 and CryPs are quickly degraded on foliage and are not expected to accumulate to high levels in the environment, (2) the quantitative risk assessment indicates an acceptable risk from all proposed uses of Foray® 76B to non-target arthropods based on endpoints for *A. rhopalosiphi* and *T. pyri* with high MoS, (3) no effects > 50% were observed in the available laboratory studies with *C. carnea*, *M. occidentalis* and *T. urticae*, (4) *Btk* ABTS-351 has a highly specific MoA against insect species of the order Lepidoptera, (5) open literature studies do not provide evidence that *Btk* ABTS-351 is pathogenic or infective in non-target arthropods (orders other than Lepidoptera) or that toxicity of CryP to arthropods other than the target pest is likely, and (6) non-target lepidopteran species are expected to recover quickly due to multiple life cycles per year, the risk from *Btk* ABTS-351 and CryP to non-target arthropods (incl. beneficial arthropods) is considered acceptable for all proposed uses of Foray® 76B. This is in line with the RMS’ conclusions during the previous EU Renewal (RAR, 2020 Vol. 3 B.9), where *Btk* ABTS-351 was considered as not toxic, not pathogenic and not infective to arthropods other than target pests, albeit EFSA concluded that available information was not sufficient to address the potential infectivity and pathogenicity of *Btk* ABTS-351 in non-target arthropods.

**Earthworms**

The risk from *Btk* ABTS-351 to earthworms was quantitatively assessed for the risk envelope application of 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens) by calculating the MoS as ratio of the available effect data (i.e., 30-d NOEL of ≥ 1.1 × 1010 CFU/kg dry soil) and the maximum Predicted Environmental Density in soil (i.e., PEDsoil of 2.26 × 108 CFU/kg dry soil). A MoS of > 48.7 was calculated indicating a low risk from *Btk* ABTS-351 to earthworms for all proposed uses of Foray® 76B. In addition, *Btk* ABTS-351 showed no signs of toxicity, pathogenicity or infectivity in the available laboratory and open literature studies with earthworms, which is in line with the fact that earthworms are well adapted to ubiquitous soilborne bacteria such as *B. thuringiensis* and are equipped with adequate immune systems to cope with microorganisms. Furthermore, *Btk* ABTS-351 and CryP are not expected to multiply or accumulate to high levels in soil. Therefore, the risk from *Btk* ABTS-351 and CryP to earthworms following the proposed uses of Foray® 76B is low, which is in line with conclusions for DiPel® DF in EFSA Journal 2021;19(10):6879.

**Soil microbial activity**

The risk from *Btk* ABTS-351 to soil microorganisms was quantitatively assessed for 4 x 2.5 L Foray® 76B/ha on deciduous and coniferous forest, pine trees, ornamental trees and shrubs or amenity areas (parks, gardens). A risk quotient of 0.63 was calculated as ratio of the available effect data (i.e., 8-wk NOEL of 1.42 × 108 CFU/kg dry soil) and the maximum Predicted Environmental Density in soil (i.e., PEDsoil of 2.26 × 108 CFU/kg dry soil). However, as the quantitative risk assessment is based on worst-case exposure estimates (e.g., yearly total dose application as one single application assuming no decline of *Btk* ABTS-351 in soil), the calculated risk quotient overestimates the potential risk from *Btk* ABTS-351 to soil microorganisms. In addition, tests on adverse effects of MPCA on soil microflora activity are of limited relevance, while natural microbial communities in soil are well adapted to their habitat, show good resilience and recovery potential. Moreover, *Btk* ABTS-351 is an ubiquitous soilborne microorganism that is not expected to accumulate to high levels in soil following application of Foray® 76B. Therefore, the risk from *Btk* ABTS-351 and CryP to microorganisms from the proposed uses of Foray® 76B is low, which is in line with conclusions for DiPel® DF in EFSA Journal 2021;19(10):6879.

**IIIM 11.3 Precautions necessary to minimise environmental contamination and to protect non-target species**

Precautions to minimise environmental contamination and to protect non-target species are not considered necessary.

