May 20, 2002

MEMORANDUM

SUBJECT: Review of ecological non-target insect studies for *Bacillus thuringiensis* Cry3Bb1 protein. EPA Reg. No. 524-LRA; Barcode No. D262045; Case No. 066221; Submission No. S572997. MRID Nos 449043-10, 449043-11, 449043-12, 449043-13, 449043-17, 455382-04, 455382-05, 455382-06, 455770-03.

TO: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

FROM: Robyn Rose, Entomologist
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Zigfridas Vaituzis, Ph.D., Microbiologist
Biopesticides and Pollution Prevention Division, 7511C

CLASSIFICATION: Supplemental to submitting the final field survey study. All other studies are **Acceptable**.

BACKGROUND:
Monsanto Company has requested a registration for *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material (ZMIR13L) necessary for its production in all corn lines and varieties. The Cry3Bb1 protein is intended to control corn rootworms (CRW, *Diabrotica* spp.), a coleopteran pest of corn. Studies were submitted to support registration of the transformation event MON 863. CRW, a primary pest of corn in the U.S., feeds on corn roots as larvae leading to a reduction in the plant's ability to absorb water and nutrients from soil and lodging. In areas where CRW is a pest (e.g., Corn Belt), significant financial losses are realized from a decrease in production and chemical insecticide usage. Significant acres of corn are treated annually to control CRW with organophosphate, carbamate and pyrethroid insecticides.

Nontarget insect studies are required to register a new pesticide (40 CFR §158.740). Monsanto Company submitted studies on nontarget adult lady beetles (MRID No 449043-14), green lacewing larvae (MRID No 449043-12), a parasitic Hymenoptera (MRID No 449043-13), adult and larval honey bees (MRID Nos 449043-11, 449043-10) and Collembola (MRID No 449043-17). Additional studies were submitted including field census evaluations (MRID No 455382-06) and an endangered species risk assessment (MRID No 455770-03). In a March 10, 2000

memorandum from Robyn Rose to Mike Mendelsohn, the adult lady beetle study was reviewed. This review concluded that a test demonstrating the effect of lady beetles feeding on corn pollen containing Cry3Bb1 should be conducted. This study was conducted by Monsanto and submitted to the Agency (MRID No 455382-04). Although Cry3Bb1 is active against coleopterans, a study was also conducted to alleviate any potential public concerns on potential effects of MON863 expressing pollen to monarch butterflies (MRID No 455382-05).

CONCLUSIONS:

Green Lacewing Larvae (MRID No 449043-12)
An acceptable study was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I. The NOEC for green lacewing larvae was determined to be >8,000 ppm Cry3Bb1 protein.

Parasitic Hymenoptera (449043-13)
An acceptable test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I. The NOEC for parasitic Hymenoptera was determined to be 400 ppm Cry3Bb1 protein. Although 400 ppm Cry3Bb1 protein is only 1X field concentrations in plant rather than a 10-fold safety factor, parasitic Hymenoptera are not expected to feed directly on corn plant tissue. Therefore, minimal exposure of parasitic Hymenoptera to Cry3Bb1 protein is expected.

Honey Bee Larvae (MRID No 449043-10)
An acceptable test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. The NOEC for honey bee larvae was determined to be 1,790 ppm Cry3Bb1 protein.

Honey Bee Adults (MRID No 449043-11)
An acceptable test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. It can be concluded from this test that the NOEC of the Cry3Bb1 protein for adult honey bees is 360µg/mL.

Collembola (MRID No 449043-17)
An acceptable test was conducted. It can be concluded from this test that the NOEC of the Cry3Bb1 protein for Collembola is #872.5µg.

Lady Beetle Larval Pollen Feeding Study (MRID No 455382-04)
the NOEC for Cry3Bb1 expressed in pollen is #93 µg/g fresh pollen weight. It can be concluded from this study that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

Monarch Butterfly Larval Pollen Feeding Study (MRID No 455382-05)

This study was not required nor requested for Cry3Bb1 because it is a coleopteran active protein that is not expected to affect lepidopterans such as the monarch butterfly. In addition, extensive research conducted on the potential affects of monarch feeding on Bt corn pollen has shown a lack of concern of acute toxicity. However, due to recent public concern of possible adverse effects of Bt corn on monarchs, Monsanto sponsored this study and submitted it to the Agency for review. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in acute toxic or developmental effects to monarch larvae.

Field Evaluation of Ecological Impacts (MRID No 455382-06)
Although this study was not conducted according to FIFRA GLP compliance, this study was conducted according to a scientifically valid, peer reviewed protocol that is acceptable. Results reported in this submission suggest that planting event MON 863 CRW protected corn will not adversely affect non-target beneficial invertebrate abundance in the field. On the contrary, a reduction in pesticide use that may result from planting MON 863 corn will probably result in increased numbers of natural enemies in CRW protected Bt corn.

This submission only reported results from the 2000 growing season. A final report will be submitted to the Agency after the 2001 data is collected and analyzed. The final report should include the location of the 2000 and 2001 test sites as well as the additional data on soil fertility, microbial populations and soil Bt protein collected during this study but not reported at this time. This study should also be conducted at additional test sites in different geographic locations to account for regional differences in insect populations.

Endangered Species Impact Assessment (MRID No. 455770-03)
Based on a 10-fold safety factor for terrestrial species and a 20-fold safety factor for aquatic species and a lack of exposure, no unreasonable adverse effects of MON 863 to endangered Coleoptera are expected. According to Monsanto's submission, the LC_{50} for the CPB, the most sensitive species to Cry3Bb1 protein, is 2.4 µg/mL diet. A maximum exposure of 0.23 µg/g (10-fold safety margin) is expected 1 m from the field edge, 0.09 µg/g (28-fold safety margin) is expected 2 m from the field edge and 0.03 µg/g (82-fold safety margin) is expected 4-5 m from the field edge. A 1-ha pond with a 2 m depth located 1 m from the edge of the corn field that has 500 grains/cm^2 would have a maximum exposure of 5.81250×10^{-5} which represents a 41290 safety margin. These data indicate that pollen drift from MON 863 corn fields will not pose a terrestrial or aquatic exposure risk.

Many of the endangered and threatened beetles occur in cave or aquatic habitats. None of the endangered beetles are expected to occur in or near corn fields. The American burying beetle may occur in old fields or cropland hedge rows. However, based upon the feeding habits of the American burying beetle, it is not expected to occur within corn fields nor will it be exposed to Cry3Bb1 protein. Adult American burying beetles are classified as opportunistic scavengers that feed on anything dead and bury vertebrate carcasses which larvae feed on. Carrion regurgitated by adults is fed to larvae until they are able to feed directly on a carcass.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on green lacewing larvae
MRID NO.:	449043-12
STUDY NO.:	WL-98-298
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Wildlife International Ltd., 8598 Commerce Dr., Easton, MD 21601
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Susan J. Palmer and Henry O. Krueger, Ph.D
STUDY COMPLETED:	July 28, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "*Bacillus thuringiensis* Protein 11231: A Dietary Toxicity Study with Green Lacewing Larvae (*Chrysoperla carnea*)"

Authors: Susan J. Palmer and Henry O. Krueger, Ph.D (Wildlife International Ltd.).

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) in diet to green lacewing larvae (*Chrysoperla carnea*).

Methods:

This test was based on protocols outlined in EPA's Series 154A *Pesticide Assessment Guidelines, FIFRA Subdivision M, Microbial Pest Control Agents* that are superceded by OPPTS 885.4340 Nontarget insect testing, Tier I and conducted according to a protocol titled "Cry3Bb2 Protein: A Dietary Toxicity Study with Green Lacewing larvae (*Chrysoperla carnea*)." Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 34.5 mg active protein/mL water) held at -80°C prior to test initiation. Current nomenclature refers to this protein as Cry3Bb1. During the test, green lacewing larvae were fed the Cry 3Bb1 protein in a moth egg (*Sitotroga* sp.) and water meal diet at rates of 400 and 8,000 ppm which is approximately equivalent to 1X and 20X the maximum protein concentration in plant tissue. Test concentrations were confirmed by a bioassay using Colorado potato beetles, s known sensitive species to the Cry3Bb1 protein.

This study included 30 test chambers (1 oz semitransparent plastic cups with lids) containing 1 newly emerged lacewing larvae that were allowed *ad libitum* access to Cry3Bb1 in diet, 1 control group given water prepared by reverse osmosis and 2 reference groups given 1,000 and 10,000 ppm potassium arsenate (41.7% arsenic). Larvae were transferred to new chambers with fresh diet weekly and kept in incubators set at an average of 20.6°C, 74% ±9% relative humidity

and a photoperiod of 12 hours of light. Rates of mortality, pupation and other clinical signs of abnormal behavior or toxicity were recorded within 2 to 4 hours after test initiation and continued daily until there was 20% mortality in the control group on Day 10. Significant differences of mortality at test termination between treated and control groups were determined using Fisher's exact test.

Results:
No pupation occurred in any of the treated, control or reference groups at test termination. There was 27% mortality (8 of 30) in the control group and no signs of clinical abnormalities at test termination. There was 27% mortality (8 of 30) in the 400 ppm Cry3Bb1 treatment group and 23% mortality (7 of 30) in the 8,000 ppm Cry3Bb1 treatment group. Differences in mortality between the control and treatment groups were not significantly different ($p>0.05$). The 1,000 and 10,000 ppm potassium arsenate groups resulted in 10% (3 of 30) and 53% mortality (16 of 30) respectively on Day 1 of testing. At test termination mortality for the 1,000 ppm group was 43% (13 of 30) and 100% mortality in the 10,000 ppm reference group.

