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DATA EVALUATION REPORT

STUDY TYPE: Acute Toxicity Study- Earthworm (OECD Guideline 207)

CITATION: Hoxter, K.A., S.J. Palmer, H.O. Kreuger. *Bacillus thuringiensis* Protein 11231: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate. Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Proj. No.139-443A. Monsanto Study No. WL-99-013, July 30, 1999. MRID 449043-16**.**

DP BARCODE: D262045 **CASE**:066221
REG./FILE#:000524-LRA **CHEMICAL/BIOL#**: 006482 Cry3Bb (Vector ZMIR14L)

COMPANY/SPONSOR: Monsanto Co., Regulatory Sciences, 700 Chesterfield Pkwy North, St. Louis, MO 63198

TEST MATERIALS: The test substance was Cry3Bb1 protein (also known as *B.t.* protein 11231; or CryIIIB2, Cry3B2 or CryIIIC). The negative control was a buffer salt; the positive control was chloroacetamide.

REVIEW CONCLUSION: The study was procedurally sound and the data show that no adverse effects to earthworms are expected at ten times the maximum environmental concentration of Cry3Bb1 protein.

ADEQUACY OF STUDY:

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA Guideline requirements for assessing acute pesticidal risks to earthworms.

MATERIALS & METHODS: The methods used in conducting the study followed procedures specified in the Organization for Economic Cooperation and Development (OECD) Guideline 207.

The test material, consisted of purified Cry3Bb1 protein in purified water suspension as prepared by the Sponsor. The frozen liquid test substance was received from Monsanto Company and was identified as: Cry3B2.11231; Lot no. 6312812; and reported purity of 96 %. The reported activity was 16.8 mg active protein/mL water (adjusted for purity). The test substance was assigned Wildlife International Ltd. identification number 4799, and frozen at -80° C. Cry3Bb1 is the current accepted designation for the nomenclature of this insecticidal protein (Crickmore, N., et al. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62:807-13.)

A buffer salt was used in preparing and isolating the test substance. It was received from Monsanto and assigned Wildlife International Ltd. identification number 4800. The frozen liquid was identified as Sodium Bicarbonate Salt Solution from Lyophilized solution; Lot no. 6436620. The control substance contained 33.6 mg sodium bicarbonate/mL deionized water. The control substance was stored at -80° C.

The reference substance was chloroacetamide, known to be toxic to earthworms and received from Chem Service and assigned Wildlife International Ltd. identification number 4058. It was described as a crystalline solid, identified as 2-chloroacetamide; Lot no. 167-54B, with a reported purity of 98%, expiration date: Feb. 2002. The reference substance was stored under ambient conditions.

The test organisms were earthworms (*Eisenia fetida*) supplied by Willingham Worm Farm, Butler, Georgia. Prior to the acclimation period and test, the worms were placed in culture chambers containing a mixture of moist peat and manure for 19 days. During this time, they were fed saturated alfalfa. About 24 hours before test initiation, 325 adult worms with clitellum were selected and placed in a container of prepared artificial soil (peat, sand and Kaolin based), adjusted to about 33% soil moisture. On the day of test initiation, worms were rinsed with deionized water, carefully blotted dry and impartially distributed by pairs into groups of 10 worms. Each group of worms was weighed and placed on the soil mixture in the test chamber. Worms were not fed during the test.

The test chambers consisted of 1-L glass beakers covered with plastic wrap and perforated for air exchange. There were four chambers, with 10 adult worms for each of 7 test groups (a total of 40 worms per treatment).

The test soils were prepared with a bulk artificial soil, which consisted of 70% sand, 20% Kaolin clay, and 10% sphagnum peat. Prior to hydration, the pH of the artificial soil was adjusted to 5.8 with calcium carbonate and stored in a sealed container under ambient conditions until needed for test soil preparation. To prepare the test soils, the appropriate test, control or reference substance was mixed with deionized water and then mixed into the moistened artificial soil and adjusted to a moisture content of 33 %. To ensure homogeneity, the soil was mixed for 15 min with the appropriate test, reference or control substances.

Concentrations of the test, control and reference substances were calculated based on the dry weight of the soil. The test substance and buffer control substance concentrations were based on the reported concentration of the protein or salt solution. The reference substance concentrations were adjusted for the purity of the reported purity of chloroacetamide. The assay control soil was mixed also for 15 minutes but without the addition of the test or control substances. Seven hundred fifty grams of the test soils were added to each test chamber.

Environmental conditions for the test chambers were maintained in an environmental chamber set to maintain a temperature of 20 ± 2°C. Air temperature was monitored continuously; the soil temperature was measured in each group on Days 0 and 14 of the test. The photoperiod during the test was 24 hrs of continuous light each day provided by overhead fluorescent bulbs. The intensity was approximately 400 to 800 lux.