**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 10.3/01 | Vergé, E | 2010a | Foray 76B Acute Oral Toxicity to the Honeybee *Apis* *mellifera* L. in the Laboratory.  Eurofins Agroscience Services GmbH, Germany  Report No.: S09-03398  GLP: Yes  Unpublished | N | XXXX |
| IIIM 10.3/02 | Vergé, E | 2010b | Foray 76B – Acute Contact Toxicity to the Honeybee *Apis mellifera* L. in the Laboratory.  Eurofins Agroscience Services GmbH, Germany  Report No.: S10-02724  GLP: Yes  Unpublished | N | XXXX |
| IIIM 10.3/03 | Leza, M.M., Llado, G., Petro, A.B., and Alemany, A. | 2014 | First field assessment of *Bacillus thuringiensis* subsp. *kurstaki* aerial application on the colony performance of *Apis mellifera* L. (Hymenoptera: Apidae.  Spanish Journal of Agricultural Research 2014 12(2): 405-408  GLP: No  Published | N | Open literature |
| IIIM 10.3/04 | Rose, R., Dively, G. P., and Pettis, J. | 2007 | Effects of *Bt* corn pollen on honey bees: Emphasis on protocol development.  Apidologie, 38(4), 368-377.  GLP: No  Published | N | Open literature |
| IIIM 10.3/05 | Malone, L. | 2004 | Potential effects of GM crops on honey bee health.  Bee World, 85(2), 29-36.  GLP: No  Published | N | Open literature |
| IIIM 10.3/06 | O'Callaghan, M., Glare, T. R., Burgess, E. P., and Malone, L. A. | 2005 | Effects of plants genetically modified for insect resistance on nontarget organisms.  Annu. Rev. Entomol., 50, 271-292.  GLP: No  Published | N | Open literature |
| IIIM 10.4/01 | Bai, Y. Y., Jiang, M. X., Cheng, J. A., and Wang, D. | 2006 | Effects of Cry1Ab Toxin on *Propylea japonica* (Thunberg) (Coleoptera: Coccinellidae) Through Its Prey, *Nilaparvata lugens* Stål (Homoptera: Delphacidae), Feeding on Transgenic *Bt* Rice.  Environmental Entomology, 35(4), 1130-1136  GLP: No  Published | N | Open literature |
| IIIM 10.4/02 | Sisterson, M. S., Biggs, R. W., Olson, C., Carrière, Y., Dennehy, T. J., and Tabashnik, B. E. | 2004 | Arthropod Abundance and Diversity in *Bt* and Non-*Bt* Cotton Fields.  Environmental Entomology, 33(4), 921-929.  GLP: No  Published | N | Open literature |
| IIIM 10.4/03 | Truter, J., Van Hamburg, H., and Van Den Berg, J. | 2014 | Comparative Diversity of Arthropods on *Bt* Maize and Non-*Bt* Maize in two Different Cropping Systems in South Africa.  Environ. Entomol. 43(1): 197-208 (2014).  GLP: No  Published | N | Open literature |
| IIM 10.5/01 | Çotuk, A. and Dales, R.P. | 1984 | The effect of the coelomic fluid of the earthworm *Eisenia foetida* Sav. on certain bacteria and the role of the coelomycetes in internal defence.  Comp. Biochem. Physiol. 78A (2): 271 275.  GLP: No  Published | N | Open literature |
| IIIM 10.5/02 | Shu, Y., Ma, H., Du, Y., Li, Z., Feng, Y. and Wang, J. | 2011 | The presence of *Bacillus thuringiensis* (*Bt*) protein in earthworms *Eisenia fetida* has no deleterious effects on their growth and reproduction.  Chemosphere 85: 1648-1656.  GLP: No  Published | N | Open literature |
| IIM 10.5/03 | Zeilinger, A.R., Andow, D.A., Zwahlen, C., and Stotzky, G. | 2010 | Earthworm populations in a northern U.S. Cornbelt soil are not affected by long-term cultivation of *Bt* maize expressing Cry1Ab and Cry3Bb1 proteins.  Soil Biology & Biochemistry 42: 1284-1292  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 |

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| --- | --- | --- | --- |
| **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 |

1. PLoS One, 7(3), e33687; Latin American Journal of Aquatic Research, 46(5), 1083-1090; Marine Ecology Progress Series, 118(1), 37-42; Journal of Ichthyology, 55, 251-258 [↑](#footnote-ref-2)
2. Ctgb, 2022. Evaluation Manual for the Authorisation of Biopesticides according to Reg. (EC) No 1107/2009, Part I: Microorganisms, Version 2.0, December 2022. [↑](#footnote-ref-3)
3. Flores JM, Ruíz JA, Ruz JM, Puerta F, Campano F, Padilla F, Bustos M, 1998. Cría controlada de abejas reinas de

   *Apis mellifera ibérica*. Arch Zootec 47: 347-350. [↑](#footnote-ref-4)