Study Conclusions:
There was no significant increase in green lacewing larval mortality when fed 1X (400 ppm) and 20X (8,000 ppm) the maximum Cry3Bb1 protein concentration found in plant tissue. Based on this test, the NOEC for green lacewing larvae exposed to Cry3Bb1 in diet is >8,000 ppm .

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. According to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I, nontarget insects should be tested with does of 10-100X the field dosage. This test was conducted at an acceptable level 20X field concentrations in plant tissue or >8,000 ppm Cry3Bb1 protein. These guidelines also recommend testing bacteria against nontarget insects for 21 to 30 days. If it is not possible to continue the test for 21 to 30 days, then testing should cease when control mortality reaches 20%. Although this test could have continued for a longer duration, terminating at 20% mortality in the control group is acceptable.

This study was submitted in support of registering CRW protected Bt corn event MON 863. However, green lacewing were fed Cry3Bb1.11231 (event MON 859) rather than Cry3Bb1.11098 (event MON 863) in this study. According to Monsanto, Cry3Bb1.11231 "demonstrates an eight-fold increase in activity against the SCRW as a result of four strategically placed amino acid substitutions in the wild type protein sequence." Cry3Bb1.11098 "demonstrates an eight-fold increase in activity against the SCRW as a result of five strategically placed amino acid substitutions in the wild type protein sequence." It is acceptable that this test was conducted with event MON 859 rather than MON 863 because they produce nearly identical Cry3Bb1 protein variants.

It can be concluded from this test that the NOEC for green lacewing larvae fed Cry3Bb1 protein

is >8,000 ppm.

DATA EVALUATION REPORT

REVIEWED BY: Robyn Rose, Entomologist
Biopesticides and Pollution Prevention Division

SECONDARY REVIEWER: Zigfridas Vaituzis, Ph.D., Microbiologist
Biopesticides and Pollution Prevention Division
STUDY TYPE: Non-target effects of Bt Protein 11231 on parasitic Hymenoptera
MRID NO.: 449043-13
STUDY NO.: WL-98-300
SPONSOR: Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB: Wildlife International Ltd., 8598 Commerce Dr., Easton, MD 21601
TEST MATERIAL: *Bacillus thuringiensis* Protein 11231
AUTHOR: Susan J. Palmer and Henry O. Krueger, Ph.D
STUDY COMPLETED: July 28, 1999
CLASSIFICATION: **Acceptable**

Study Summary
Title: "*Bacillus thuringiensis* Protein 11231: A Dietary Toxicity Study with the Parasitic Hymenoptera (*Nasonia vitripennis*)"

Authors: Susan J. Palmer and Henry O. Krueger, Ph.D (Wildlife International Ltd.).

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) in diet to a representative parasitic wasp (*Nasonia vitripennis*).

Methods:
This test was based on protocols outlined in EPA's Series 154A *Pesticide Assessment Guidelines, FIFRA Subdivision M, Microbial Pest Control Agents* that are superceded by OPPTS 885.4340 Nontarget insect testing, Tier I and conducted according to a protocol titled "Cry3Bb2 Protein: A Dietary Toxicity Study with the Parasitic Hymenoptera (*Nasonia vitripennis*)." Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 34.5 mg active protein/mL water) held at -80°C prior to test initiation. Current nomenclature refers to this protein as Cry3Bb1.

Twenty five adult wasps were kept in ½ pt rolled paper containers (9 cm in diameter and 5 cm high) covered with a plastic petri dish and a 20 mL glass vial with deionized water inserted through it during testing. Diet, including honey combined with Cry3Bb1 protein, was administered to wasps on a cotton swab inserted through the side of test chambers. Test diets were prepared at test initiation and weekly thereafter. Wasps were tested at rates of 400 and 8,000 ppm Cry3Bb1 protein which is approximately equivalent to 1X and 20X the maximum protein concentration in plant tissue. Test concentrations were confirmed by a bioassay using Colorado potato beetles, a known sensitive species to the Cry3Bb1 protein. In addition to Cry3Bb1 treated groups, one control group given water prepared by reverse osmosis and two reference groups given 100 and 1,000 ppm potassium arsenate (41.7% arsenic). Wasps were kept in incubators set at an average of 26.7±0.7°C, 54% ±4% relative humidity and a

photoperiod of 12 hours of light and allowed *ad libitum* access to diets. Rates of mortality, pupation and other clinical signs of abnormal behavior or toxicity were recorded within four hours of test initiation and continued daily until there was 20% mortality in the control group on Day 16. Significant differences of mortality at test termination between treated and control groups were determined using Dunnett's test.

Results:
Some wasps were not included in the data because of mortality in the control, reference and 8,000 ppm treatment group that was due to wasps being trapped under the container lids. There was 23% mortality (17 of 73) in the control group at test termination and one immobile wasp recorded on Day 15. There was 24% mortality(18 of 75) in the 400 ppm Cry3Bb1 treatment group and 58% mortality (42 of 73) in the 8,000 ppm Cry3Bb1 treatment group. Additional wasps were observed to be immobile during the test and one wasp was "near death" in each treatment group at test termination. If the wasps "near death" are included in the mortality group, then mortality rates are increased to 25% and 59% for the 400 and 8,000 ppm treatments respectively. If an adjustment for mortality in the control group is considered, mortality in the 8,000 ppm treatment group is 45%. Although differences in mortality between the control and treatment groups were not significantly different ($p>0.05$), a treatment effect could not be precluded in this study. At test termination mortality for the 100 ppm potassium arsenate reference group was 33% (24 of 73) and 100% mortality (70 of 70) in the 1,000 ppm reference group.

Conclusions:
Based on this test, the authors concluded that the NOEC for adult parasitic Hymenoptera exposed to Cry3Bb1 in diet is 400 ppm and the LC_{50} is 8,000 ppm.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. According to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I, nontarget insects should be tested with doses of 10-100X the field dosage. This test was conducted at an acceptable level 20X field concentrations in plant tissue or 8,000 ppm Cry3Bb1 protein. These guidelines also suggest testing bacteria against nontarget insects for 21 to 30 days. If it is not possible to continue the test for 21 to 30 days, then testing should cease when control mortality reaches 20%. Although this test could have continued for a longer duration, terminating at 20% mortality in the control group is acceptable.

It can be concluded from this test that the NOEC for the Cry3Bb1 protein is 400 ppm. Although these results are based on 400 ppm Cry3Bb1 protein which is only 1X field concentrations in plant rather than a 10-fold safety factor, parasitic Hymenoptera are not expected to feed directly on corn plant tissue. Therefore, minimal exposure of parasitic Hymenoptera to Cry3Bb1 protein is expected.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on honey bee larvae
MRID NO.:	449043-10

STUDY NO.:	CA-98-169
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	California Agricultural Research, Inc., 4141 N. Vineland, Kerman, CA 93630
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Victor L. Maggi, M.S.
STUDY COMPLETED:	August 2, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Evaluation of the Dietary Effects of Purified *Bacillus thuringiensis* Protein 11231 on Honey Bee Larvae"

Author: Victor L. Maggi (California Agricultural Research, Inc.)

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) in diet to larval honey bees (*Apis mellifera*).

Methods:

This test was based on protocols outlined in EPA's OPPTS 885.4380, Honey Bee Testing, Tier I and conducted according to a protocol titled *Evaluation of the Dietary Effects(s) of Purified Bacillus thuringiensis Cry3Bb2 Protein in Honey Bee Larvae.* Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 1.79 mg active protein/mL water) held at -80°C prior to test initiation. Current nomenclature refers to this protein as Cry3Bb1.

Four honey bee hives with at least 2 frames with large, uniformly-sized larval brood populations were treated with Terabee Mix 2X Terramycin (50 g; 5.5% oxytetracycline) and Apistan strips (10.0% Fluvalinate) to control foulbrood diseases and varroa mites respectively and acclimated 30 days prior to testing. Honey bees (*Apis mellifera lingustica* Spin.) were fed a 30% sucrose solution prior to testing.

Each hive represented a replicate containing each treatment group. Twenty honey bee larvae (2-3 days old) per replicate (80 bees/treatment) were treated with a reference substance (0.5 mg/mL (500 ppm) potassium arsenate + 3µL deionized water), a control substance (3µL deionized water) and a test substance (1.79 mg/mL (1,790 ppm) Cry3Bb1 protein + 3µL deionized water). The reference substance was administered to separate frames from treatments and controls to avoid cross-contamination. The treatment concentration, 1.79 mg/mL Cry3Bb1 protein, was based on a 100-fold safety factor over the maximum concentration of Cry3Bb1 protein expressed in pollen.

Larval brood cells intended to receive treatments were mapped on an acetate sheet laid over the

frame. In the laboratory, treatments were administered to brood cells using a micropipette and frames were left undisturbed for 30 minutes to allow larvae to feed prior to returning frames to their original hive.

Capping and the presence of larvae were evaluated on Days 8 and 12 after treatment. On Day 12 treated frames were removed from hives and placed in emergence cages consisting of 3.2 cm wire mesh cage (3 × 6 cm) fit around each group of treated brood cells. Cages were kept in chambers at 28 to 32°C and 25 to 85% relative humidity. Observations of adult emergence occurred twice a day until all bees emerged. When the test was terminated two samples of the test substance were removed and bioassayed with the Colorado potato beetle, a known sensitive species to Cry3Bb1 protein, to verify bioactivity of the protein. A *t*-Test was conducted using Pesticide Research Manager (PRM) software, version 5.

Results:
Within 18 days after treatments were administered, all larvae emerged from capped brood cells. On Day 18, larvae were 20 to 21 days old. All of the larvae (100%) treated with Cry3Bb1 protein survived to pupation or "capping"; whereas, 97.5% (2.5% mortality) of the honey bee larvae in the control group survived to pupation. There was no statistical difference (p=0.05) in total percent mortality during the larval development or adult emergence stages between treated and control groups.