Soil samples and analyses were collected for measurements of pH, moisture content and verification of test concentrations and homogeneity of test substances in the soil. At the start and

end of the test, soil samples were collected from each test, control and reference substance group and were used to measure the initial and final pH and moisture content of the soils. Moisture content was determined by measuring the initial weight of the soil sample, and weighing the soil sample after drying for 18 hrs at 105°C. Percent moisture was calculated using the formula:

%moisture = [(net wet weight - net dry weight) ÷ net wet weight] x100

Samples of treated soils were also collected to verify test concentrations and to confirm the homogeneity and stability of the test, buffer control or reference substances in the soil. On Day 0, samples for homogeneity (or verification) were collected from the top, middle and bottom of the mixing vessel with the 570 mg/kg test soil; and verification samples also were collected from the assay control , the 57.0 mg/kg test, and the control substance soils at preparation. Upon termination of the study, one sample was collected from the assay, and each of the test and control substance soils for stability analyses. All samples were stored in 4-oz polypropylene bottles and frozen (-80 °C) for a maximum of 5 days before shipment to the Sponsor for analysis. Analyses of the samples were based on Monsanto Company procedures SOP No. BR-ME-0044-02, entitled "Diet incorporation insect bioassay for the biological activity measurement of *Bacillus thuringiensis* and other insecticidal proteins."

Other observations and statistical analyses were recorded on Days 0, 7 and 14. Worms were placed on the surface of the soil in each test chamber and were observed for burrowing behavior on Days 0 and 7. The number of surviving worms were counted and observed for behavioral abnormalities on Days 7 and 14 of the test after the contents of each test chamber were emptied onto clean paper. Mortalities, signs of toxicity or abnormal behavior were noted. On Day 14, all worms were euthanized after observations; and all carcasses and soil substrate were disposed of by incineration. Body weights by group for each replicate were determined before placement in the test chambers on Day 0. Surviving worms were removed from each replicate test chamber, rinsed with de-ionized water and weighed as a group at test termination, Day 14. Individual body weights were calculated on the basis of the group average.

Statistical analyses of the mortality and body weight data for the test substance, control substance and assay control groups were conducted using Fisher's LSD test. Comparisons were made between the test substance and the respective control substance groups; and between the control substance and the assay control groups. The reference substance groups were not included in the statistical analyses of the test and control substances.

REPORTED RESULTS:

A. Dose Confirmation: Bioactivity of Cry3Bb1 protein in the earthworm soil samples was confirmed using a bioassay with the Colorado potato beetle (CPB) , a known susceptible insect. At test initiation, the bioactivity reported in the collected samples was about 37% of the expected level of activity, whereas the activity was estimated to be between 77 to 96% of the expected level of activity. The cause of the variation was not reported in the study. The difference between Day 0 and Day 14 samples was not significant based on overlap of the 96% confidence intervals. Overall, the data demonstrated that bioactive Cry3Bb1 protein was detected "[t]hroughout the duration of the earthworm study." *Reviewers' Comment*: *Bioactive Cry3Bb1 protein data for Days 0 and 14 were the only two sampling times provided in the report.*

B. Environmental Conditions: For the 14-day study, air temperatures in the chamber ranged from 19.5 to 20.5 °C; and under continuous lighting at an average intensity of 600 ± 93 lux (range of surface of test chambers: 459 to 780 lux).

The pH of all soils ranged from 7.8 to 8.5 on Day 0 , and from 7.9 to 8.7 on Day 14. The soil temperature "[d]uring the 14-day test remained within the desired range of 20 ± 2 °C, and the moisture content of the soils remained relatively constant during the test." *Reviewers' Comment*: *Soil temperatures and moisture data were reported only for two times – Days 0 and 14.*

C. Mortalities and Clinical Observations.

Assay (Negative Control) Group. In the assay control group, there were 7 deaths (of 40; 18%). One worm was reported as remaining on the soils surface almost 2 hrs after Day 7 observations, and one worm was found dead on top of the soil surface on the last day (Day 14). All other surviving worms were normal in appearance and behavior throughout the test.

57.0 mg a.i./kg soil Group. The percent mortality reported was 8% (3 of 40) on Day 14. One worm in the 57.0 mg/kg group inadvertently was injured during the Day 7 observation procedure; and other surviving worms appeared normal. Worms in the 57.0 mg /kg group burrowed beneath the surface within ½ hr on Day 0, and within one hr on Day 7. The study authors attributed such behavior as signs of aversion to the soil. The number of mortalities in this group was less than that in the assay control group, and was not significantly different ($p>0.05$) from the [a]ppropriate buffer salt control group (117.5 mg/kg). Accordingly, the mortalities were considered not to be related to treatment with the test substance.