Although 3 bees among 2 replicates of the test substance were not accounted for, no capped cells remained. This suggests that these bees emerged but were somehow missed during counting. An additional error occurred in the 4th replicate of the reference treatment where an additional bee emerged (#21).

Conclusions:
Based on the results presented in this study, it can be concluded that honey bee development and survival are not affected by exposure to the Cry3Bb1 protein. There was 88.8% mortality of larvae treated with the reference substance which indicated that bees were exposed to the treatments.

Reviewer Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. This test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. According to the guidelines, nontarget insects should be tested with does of 10-100X the field dosage. This test was conducted at an acceptable level 100X the concentration in pollen or 1,790 ppm Cry3Bb1 protein. Since potential exposure of honey bees to Cry3Bb1 will be from pollen, this test was conducted at an appropriate maximum hazard dose.

An unclear result was reported on page 15 in the Results and Discussion section of this report. A

21st emerged bee was noted from the 4th replicate of the reference group; however, Table 1 on page 17 does not report this finding. On the contrary, #3 bees emerged from each of the 4 reference group replicates resulting in 88.8% overall mortality. The 21st emerged bee is probably from the treated group (replicate 1 or 2) or the control group (replicate 2 or 3) and was likely counted due to error.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for honey bee larvae is >1,790 ppm.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on honey bee adults
MRID NO.:	449043-11
STUDY NO.:	CA-98-171
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis,

	MO 63198
TESTING LAB:	California Agricultural Research, Inc., 4141 N. Vineland, Kerman, CA 93630
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Victor L. Maggi, M.S.
STUDY COMPLETED:	August 2, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Evaluation of the Dietary Effect(s) of Purified *Bacillus thuringiensis* Protein 11231 on Adult Honey Bees (*Apis mellifera* L.)"

Author: Victor L. Maggi (California Agricultural Research, Inc.)

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) administered in diet to adult honey bees (*Apis mellifera*).

Methods:
This test was based on protocols outlined in EPA's OPPTS 885.4380, Honey Bee Testing, Tier I and conducted according to a protocol titled *Evaluation of the Dietary Effects(s) of Purified Bacillus thuringiensis Cry3Bb2 Protein on Adult Honey Bees2*. Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 0.36 mg active protein/mL water). Current nomenclature refers to this protein as Cry3Bb1.

Treatment groups consisted of a control group fed 30% sucrose in deionized water, a reference group fed 100µg/mL potassium arsenate, and a test group fed 360 µg/mL of Cry3Bb1 protein and a water only group. The 360 µg/mL Cry3Bb1 protein used in the treatment group was gotten from mixing 100 mL volume of 0.72 mg/mL purified protein with an equal volume of 60% sucrose:40% deionized water solution. The potassium arsenate and 360 µg/mL Cry3Bb1 protein were fed to bees in a 30% sucrose:70% deionized water solution. Stability and dose of the Cry3Bb1 protein was confirmed from samples collected before test initiation, twice during the study, and after completion via bioassay of Colorado potato beetles, a known sensitive species.

In March and October of each year, honey bee (*Apis mellifera*) hives received ~50 g of 5.5% oxyteteracycline hydrochloride to prevent and control foul brood diseases and were treated with an acaricide containing 10.0% Fluvalinate in November to control Varroa mites. Three hives were allowed to acclimate 30 days prior to treatments. The 9 frames used in this study were chosen from hives that were evaluated 2 months prior to testing for the presence of 2 or more frames with large populations of capped brood about to emerge. Two frames from one hive were treated on August 7, 1998 and the other two hives (2 and 3 frames) were treated on August 13. Since the test began late in the season, hives were fed a 30% sucrose solution to ensure bees would accept treated diets that were fed to hives three times a week during the study. Diet was

administered to hives in a 12 mL glass vial.

Frames (12.7 cm cube-shaped 3.2 mesh/cm hardware cloth cage with a 10 × 10 cm cardboard door) with capped brood and no adults were put in the cages that were kept in chambers set at 24 hours dark, 26 to 40°C and 22 to 84% relative humidity prior to administering treatments. Forty bees that appeared normal and were #6 days old were taken from these frames and placed in hive boxes to be treated. There were 4 replicates of the 3 treated groups (160 bees each) and one water only group for a total of 13 cages and 520 bees used in this study. Treated cages were kept in a chamber set at 24 hours dark, 16 to 18°C and 41 to 49% relative humidity.

Observations of mortality and agitation of the feeding vials were made 5 hours after test initiation and daily thereafter. Test substance solutions (< 8 days old) were replenished every 48 hours. However, since the reference and water only groups resulted in high mortality rates within 48 hours of test initiation and 100% mortality by Day 4, they did not receive new diet. The study was terminated on Day 11 when there was 40% mortality in the control group.

The cumulative percent morality for each replicate was determined by calculating # of dead bees/# bees at study initiation × 100. Each test groups cumulative mean mortality was determined by calculating the cumulative # of dead bees/# bees at study initiation × 100. The Pesticide Research Manager Software , Version 5 was used con conduct a *t*-Test.

Results:
In the arsenic treated reference group, there was 60% mortality on Day 1, 90% mortality on Day 2, and 100% mortality on Day 3 indicating that bees were consuming the diet. There was 100% mortality of the bees in the water only group suggested bees require a dietary component to survive. In the control group, there was 20% mortality on Day 4 and 40% mortality on Day 11 when the test was terminated. The test was continued beyond 20% mortality in the control for a more comprehensive comparison between treated and control groups.

The dose of the Cry3Bb1 protein was confirmed to be bioactive toward susceptible insects. There was no statistically significant difference (p=0.05) between mortality observed in the treated and control groups on a daily basis. There was also no abnormal behavior of bees observed in the treated and control groups.

Table 1. Cumulative mortality from Day 0 (test initiation) to Day 11 (test termination) for the treated and control groups

Treatment	Day											
	0	1	2	3	4	5	6	7	8	9	10	11
Control	0	3.3	6.5	8.3	9.3	9.8	11.0	11.8	14.3	16.3	19.5	19.5
Cry 3Bb1	0.3	3.5	6.0	8.8	10.8	11.5	12.5	14.0	14.5	15.3	16.0	17.3

Table2. Cumulative percent mortality from Day 1 to Day 11 (test termination) for the treated and control

groups

Treatment	Day										
	1	2	3	4	5	6	7	8	9	10	11
Control	6.92	14.07	18.08	20.26	21.38	24.24	25.9	31.67	36.37	43.8	43.8
Cry 3Bb1	8.22	14.08	20.50	25.21	26.95	29.29	32.79	33.96	35.7	37.46	40.37

Conclusions:
The study authors concluded that 360 µg/mL Cry3Bb1 protein did not affect survival or behavior of adult honey bees. The 360 µg/mL test concentration is 20X the concentration found in pollen. Therefore, there will be minimal risk from the Cry3Bb1 protein to honey bees.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. This test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. According to the guidelines, nontarget insects should be tested with does of 10-100X the field dosage. This test was conducted at an acceptable level 20X the concentration in pollen or 360µg/mL Cry3Bb1 protein. Since potential exposure of honey bees to Cry3Bb1 will be from pollen, this test was conducted at an appropriate maximum hazard dose.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for adult honey bees is >360µg/mL.

DATA EVALUATION REPORT

REVIEWED BY: Robyn Rose, Entomologist
Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER: Zigfridas Vaituzis, Ph.D., Microbiologist
Biopesticides and Pollution Prevention Division
STUDY TYPE: Non-target effects of Bt Protein 11098 on Collembola
MRID NO.: 449043-17
STUDY NO.: SB-98-296
SPONSOR: Monsanto Company, 700 Chesterfield Parkway North, St Louis,

	MO 63198
TESTING LAB:	Springborn Laboratories, Inc., 790 Main Street, Wareham, MA 02571-1075.
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11098
AUTHOR:	Debra Teixeira
STUDY COMPLETED:	August 13, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Assessment of Chronic Toxicity of Corn Tissue Containing the *Bacillus thuringiensis* Protein 11098 to Collembola (*Folsomia candida*)"

Author: Debra Teixeira

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11098) administered in diet to Collembola (*Folsomia candida*).

Methods:

Protocols described in Springborn Protocol # 110398/OECD/123/Monsanto were followed for this study. Treatments consisted of 0.50, 5.0, and 50% Bt corn leaf tissue or non-transgenic corn tissue combined with yeast. Lyophilized leaf tissue was used rather than purified protein because initial results indicated that the buffer salt was toxic to Collembola. The Lyophilized corn tissue expressed the Cry3Bb1 protein from the MON 859 line. The concentration of Cry3Bb1 protein in leaf tissue was determined by ELISA to be 1,745 µg Cry3Bb1 protein/g leaf dry weight. The control groups (non-transgenic lyophilized corn tissue) was from the MON 846 corn line which has a similar genetic background to the MON 859 line. MON 859 and MON 846 corn tissue were kept in a freezer at -80°C prior to testing.

Prior to testing, Collembola (*Folsomia candida*) were placed in 4 oz glass jars with approximately 1 cm of a 8:1 plaster:coal breeding substrate and kept in an incubator set at 20 to 22°C and 70 to 100% relative humidity. Collembola were 10 days old at test initiation. There were 10 Collembola added to each test jar for a total of 40 Collembola per treatment. These organisms were allowed *ad libitum* access to dry granulated yeast once or twice weekly. Temperature and humidity were similar during testing plus the photoperiod was set at 16 hours of light 8 hours of dark.