570 mg a.i./kg soil Group. The percent mortality reported was 38% (15 of 40) at the end of the test. A marked increase in the number of mortalities and in the number of worms exhibiting signs of toxicity were noted in the 570 mg/kg test substance group. Reduced reaction to mechanical stimuli and worms which appeared soft, thin and necrotic were considered signs of toxicity. There was no indication of an aversion to the soil on Day 0. Two worms were noted to remain on the soil surface for about 1 hr on Day 7 in this test group. The increase in mortalities in this group was statistically significant [($p<0.05$)] when compared to the appropriate buffer salt control group (117.5 mg/kg); 38% and 3% mortalities, respectively. The mortalities were likely due to a miscalculation of salt concentrations in the test soil [The actual concentration of sodium bicarbonate salt in the 57.0 and 570 mg Cry3Bb1 protein/kg treatment groups was 70 and 699 mg/kg, respectively.]

Buffer salt control Groups. In both of the buffer salt control groups, mortality on Day 14 was 3% (1 out of 40). The mortality in these groups was not significantly different ($p>0.05$) from the assay control group. Surviving worms appeared normal and exhibited no signs of toxicity or aversion to the soils for the test duration.

Reference substance Groups. At the end of the study, mortality in the 10 and 20 mg chloroacetamide/kg soil was 3%(1 of 40) and 100% (40 of 40), respectively. All surviving worms in the soils treated with the lower concentration of chloroacetamide were normal in appearance and behavior throughout the test; all worms had died by Day 7 in the 20 mg chloroacetamide/kg group.

D. Body Weight and Feed Consumption. Average individual body weights for surviving worms were calculated from the measurements taken on Days 0 and 7. There was no significant

difference ($p>0.05$) in the average individual body weights for the 57.0 mg/kg test substance group ($\bar{0}_{n=40} = 0.30$ g), compared to the appropriate buffer control substance group (117.5 mg salt/kg soil control group; $\bar{0}_{n=40} = 0.31$ g). However, the average individual body weights of worms exposed to 570 mg/kg test substance ($\bar{0}_{n=40} = 0.20$ g) was significantly different [($p<0.05$)] from the appropriate buffer control substance group (117.5 mg buffer salt/kg; $\bar{0}_{n=40} = 0.32$ g). *Reviewers' Comment*: *Body weight comparisons were statistically significant; probability of this significance is likely $p>0.05$, and not as reported*. Comparison of the control substance treatment levels with the assay control indicated a relatively slight, yet statistically significant increase in body weight.

DISCUSSION: This study was conducted according to EPA approved guideline procedures for acute toxicity testing of pesticidal substances to earthworms. The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein in an artificial soil substrate was determined to be greater than 570 mg Cry3Bb1 protein/kg dry soil, or greater than 10 times the maximum EEC of the protein. Based on the effects observed among worms in the 570 mg Cry3b1 protein/kg dry soil treated group, the NOEC was 57.0 mg Cry3Bb1 protein/kg dry soil. There was no apparent effect of the buffer on the earthworms. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

REVIEWER'S COMMENTS:

A. **Test Procedures:** The procedures used follow those recommended by EPA Pesticide Testing Guidelines for Microbial and Biochemical Pest Control Agents, Subdivision M. It was noted in the study design that the levels of buffer salt in the control substance treatment groups were higher than the buffer salt levels in the test substance treatment groups, because of a miscalculation. The actual concentration of sodium bicarbonate salt in the 57.0 and 570 mg Cry3Bb1 protein/kg treatment groups was 70 and 699 mg/kg, respectively. The higher concentrations did not appear to have any influence on the overall conclusions of the study**.**

Regulatory Compliance: The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. EPA in 40 CFR Parts 160 and 792; OECD Principles of Good Laboratory Practice; and Japan MAFF, except:

*Reference substance soils were not analyzed to verify concentrations, homogeneity, or stability of the reference substance in the carrier. This did not affect the results of this study.

*The test and control substance characterization, derivation of the maximum hazard dose, and dose confirmation portions of the study were conducted by the Sponsor in accordance with the GLPs, except:

–Egg receipt, sterilization and incubation to the first instar larvae stage for the insect bioassay portion of the analytical procedure was not conducted in accordance with the GLPs. There was no impact on the outcome of the bioassay.

–SDS-PAGE analysis was performed to reevaluate the molecular weights of the test substance after the initiation of the study. The resulting values were a more accurate assessment of the molecular weights than reported previously. This did not affect the integrity of the study.

B. **Statistical Analysis:** The LC_{50} value could not be subjected to statistical analysis because only one test concentration yielded adverse effects attributable to the test substance. The no-mortality and no-observed-effect concentrations were determined using a visual examination of the mortality and clinical observation data.

C. **Discussion/Hazard Assessment:** The study was procedurally sound and the data show that no adverse effects to earthworms are expected at ten times the maximum environmental concentration of Cry3Bb1 protein.

D. **Adequacy of the Study:**

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA Guideline requirements for assessing acute pesticidal risks to earthworms.