Treated Collembola were fed approximately 2 mg of diet every other day which allowed for an excess of food. Diets consisted of transgenic corn leaf tissue containing Cry3Bb1 protein mixed with dry granulated Brewer's yeast. Diets contained a ratio of 0.50, 5.0 and 50% corn leaf tissue in Brewer's yeast which was equivalent to 8.73, 87.3 and 872.5 µg corn leaf tissue per gram diet respectively (Table 1). Corn leaf tissue dilutions were 1,745 µg Cry3Bb1 protein/g dried leaf tissue. Bioactivity of the Cry3Bb1 protein was determined at test initiation by conducting a 28-day bioassay with the Colorado potato beetle (*Leptinotarsa decemlineata*), a sensitive species, at

test concentrations.

Table 1. Concentration of corn leaf tissue in diet

Bt or Non-Bt Corn Leaf Tissue	Brewer's Yeast (g)	% Concentration of Leaf Tissue
0.50 g	0.50	50
0.10 g of 50%	0.90	5.0
0.010 g of 50%	.99	0.5

A control consisting of Brewer's yeast and non-transgenic corn leaf tissue at ratios of 0.50, 5.0 and 50% was conducted as well as a control using only Brewer's yeast. An additional reference test was conducted with thiodicarb (1.0, 10, and 100 mg a.i./kg) to demonstrate that the study design is able to detect toxic effects.

From test initiation to test termination on Day 28, observations of mortality and sublethal effects such as lethargic behavior were recorded. Adult and young Collembola (e.g., offspring) were removed and counted at test termination. Collembolans reproductive performance was compared between treated and control groups by conducting a Students t-Test. There was not statistical analysis performed on mortality rates since there was #5% mortality of Collembola during the study.

Results:

Table 2. Bioactivity of the test substance was verified by the Colorado potato beetle (CPB) bioassay. Results of the (CPB) bioassay

Test Substance	Week	Day	Concentration (ppm)	Initial/Survivors	Weight (mg)
Control	1	0	0	16/16	131.9
50% TS (872.5 ppm)	1	0	1	16/7	22.2
50% TS (872.5 ppm)	1	0	5	16/1	0.7
Control	4	1	0	16/15	110.4
50% TS (872.5 ppm)	4	1	1	16/12	44.9
50% TS (872.5 ppm)	4	1	5	16/1	0.7

TS= test substance

It was verified that Collembola were ingesting the corn leaf tissue in diet on Day 6 when organisms fed 50% control test substance levels were observed to have green digestive tracts. In the reference test, there was 5%, 17%, and 57% mortality of Collembola in the 1, 10 and 100 mg respectively demonstrating that Collembola would be killed if ingesting a toxic substance (e.g., thiodicarb) in diet. There was no significant difference in the mortality rate in the treated and control groups. There was also no significant difference in the cumulative number of offspring produced in the treated and control groups

Table 3. Average % survival and average cumulative number of offspring produced.

Nominal Diet Concentration (% as corn leaf tissue)	Percent Survival	Cumulative # of Offspring Produced
Assay Control	98	191
0.5% Control	98	207
5.0% Control	95	237
50% Control	95	226
0.5% Cry3Bb1	100	20
5.0% Cry3Bb1	98	185
50% Cry3Bb1	100	228

These results show a NOEC of Cry3Bb1 protein in diet for Collembola to be > 872.5 µg/g diet.

Conclusions:
Results of this study demonstrated that diet containing 50% corn leaf tissue expressing the Cry3Bb1 Bt protein or 872.5µg did not adversely affect survival or reproduction of Collembola. Therefore, it can be concluded that the NOEC of Cry3Bb1 protein in diet for Collembola is >872.5µg/g diet.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. There are no EPA guidelines developed for Collembola testing. This test was conducted at concentration levels much greater than Collembola are expected to be exposed to in the field. MON 859 is expressed in corn leaves in the range of 323-451 µg/g as compared to MON 863 which expresses 30-93 µg/g. The primary route Collembola would be exposed to Cry3Bb1 in the field is from decaying root tissue (and possibly from pollen to a much lesser degree). MON 863 is expressed in corn roots in the range of 3-66 µg/g which is significantly lower than the levels used in this test.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for Collembola is >872.5µg/g diet.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Cry3Bb1 Bt pollen on lady beetle larvae
MRID NO.:	455382-04
STUDY NO.:	00-01-39-26
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Ecological Technology Center, 800 North Lindbergh, St. Louis, MO 63141
TEST MATERIAL:	Pollen expressing *Bacillus thuringiensis* Cry3Bb1 Protein
AUTHOR:	Jian J. Duan
STUDY COMPLETED:	March 1, 2001

CLASSIFICATION: **Acceptable**

Study Summary
Title: "Dietary Effects of Transgenic *Bacillus thuringiensis* (Bt) Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Larvae of the Ladybird Beetle, *Coleomegilla maculata*"

Author: Jian J. Duan

Objective: This study was conducted to evaluate the affect of corn pollen expressing Cry3Bb1 protein on survival and development of lady beetle larvae (*Coleomegilla maculata*).

Methods:
Since corn pollen may comprise up to 50% of lady beetle larvae's diet, the effects of corn pollen containing event MON 863 which encodes a variant of the Cry3Bb1 protein on lady beetle larvae (*Coleomegilla maculata*) was evaluated. ELISA has shown there is 93µg/g fresh weight MON 863 in corn pollen. The corn pollen used in this study was stored in the freezer at -80°C for nine months and then an ELISA was conducted that showed approximately 101 µg MON 863/g fresh weight pollen. The transgenic corn pollen was compared to non-transgenic pollen from the corn line MON 864 and a reference substance containing potassium arsenate (positive control). Pollen was fed to lady beetle larvae in a diet consisting of equal amounts of lyophilized tephritid fruit fly eggs and bee pollen. Diets contained 50% pollen since this is the potential level of field exposure and an equal amount of the tephritid fruit fly diet. Diets were thoroughly mixed in a centrifuge tube by rolling contents until they appeared uniform. Between uses diets were stored at -20°C and stability of the Cry3Bb1 protein was determined at test termination.

Three - 6 mg subsamples of diets were taken and the number of pollen grains were counted at the end of the experiment to verify test and control concentrations. Pollen grains were counted from a 5 µL subsample of 1 mg of diet samples suspended in 0.5 mL of 0.2% agar solution. The total number of pollen grains was estimated from 15 subsamples of the 5 µL diet-agar suspension. An additional evaluation of the number of pollen grains in 1 mg diet/1 mL agar was conducted to determine if the total number of pollen grains in the test and control substances were at expected levels. An ELISA was also conducted to verify the presence of Cry3Bb1 protein in the diet mixes.

First instar lady beetle larvae were individually placed in test arenas to avoid cannibalism. Beetle larvae were given diet mix containing either test pollen, control pollen, reference pollen (potassium arsenate), or bee pollen. Larvae were fed daily and a water moistened sponge was presented as needed on a microscope slide. Diet and water were never depleted during the test. Arenas consisted of 10 cm in diameter and 1.5 cm deep polystyrene petri dishes with a ventilated lid and filter paper on the bottom. Each assay consisted of 10 larvae per treatment replicated 3 times for a total of 30 larvae per treatment. Test arenas were kept in incubators during the test set at - 27°C and a photoperiod of 14 hours of light and 10 hours of dark. Larvae were kept in the arenas until they died or pupated.

Observations were made daily for developmental stage or mortality and newly emerged adults were weighed. An LSD (least significant difference) was determined from a PROC ANOVA conducted by SAS to compare survival rates among the different treatments. Since all pupae developed adults, the survival rate for pupae and adults were identical. An LSD was determined from PROC MIXED conducted by SAS to compare developmental time and weight among the different treatments. There was 100% morality in the reference group so this group was not included in the developmental time or adult weight analysis.

Results:

ELISA of the Bt corn pollen determined that the level of variant Cry3Bb1 protein was approximately 135 µg/g fresh weight pollen in the test pollen stored at -20°C for 35 days. This verified that there was no reduction in the level of variant Cry3Bb1 protein in test pollen which was previously estimated at 93-101µg/g fresh weight pollen. There was no Bt detected in the control pollen. The number of pollen grains in treated and control groups were similar. Pollen grain counts resulted in 2093-2987 grains/mg diet mix for Bt pollen and 2773-2920 grains/mg diet mix for non-transgenic pollen. The number of pollen grains counted in the pure test and control samples were 3907 and 3387 grains/mg test and control pollen samples respectively. Pollen grain levels in test and control diet mixes were within 54-76% and 82-86% of the expected ranges respectively.

There was not a statistically significant difference between survival of lady beetle larvae fed bee pollen or corn pollen nor was there a difference in survival between larvae fed Bt and non-Bt pollen. There was a significant difference between the reference group (potassium arsenate) and other test groups since no larvae survived in the reference group. The 100% mortality observed in the reference group verified that the lady beetles were ingesting the diet (Table 1).

Table 1. Lady beetle larval survival to pupation and/or adult emergence

Treatment	% Survival
test pollen (MON 863)	96.7
control pollen (MON 864)	90
bee pollen (assay control)	93.3
reference pollen (potassium arsenate)	0

There was also no significant difference between the bee pollen and non-Bt and Bt pollen in developmental time of larvae to pupae and/or adults; nor was there a difference in adult weight (Table 2).

Table 2. Survival and development of lady beetle larvae to pupae and adults.

Treatment	# Larvae Surviving to Pupae &/or Adults	Avg Days to Pupation ± SE	Avg Days to Adult ± SE	Adult Weight ± SE (mg)

test pollen (MON 863)	29	12.7 ± 0.54	16.8 ± 0.55	10.9 ± 0.39
control pollen (MON 864)	27	12.9 ± 0.56	16.9 ± 0.57	10.7 ± 0.40
bee pollen (assay control)	28	13.8 ± 0.55	17.8 ± 0.56	10.4 ± 0.39

Conclusions:
Development and survival of *C. maculata* were not affected by feeding on pollen containing the variant Cry3Bb1 protein at the level of ~93 µg/g fresh pollen weight.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. This study was conducted at EPA's request. This test was conducted with pollen levels greater than or equal to levels lady beetle larvae are expected to be exposed to in the field. Therefore, the NOEC for Cry3Bb1 expressed in pollen is >93 µg/g fresh pollen weight. It can be concluded from this study that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

DATA EVALUATION REPORT

REVIEWED BY: Robyn Rose, Entomologist
Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER: Zigfridas Vaituzis, Ph.D., Microbiologist
Biopesticides and Pollution Prevention Division
STUDY TYPE: Non-target effects of Cry3Bb1 on monarch butterfly larvae
MRID NO.: 455382-05
STUDY NO.: 01-01-39-26
SPONSOR: Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB: Department of Environmental Biology, University of Guelph, Guelph, Ontario, NIG 2W1, Canada
TEST MATERIAL: Pollen expressing *Bacillus thuringiensis* Cry3Bb1 Protein
AUTHOR: Mark Sears, PhD and Heather Mattila
STUDY COMPLETED: July 27, 2001
CLASSIFICATION: **Acceptable**

Study Summary

Title: "Determination of the Toxicity of Corn Pollen Expressing a Cry3Bb1 Variant Protein to First Instar Monarch Butterfly Larvae (*Danaus plexippus*) via Laboratory Bioassay"

Authors: Mark Sears and Heather Matilla

Objective: This study was conducted to evaluate the affect of corn pollen expressing a Cry3Bb1 variant protein on survival and development of the monarch butterfly.

Methods:

This study was conducted to evaluate the lethal and sublethal effects of Bt corn pollen event MON 863 expressing Cry3Bb1.11098 (referred to as Cry3Bb1) protein on young monarch butterfly larvae (*Danaus plexippus*). Effects of corn pollen expressing the *cry3Bb1* gene was compared to non-Bt corn pollen from the MON 846 corn line. MON 863 and MON 846 identities were confirmed at the molecular level by Monsanto. An ELISA showed that there was 89.2 µg Bt/g pollen for MON 863 and no detectable Bt (<0.55 µg/g pollen) in the MON 846 corn pollen. Pollen was shipped from Monsanto to the University of Guelph and stored at -80°C until it was needed for the study.

Prior to testing, pollen was throughly sifted through a 150 mesh (90 microns) screen and subsequently a 250 mesh (63 microns) screen to remove debris, particularly anthers which may have a higher toxin concentration than pollen. After sieving, Pollen samples were re-analyzed by Monsanto using ELISA which showed a level of Cry3Bb1 protein of 63.1 µg/g fresh weight and no detectable level of Bt in the MON 846 corn.

Common milkweed (*Asclepias syriaca*) plants were grown in the greenhouse. A breeding colony was started from adults purchased from Greathouse Butterfly Farm, Inc. (Earleton, FL) that were confirmed to be free of the parasite *Ophryocystis elektroscirrha*. Rearing included 60 adult monarchs kept in 1×1×1 m screened cages with milkweed plants changed daily for monarchs to oviposit on. Rearing cages were kept in chambers set at 25°C, 75% relative humidity and a photoperiod of 16 hours light and 8 hours of dark.

There were 4 replicates for each bioassay and 18 bioassay arenas per replicate that included a milkweed leaf and 10 neonate monarchs. Replicates were blocked by initiating each bioassay on consecutive days. The 18 bioassays included 7 with Bt pollen, 7 with non-Bt pollen and 4 with no pollen. Pollen was evenly spread on milkweeds by an air pressurized (20 psi) Venturi-shaped tube. Even distribution of pollen was visually verified under a dissecting microscope and the number of grains in a 1 cm^2 area on each side of the upper leaf surface were counted. Leaves with pollen densities 10% above or below the desired testing level were discarded. Doses of the 4 replicated were determined to be 55.7±2.1, 107.9±3.6, 199.1±5.5, 423.4±11.3, 847.4±21.2, 1652.3±48.1 and 3279.0±60.5 grains/cm^2.

Neonate larvae (1st instars <24 hrs old) were used in this bioassay. Bt and non-Bt pollen was applied to milkweed leaves in the laboratory at rates of 50, 100, 200, 400, 800, 1600 and 3200 grains/cm^2. A maximum of 3200 pollen grains/cm^2 was chosen because previous studies conducted by this laboratory demonstrated that high levels of pollen deterred monarch feeding on milkweed leaves leading to artificially inflated mortality. Larvae were weighed prior to placing them on milkweed leaves kept in ventilated plastic arenas placed in a growth room set at 20°C, 50% relative humidity, and 16 hours of light and 8 hours of dark. Larvae were allowed to feed on pollen dusted milkweed leaves for 4 days and then moved to clean leaves for an additional 6 days. Observations of larval survival and development were evaluated 48 hours, 96 hours and 10 days after test initiation. Larval weights were determined on Days 0, 4 and 10 to determine weight gain in mg/larva by calculating [(weight$_{timeX}$/larva) - (weight$_{time0}$/larva)]. Leaf consumption was also evaluated 48 and 96 hours after test initiation and reported in cm^2/larva.

Percent survival, development, weight gain and leaf consumption were statistically analyzed (SAS) as a three-factor design (pollen type, dose and time) with a repeated measure on the last factor. A two-way ANOVA (pollen type and dose) was conducted from daily sampling data when the repeated measure model could not be fitted due to significant time interactions. The survival data was log transformed to equalize variance and significant differences (p=.05) were determined with Tukey's test.

Results:
Overall larval survival decreased over time; however, there was no statistical difference in survival of larvae feeding on the Bt or non-Bt pollen at the different doses. This implies that the reduction in larval survival was not due to feeding on Bt. On average, there was 95 to 100% larval survival after 48 and 96 hours of feeding on the milkweed leaves and 87.5 to 100% survival after 10 days (Table 1). Ten days after testing initiation, 71% of the arenas containing 10 larvae had 100% survival.

Table 1. Mean percent survival of larval cohorts ± S.E. after 48 hours, 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm^2)	48 hour Post-Treatment	96 hour Post-Treatment	10 Days Post-Treatment
Control	0	99.4 ± 0.6 %	98.8 ± 0.9 %	96.3 ± 1.8 %
Bt	50	100 ± 0 %	100 ± 0 %	100 ± 0 %
Non-Bt	50	100 ± 0 %	100 ± 0 %	100 ± 0 %
Bt	100	95 ± 2.9 %	95.5 ± 2.9	95 ± 2.9 %
Non-Bt	100	97.5 ± 2.5 %	95.5 ± 2.9	95 ± 2.9 %
Bt	200	100 ± 0 %	100 ± 0 %	97.5 ± 2.5 %
Non-Bt	200	100 ± 0 %	97.5 ± 2.5 %	92.5 ± 2.5 %
Bt	400	100 ± 0 %	100 ± 0 %	100 ± 0 %

Non-Bt	400	97.5 ± 2.5%	100 ± 0 %	100 ± 0 %
Bt	800	97.5 ± 2.5 %	97.5 ± 2.5 %	95 ± 5 %
Non-Bt	800	97.5 ± 2.5 %	97.5 ± 2.5 %	95 ± 2.9 %
Bt	1600	100 ± 0 %	100 ± 0 %	100 ± 0 %
Non-Bt	1600	100 ± 0 %	100 ± 0 %	92.5 ± 4.8 %
Bt	3200	97.5 ± 2.5 %	95 ± 2.8 %	92.5 ± 4.8 %
Non-Bt	3200	100 ± 0 %	95 ± 5 %	87.5 ± 7.5 %

Mean development of larvae were effected by time; however there was no statistical difference between larvae feeding on Bt and non-Bt pollen. After ten days, average larval instar stage ranged from 1.28 to 1.55, 1.98 to 2.26 and 4.31 to 4.68 at 48 hours, 96 hours and 10 days respectively after test initiation (Table 2).

Table 2. Mean larval development of <24 hour old 1st instar larvae ± S.E. after 48 hours, 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm^2)	Mean Larval Instar Stage 48 hour Post-Treatment	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 days Post-Treatment
Control	0	1.34 ± 0.6	2.12 ± .04	4.53 ± .07
Bt	50	1.55 ± .09	2.00 ± .04	4.53 ± .21
Non-Bt	50	1.28 ± .06	2.10 ± .07	4.40 ± .12

Table 2. Continued

Pollen Type	Pollen Density (grains/cm^2)	Mean Larval Instar Stage 48 hour Post-Treatment	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 days Post-Treatment
Bt	100	1.39 ± .11	2.05 ± .05	4.31± .18
Non-Bt	100	1.31 ± .10	2.05 ± .05	4.50 ± .16
Bt	200	1.45 ± .06	2.08 ± .05	4.60 ± .24
Non-Bt	200	1.30 ± .06	2.05 ± .05	4.38 ± .13
Bt	400	1.43 ± .01	2.18 ± .09	4.68 ± .20
Non-Bt	400	1.40 ± 0.17	2.23 ± .19	4.50 ± .22
Bt	800	1.44 ± .06	2.05 ± .05	4.49 ± .17
Non-Bt	800	1.37 ± 0.13	2.26 ± 0.12	4.66 ± .14
Bt	1600	1.33 ± .06	2.13 ± .10	4.35 ± .13
Non-Bt	1600	1.35 ± 0.19	2.03 ± .10	4.44 ± .23

Bt	3200	1.41 ± 0.11	1.98 ± .05	4.44 ± .16
Non-Bt	3200	1.35 ± 0.17	2.03 ± .03	4.40 ± .23

Although larval weight gain significantly changed over time, there was no statistical difference in larval weight gain or pollen consumption between Bt and non-Bt pollen 96 hours and 10 days after test initiation (Table 3). Additional analysis showed no statistical difference in the pollen and dose interaction.

Table 3. Mean larval weight gain ± S.E. (mg/larva) of <24 hour old 1st instar larvae after 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm^2)	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 Days Post-Treatment
Control	0	9.15 ± 0.62	321.07 ± 13.08
Bt	50	9.40 ± 1.07	341.16 ± 26.76
Non-Bt	50	7.94 ± 1.26	306.67 ± 23.80
Bt	100	7.51 ± 0.34	333.88 ± 36.21
Non-Bt	100	7.88 ± 0.90	294.12 ± 25.00
Bt	200	8.58 ± 1.56	351.12 ± 53.52
Non-Bt	200	6.92 ± 1.24	295.47 ± 42.16
Bt	400	9.65 ± 1.15	307.23 ± 30.04
Non-Bt	400	8.37 ± 1.20	349.21 ± 69.60

Table 3. Continued

Pollen Type	Pollen Density (grains/cm^2)	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 Days Post-Treatment
Bt	800	9.28 ± 0.64	330.97 ± 18.98
Non-Bt	800	8.17 ± 1.66	410.94 ± 69.89
Bt	1600	8.56 ± 1.17	319.40 ± 29.21
Non-Bt	1600	7.64 ± 1.68	274.78 ± 32.73
Bt	3200	7.44 ± 0.64	289.35 ± 31.74
Non-Bt	3200	7.03 ± 1.88	305.12 ± 31.66

There was not a statistically significant difference in larval milkweed leaf consumption between Bt and non-Bt treatments. However, there was a downward trend in consumption of both Bt and non-Bt pollen as the dose increased (Table 4). This trend was not statistically significant. In general, results of this study showed that monarch larvae consumed more leaf tissue dusted with Bt pollen than non-Bt pollen.

Table 4. Mean leaf consumption ± S.E. (cm^2/larva) after 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm^2)	96 hour Post-Treatment	10 Days Post-Treatment
Control	0	0.37 ± 0.02	1.26 ± 0.07
Bt	50	0.40 ± 0.04	1.37 ± 0.21
Non-Bt	50	0.40 ± 0.04	1.24 ± 0.12
Bt	100	0.37 ± 0.02	1.40 ± 0.13
Non-Bt	100	0.41 ± 0.06	1.11 ± 0.12
Bt	200	0.35 ± 0.03	1.29 ± 0.13
Non-Bt	200	0.31 ± 0.03	1.17 ± 0.20
Bt	400	0.34 ± 0.03	1.27 ± 0.05
Non-Bt	400	0.37 ± 0.02	1.28 ± 0.11
Bt	800	0.35 ± 0.03	1.37 ± 0.13
Non-Bt	800	0.29 ± 0.04	1.13 ± 0.23
Bt	1600	0.32 ± 0.02	1.31 ± 0.15
Non-Bt	1600	0.29 ± 0.04	1.04 ± 0.22
Bt	3200	0.32 ± 0.02	1.11 ± 0.08
Non-Bt	3200	0.30 ± 0.02	0.84 ± 0.07

Conclusions:
Neonate monarch survival was not affected after feeding on milkweed dusted with corn pollen expressing Cry3Bb1 event MON 863 for 2, 4 or 10 days of pollen exposure. Larval weight gain and milkweed leaf consumption were also not affected by feeding on Bt pollen 96 hours and 10 days after exposure. In fact, there was an increase in leaf consumption 96 hours after test initiation in the Bt group. In addition, increasing doses of corn pollen (Bt or non-Bt) did not effect larval survival, development, or weight gain during the 10-day bioassay. The only effect seen was an increase in leaf consumption 48 hours after test initiation in both the Bt and non-Bt groups.

Pollen densities in the field are not expected to be as great as 1600 or 3200 grains/cm^2. Levels of 400 and 800 pollen grains/cm^2 would probably be rare. Therefore, results of this study indicate that young monarch larva (the most sensitive stage) will not be adversely affected by exposure to Bt corn pollen expressing Cry3Bb1 in the field.

Reviewer's Comments:
Although this study was not conducted in accordance with FIFRA GLP compliance as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160, a scientifically valid study was performed by a university laboratory and is considered

acceptable. This study was not required nor requested to register Cry3Bb1 because it is a coleopteran active protein that is not expected to affect lepidopterans such as the monarch butterfly. In addition, extensive research conducted on the potential affects of monarch larvae feeding on Bt corn pollen has shown a lack of concern of acute toxicity. However, due to recent public concern of possible adverse effects of Bt corn on monarchs, Monsanto sponsored this study and submitted it to the Agency for review. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in acute toxic or developmental effects to monarch larvae.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Field evaluation of exposure to Cry3Bb1 protein effects on non-target organisms
MRID NO.:	455382-06
STUDY NO.:	01-01-39-16
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Monsanto Agronomy Center, Monmouth, IL 61462, Monsanto Company, 800 N Lindbergh Blvd., St. Louis, MO 63141
TEST MATERIAL:	*Bacillus thuringiensis* Event MON 863 Cry3Bb1 Protein
AUTHORS:	Muhammad A. Bhatti, Carol L. Pilcher, Michael J. McKee, Thomas E. Nickson, Graham P. Head and Clinton D. Pilcher
STUDY COMPLETED:	July 19, 2001
CLASSIFICATION:	**Supplemental to submitting the full report after completion of**

this study.

Study Summary

Title: "Field Evaluation of the Ecological Impact of Corn Rootworm Insect-Protected Corn on Non-Target Organisms"

Authors: Muhammad A. Bhatti, Carol L. Pilcher, Michael J. McKee, Thomas E. Nickson, Graham P. Head and Clinton D. Pilcher

Objective: This study was conducted to evaluate the ecological impact of MON 863 Bt corn grown under different insecticide regimes on abundance of non-target organisms relative to non-transgenic corn.

Methods:

Transgenic Bt corn and non-transgenic corn were monitored for two years (2000-2001) for the stability and abundance of non-target organism communities in soil, soil surface and foliage including arthropods, earthworms and soil microbes as well as plant diseases. Additional data on soil fertility, microbial populations and soil Bt protein was collected during this study but not reported at this time. Cry3Bb1 corn rootworm (*Diabrotica* spp.; CRW) protected corn event MON 863 (RX670 pedigree) was compared to a non-transgenic control hybrid (RX670) with a similar genetic background.

All experimental plots were managed according to typical cultural practices of commercially grown corn in the region and included the application of the herbicides acetochlor and atrazine after planting and before emergence. Bt and control hybrids were planted in a split-plot design with four replications planted 20 ft apart. Rows were planted 30 in apart, seeded at a rate of approximately 1.7 seeds/ft and planted 1.5 - 1.75 inches deep. Plots (240 ft × 60 ft) were divided into 24 row subplots (60 ft × 60 ft) that served as replications receiving one of 4 insecticide regimes. Insecticide treatments of the Bt and non-Bt plots included: 1. No insecticide; 2. Seed treated with Gaucho prior to planting; 3. The granular insecticide Force 3G applied and incorporated in furrows at planting; and 4. A foliar insecticide, Pounce 3.2 EC, applied at the V10 and R2 corn growth stages to control 1st and 2nd generation CRW adults. A 4-row buffer of non-transgenic corn was planted around each plot to minimize edge effects from adjacent subplots.

Invertebrates were sampled from the soil, soil surface and foliage. Soil-dwelling invertebrates were collected using a "pan trap" which utilized a modified Burlese extraction method. At the V-6, V-10 and R-1 corn growth stages, 3 root balls including an 8 in diameter of root mass and soil were randomly selected from rows 8, 12 and 16 of each subplot. Root balls were transported to the greenhouse in an onion sack where they were suspended 2 in above a 10 in pan containing 500 mL of a 50:50 alcohol: water solution and a small amount of antifreeze to delay alcohol evaporation. Root balls were kept in the greenhouse with the cooling system off to increase invertebrate extraction for 7 days. After samples were sifted through an 80-mesh screen, they

were placed in the refrigerator for later counting identification to the family level.

Surface-dwelling invertebrate were sampled in the field with pitfall traps. Four plastic cups (474 mL, 110 mm rim diameter) were placed rows 9 and 16 of each subplot to trap invertebrates. Cups were set inside larger plastic cups (947 mL, 110 mm rim diameter) that stayed in the field and were protected from rain by a 20 cm × 20 cm plastic plate. Invertebrates were trapped and preserved in 100 mL ethylene glycol for 3 day periods during the V-6 to R-4 growth stages. Samples were stored in ethylene glycol in the refrigerator until they were counted and identified to the family level.

Foliage-dwelling invertebrate were monitored by yellow sticky traps (Pherocon AM™) set in the field at canopy level and adjusted as the season progressed. Three traps were placed 15 ft apart in rows 6, 12 and 18 of each subplot from the V-6 to R-4 corn growth stages and switched at approximately 7 day intervals. Sticky traps were stored in the refrigerator until organisms were counted and identified to family and the genus and species level when possible.

Amendments to this study written during the 2000 season included sampling for lady beetles using a drop cloth technique. Three deviations were also noted in the methods of this study. 1) There were deviations in sampling time during the season due to physical constraints. 2) Two soil core samples were collected rather than 4. 3) Insect thresholds were not determined nor was equipment calibrated within 24 hours of insecticide applications.

Data was analyzed by SAS using a mixed linear model (PROC MIXED) to compare transgenic and non-transgenic hybrids among the 4 insecticide treatments, their interactions and effect of sampling time. Since the number of insects captured depended on the area of the trap, square root transformations to adjust for normality were done prior to data analysis. Hybrid effects were determined from main plot residual variation, insecticide treatment effects and their interaction with hybrids was determined from the subplot residual variation and effects of sample date were evaluated from the residual variation.

Results:
Interim results are reported at this time and a full report will be submitted to the Agency for review when all data is collected and analyzed. Replications of each treatment summed across sampling dates are reported as well as the mean and standard error of the most abundant species collected.

The 7 most abundant species counted and identified in the pan samples were: Araneae (spiders), Carabidae (immature and adult ground beetles), Chilopda (centipedes), Staphylinidae (rove beetles), Japygidae (diplurans) and Oligochaeta (earthworms). Preliminary results show no statistically significant difference between the Bt and non-Bt hybrids in the number of soil-dwelling organisms collected from pan samples. There was also no significant effect on abundance of organisms from the insecticide treatments and their interaction with hybrids. The number of carabid larvae collected in the pan samples varied among Bt and non-Bt plots treated with different insecticides. For example, the abundance of Aranaea were less in the non-Bt plots

treated with soil insecticide and the number of Oligochaeta and Japygidae were very variable but with no trends observed. On the other hand, the number Chilopods and Staphaylinds were somewhat consistent among insecticide treatments. Since the number of pest species (e.g., scarab beetles, sap beetles, wireworms/click beetles and immature CRW) in the pan samples were low, no comparison between treatments was made.

The most abundant surface-dwelling species collected in pitfall traps were Araneae (spiders), Carabidae (immature and adult ground beetles), Gryllidae (crickets), Cicindellidae (tiger beetles), Chilopoda (centipedes), Diplopoda (millipedes), Formicidae (ants), Staphylinidae (rove beetles) and Silphidae (carrion beetles). Of these organisms, spiders, Carabids, and crickets were the predominant species sampled respectively and were not significantly different between the two hybrids. There was also no interaction effect of insecticide and hybrid found for any of the species evaluated. Insecticide treatments did not significantly effect the number of Carabids or crickets but did effect spider abundance. There were 30% fewer spiders found in soil treated plots and 50% fewer in foliar treated plots then the seed and no insecticide treated plots. Effects of individual insecticides will be evaluated and presented in the final report.

In addition to non-pest species, the abundance of pest insects including Nititulidae (sap beetles), Scarabaeidae (scarab beetles), Chrysomelidae (corn flea beetles) and a minimal number of Elateridae (click beetles) was determined in the pitfall traps. There was no differences in the number of pest insects between hybrids but there were differences between insecticide treatments. There was a large number of sap beetles collected and abundance was highly variable in all treatments. Scarab beetle abundance was consistent among plots except for foliar treatments which resulted in fewer beetles. Soil and foliar insecticides resulted in fewer corn flea beetles.

The 5 most abundant pest species captured on sticky traps were *Diabrotica barberi* (northern corn rootworm), *Diabrotica virgifera* (western corn rootworm), *Chaetocnema pulicaria* (corn flea beetle), Nititulidae (sap beetle) and *Rhopalosiphium maidis* (corn leaf aphids). The predominant foliage-dwelling beneficial insects collected on sticky traps were lady beetle species including *Harmonia axyridis* (Asian lady beetle), *Coccinella septempunctata* (7-spotted lady beetle), *Hippodamia convergens* (convergent lady beetle) and *Cycloneda munda* (lady beetle). Other non-target beneficial invertebrate found on sticky traps were spiders, parasitic Hymenoptera, Syrphidae (syrphid flies), *Chrysoperla carnea* (green lacewing), Hemerobiidae (brown lacewings), Carabidae (ground beetles), Fomicidae (ants) and Nabidae (damsel bugs).

The key natural enemies in corn fields *Coleomegilla maculata* (12-spotted lady beetle), *Orius insidiosus* (minute pirate bug) and the parasitic Hymenoptera *Macrocentrus grandii* (Macrocentrus), were also abundant in the treated and control plots. The number of beneficial insects was not significantly different between the Bt and non-Bt plots nor was the interaction between insecticide treatment and hybrid. Although *C. maculata*, a generalist lady beetle predator that feeds on ECB eggs and larvae, abundance was not significantly effected by the insecticide treatments, there were less observed in the foliar treated plots. In general, the number of *C. maculata* increased during the growing season. The number of *M. grandii* were

significantly less in plots receiving foliar insecticide treatments. This affect was probably due to the insecticide directly affecting the parasitoid or from reducing ECB populations. There were no differences between treated and control plots in the abundance of *O. insidiosis*, a generalist predator that feeds on ECB eggs and larvae. However, foliar insecticide treatments reduced the number of *O. insidiosis* by 50% and the population never fully recovered. *O. insidiosis* numbers also differed among sample dates; abundance increased from the first to the third date and dropped on the final sample date.

Conclusions:
These results are preliminary and this study will be continued for a second year. According to the pan, pitfall and sticky trap sample methods used in this study, abundance of the predominant species were not significantly different between the Bt treated and non-Bt control plots. The most abundant soil-dwelling organisms found in pan and pitfall traps were spiders and Carabids. The abundance of most important foliage-dwelling natural enemies captured on sticky traps, *C. maculata*, *O. insidiosis* and *M. grandii*, did not differ between treated and control plots. These preliminary results indicate that planting event MON 863 does not negatively impact the abundance of several beneficial non-target invertebrate. Data also indicated that planting event MON 863 results in less impact on non-target invertebrate than conventional pest management practices.

Reviewer's Comments:
Although this study was not conducted according to FIFRA GLP compliance as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160, this study was conducted according to a scientifically valid, peer reviewed protocol that is acceptable. Results reported in this submission suggest that planting event MON 863 CRW protected corn will not adversely affect non-target beneficial invertebrate abundance in the field. On the contrary, a reduction in pesticide use that may result from planting MON 863 corn will probably result in increased numbers of non-target invertebrate in CRW protected Bt corn.

This submission only reported results from the 2000 growing season. A final report will be submitted to the Agency after the 2001 data is collected and analyzed. The final report should include the location of the 2000 and 2001 test sites as well as the additional data on soil fertility, microbial populations and soil Bt protein collected during this study but not reported at this time. This study should also be conducted at additional test sites in different geographic locations to account for regional differences in insect populations.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Endangered Species Impact Assessment
MRID NO.:	455770-03
REPORT NO.:	MSL-17614
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TEST MATERIAL:	*Bacillus thuringiensis* Event MON 863 Cry3Bb1 Protein
AUTHORS:	Jian J. Duan, Michael J. McKee, Graham Head, Christopher R. Brown
STUDY COMPLETED:	January 4, 2002
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Endangered Species Impact Assessment for Cry3Bb1 Protein in Transgenic Corn Event MON 863"

Authors: Jian J. Duan, Michael J. McKee, Graham Head, Christopher R. Brown

Objective: This report evaluated the potential risk Cry3Bb1 corn rootworm protected corn poses to endangered species.

Endangered Species Assessment

Cry3 proteins including Cry3Bb1 are known to be highly specific against coleopteran insects and are not hazardous to vertebrate animals. It has been generally demonstrated that Cry3 proteins do not pose a risk to non-target animals or invertebrates. The Cry3Bb1 protein is specifically toxic to Chrysomelid beetles including corn rootworms (*Diabrotica* spp.) and Colorado potato beetles (*Leptinotarsa decemlineata*). Currently, there are no Chrysomelid species listed on the endangered species list and no other species are expected to be sensitive to Cry3Bb1. Therefore, no adverse affects from Cry3Bb1 event MON 863 are expected against endangered species.

Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera. This endangered species assessment was based on the Hazard Evaluation Division, Standard Evaluation Procedure, Ecological Risk Assessment (U.S. EPA, 1986).

Hazard Assessment: Most Susceptible Coleoptera

According to research that has been conducted with Cry3Bb1 thus far, Chrysomellids are the most sensitive species. Of species in the Chrysomellid family, the Colorado potato beetle (*Leptinotarsa decemlineata*; CPB) has been identified as the most sensitive species to the Cry3Bb1 protein. Astwood *et al.* (2001) determined the Cry3Bb1 LC_{50} for CPB to be 2.4 µg/mL of diet. Since CPB is the most sensitive species identified to the Cry3Bb1 protein, 2.4 µg/mL of diet will be used for this risk assessment (Table 1).

Table 1. Sensitivity of tested species to the Cry3Bb1 protein.

Order/Family	Genus/Species	Stage Tested	LC_{50} (µg/mL diet)	NOEC* (µg/mL diet)
Coleoptera/Chrysomelidae	*Leptinotarsa decemlineata*	larvae	2.4	-
Coleoptera/Chrysomelidae	*Diabrotica virgifera*	larvae	75	-
Coleoptera/Bruchidae	*Callosobruchus maculatus*	larvae	>200	200
Coleoptera/Coccinellidae	*Hippodamia convergens*	adults	>8000	8000
Coleoptera/Tenebrionidae	*Tribolium castaneum*	larvae	>200	200
Coleoptera/Curculionidae	*Anthonomus grandis*	larvae	>50	50
Coleoptera/Curculionidae	*Anthonomus eugenii*	larvae	>200	200
Coleoptera/Curculionidae	*Sitophilus oryzae*	larvae	>200	200
Lepidoptera/Noctuidae	*Helicoverpa zea*	larvae	>200	200
Lepidoptera/Crambidae	*Ostrinia nubilalis*	larvae	>200	200

Hymenoptera/Brachonidae	*Nasonia vitripennis*	adults	>200	400
Hymenoptera/Aphidae	*Apis mellifera*	adults	>360	360
Hymenoptera/Aphidae	*Apis mellifera*	larvae	not reported	1790
Neuroptera/Chrysopidae	*Chrysoperla carnea*	larvae	>8000	8000
Collembola	*Folsomia candida*	nymphs/adults	>870	870

* NOEC= maximum dose tested

Exposure Assessment for Endangered Coleoptera

Terrestrial and aquatic exposure were considered in this assessment since non-target coleopterans may be exposed to the Cry3Bb1 protein within corn fields or in surrounding areas from plant tissue (e.g., pollen) movement offsite. However, the distance pollen moves outside of the corn field should be considered. Pleasants *et al.* showed that <25 grains of pollen per cm^2 are expected 4-5 meters from the corn field edge. A relative comparison of surface ratio of milkweed to other substrates (e.g., other host plants, arthropod prey, animal carrion) can be used as a basis for estimating the amount of pollen that may leave the field. The maximum concentration of Cry3Bb1 protein in corn tissue has been determined to be 93 µg/g fresh weight pollen or other tissue. Based on this concentration, <0.03 µg Cry3Bb1 protein/g of diet would be expected to be deposited 4-5 meters from the field edge.

The potential of aquatic organisms to be exposed to the Cry3Bb1 protein is minimal. Such exposure would occur from runoff of the protein (either free or sequestered in plant debris) into adjacent water bodies or pollen drift. Since movement of Cry3Bb1 in soil into water bodies is probably negligible, pollen drift was considered the primary source of potential risk to endangered aquatic Coleoptera. According to Monsanto's estimation based on studies conducted by Pleasants *et al.* (2001), if 100% of the pollen grains leaving the field were deposited in a 1 ha pond with 2 m depth and located $1 m from the edge of the corn field, <0.0001 µg Cry3Bb1/mL of water would be expected (Table 2).

Table 2. Pollen grain density

Distance from field edge	Pollen grains/cm^2 on surface H_2O	Total pollen grains in 1 ha surface H_2O	Cry3Bb1 protein (µg/1-ha pond)	Max. Cry3Bb1 protein in 1-ha, 2 m deep pond (µ/mL)
1	500	5.000×10^{10}	1.162500×10^{6}	5.81250×10^{-5}
2	187.5	1.875×10^{10}	4.359375×10^{5}	2.17969×10^{-5}
4-5	62.5	6.250×10^{9}	1.453125×10^{5}	7.26563×10^{-6}

Risk Characterization for Endangered Species

A 10-fold safety factor for terrestrial species and a 20-fold safety factor for aquatic species based on the LC_{50} is recommended by EPA for endangered species (U.S. EPA 1986 Hazard Evaluation Division, Standard Evaluation Procedure, Ecological Risk Assessment). Based on the 2.4 µg/mL diet LC_{50} for the CPB, the most sensitive species to Cry3Bb1 protein, a maximum exposure of 0.23 µg/g (10-fold safety margin) is expected 1 m from the field edge, 0.09 µg/g (28-fold safety

margin) is expected 2 m from the field edge and 0.03 µg/g (82-fold safety margin) is expected 4-5 m from the field edge. A 1-ha pond with a 2 m depth located 1 m from the edge of the corn field that has 500 grains/cm^2 would have a maximum exposure of 5.81250×10^{-5} which represents a 41290 safety margin. These data indicate that pollen drift from MON 863 corn fields will not pose a terrestrial or aquatic exposure risk.

Conclusions

According to Monsanto's risk analysis, event MON 863 field corn expressing the Cry3Bb1 protein will not pose a risk to aquatic or terrestrial endangered species. Endangered coleopterans, the most sensitive species to Cry3Bb1 are not expected to occur in or near corn fields. If an endangered coleopteran were to occur in a corn field, exposure and consumption would be minimal because of their habits and feeding biology. Plant feeding Coleoptera from the Cerambycidae and Scarabaeidea would not occur because their host plants are not present in corn fields. The endangered Cerambycid feeds on the elderberry tree (*Sambucus* sp.) which grows in riparian forests along rivers and streams. The endangered Scarab's host plant occurs in sandy habitats where corn is not likely to be grown. Other terrestrial beetles would be minimally exposed to pollen from food items such as prey and carrion.

Reviewer's Comments

Based on a 10-fold safety factor for terrestrial species and a 20-fold safety factor for aquatic species and a lack of exposure, no unreasonable adverse effects of MON 863 to endangered Coleoptera are expected. According to Monsanto's submission, the LC_{50} for the CPB, the most sensitive species to Cry3Bb1 protein, is 2.4 µg/mL diet. A maximum exposure of 0.23 µg/g (10-fold safety margin) is expected 1 m from the field edge, 0.09 µg/g (28-fold safety margin) is expected 2 m from the field edge and 0.03 µg/g (82-fold safety margin) is expected 4-5 m from the field edge. A 1-ha pond with a 2 m depth located 1 m from the edge of the corn field that has 500 grains/cm^2 would have a maximum exposure of 5.81250×10^{-5} which represents a 41290 safety margin. These data indicate that pollen drift from MON 863 corn fields will not pose a terrestrial or aquatic exposure risk.

Many of the endangered and threatened beetles occur in cave or aquatic habitats. None of the endangered beetles are expected to occur in or near corn fields (Table 3). The American burying beetle may occur in old fields or cropland hedge rows. However, based upon the feeding habits of the American burying beetle, it is not expected to occur within corn fields nor will it be exposed to Cry3Bb1 protein. Adult American burying beetles are classified as opportunistic scavengers that feed on anything dead and bury vertebrate carcasses which larvae feed on. Carrion regurgitated by adults is fed to larvae until they are able to feed directly on a carcass.

Table 3. Endangered Beetles

Common Name	Scientific Name	Family	Status	States	Host Plant/Habitat

American burying beetle	*Nicrophorus americanus*	Silphidae	E	AK, MA, MI, NE, OH, OK, RI, SD	Cropland/hedgerow; forest (conifer, hardwood,); grassland/herbaceous, old field, shrubland/chaparral; eggs laid in soil adjacent to buried carcass; adults live above ground
Coffin cave mold beetle	*Batrisodes texanus*	Pselaphidae	E	TX	Occurs in very small isolated caves within the Edwards Limestone Formation. Has only been found under rocks lightly buried in silt in total darkness
Comal Springs dryopid	*Stygoparnus comalensis*	Dryopidae	E	TX	Occurs in subterranean waters (several outlets of Comal Springs which forms the headwaters of the Comal River).

Table 3. Continued

Common Name	Scientific Name	Family	Status	States	Host Plant/Habitat
Comal Springs riffle	*Heterelmis comalensis*	Elmidae	E	TX	Aquatic; Inhabits the gravel substrates & shallow riffles in spring runs
delta green ground beetle	*Elaphrus viridis*	Carabidae	T	CA	Predators preying on softbodied arthropods particularly Collembola. Typically occurs along the margins of vernal pools within 1.5 m of the water but has been found 100 m away. Prefers sandy mud substrate that slopes into the water & low-growing vegetation that provides 25-100% cover.
Hungerford's crawling water beetle	*Brychius hungerfordi*	Halipilidae	E	MI, Canada	Prefers a warm shallow, gravel bottom outflow stream - 1-2 miles below the lake. Found with - 2 ft deep plant roots.

Kretschmarr Cave mold beetle	*Texamaurops reddelli*	Pselaphidae	E	TX	Localized & distinct cave fauna occurring in very small isolated caves within the Edwards Limestone Formation. Found under rocks buried in silt in total darkness.
Mount Hermon June beetle	*Polyphylla barbata*	Scarabaeidae	E	CA	Adults don't feed & larvae feed & occur on roots underground. Occurs in sparsely vegetated, sand parkland & other sandy areas within chaparral & ponderosa pine stands.
Tooth Cave ground beetle	*Rhadine persephone*	Carabidae	E	TX	A subterranean obligate feeding on crickets. Occurs in deep uncompacted silt in small isolated karst caves within the Edwards Limestone Formation.

Table 3. Continued

Common Name	Scientific Name	Family	Status	States	Host Plant/Habitat
valley elderberry longhorn beetle	*Desmocerus californicus dimorphus*	Cesambycidae	T	CA	Associated with elderberry trees (*Sambucus* spp.) in California's Central Valley during its entire life cycle
unnamed ground beetle	*Rhadine exilis*	Carabidae	E	TX	Subterranean obligate. A troglobitic ground beetle only found in caves in San Antonio area, Bexar County, TX feeding on cave cricket eggs & nymphs.
unnamed ground beetle	*Rhadine infernalis*	Carabidae	E	TX	Subterranean obligate. A troglobitic ground beetle only found in caves in San Antonio area, Bexar County, TX feeding on cave cricket eggs & nymphs.

Helotes mold beetle	*Batrisodes venyivi*	Pselaphidae	E	TX	Subterranean obligate. Found in caves in San Antonio area, Bexar County, TX
northeastern beach tiger beetle	*Cicindela dorsalis dorsalis*	Cincindelidae	T	CT, MA, MD, NJ, RI, VA	Adults and larvae occur in wide, undisturbed, dynamic, fine sand beaches on the east coast.
Ohlone tiger beetle	*Cincindela ohlone*	Cincindelidae	E	CA	Occurs in CA native grasslands including oatgrass & purple needlegrass where the substrate is poorly-drained clay or sandy clay soil over bedrock of Santa Cruz Mudstone.
Puritan tiger beetle	*Cincindela puritana*	Cincindelidae	E	CT, MA, MD, NH, VT	Occurs near the CT River or Chesapeake Bay. Adults and larvae found on the upper portions of sandy beaches near either fresh or salt water. Larvae found in assoc. with cliffs.

Information for Table 3 taken from
http://ecos.fws.gov/webpage/webpage_vip_listed.html?module=undefined&code=I&listings=0#I
http://shanana.berkeley.edu/essig/endins/desmocer.htm
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