UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Preliminary risk assessment for soil, soil surface and foliar invertebrates for *Bacillus thuringiensis* Cry3Bb protein, EPA Reg. No. 524-LEI; Barcode No. D262045; Case No. 066221; Submission No. S572997, submitted by Monsanto Co. for corn containing *Bacillus thuringiensis* Cry3Bb protein and the genetic material necessary for its production (vector ZMIR13L) in corn for control of corn root worm (*Diabrotica* spp.)

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Pesticide Name: Modified *Bacillus thuringiensis* Cry3Bb1 insecticidal protein and genetic material necessary for its expression in corn developed with event MON 863 (vector ZMIR13L).

Registrant: Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198

The 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 the corn rootworm (CRW, *Diabrotica* spp.), a coleopteran pest of corn. The CRW is a primary pest of corn in the U.S., it 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 the 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.

Non-target insect studies are required to register a new pesticide (40 CFR §158.590). Monsanto Co. submitted studies on adult and larval lady beetles (MRID Nos 449043-14, 455382-04, 453613-01, 453613-02), 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), Collembola (MRID No 449043-17) and earthworm (MRID No 449043-16). Additional studies were submitted including field census evaluations (MRID No 455382-06), a soil degradation study (MRID No 451568-04) and an endangered species risk assessment (MRID No 455770-03). In March 10, 2000, the adult lady beetle study was reviewed. This review concluded that tests demonstrating the effect of adult and larval lady beetles feeding on corn pollen containing Cry3Bb1 should be conducted. These studies were conducted by Monsanto and submitted to the Agency. 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). The Agency has used these data to perform a risk assessment to the invertebrate corn field fauna.

The form of the test substances used in the studies for this assessment are plant material such as leaves, pollen or purified bacterially-produced Cry protein incorporated into the diet. The Scientific Advisory Panel (SAP) has provided guidance to EPA that while actual plant material is the preferred test material, bacterially-derived protein is also a valid test material.

A. Non Target Insect Hazard Assessment

Based on the evaluation of the submitted data no unreasonable adverse effects of Cry3Bb1 protein on the invertebrate fauna of the corn field are expected. Preliminary field data suggest that some subtle population shifts may occur, but these may not be as substantial as those resulting from conventional chemical pesticide use. Specific data are cited for concerns related to Cry protein fate in soils, potential effects on soil biota represented by the earthworm and field data, effects on non-target soil Coleoptera species, foliar insects, and endangered or threatened coleopteran species. The results of these studies are presented here in both tabular and more detailed descriptive format. The complete record of the submitted data can be found in the attached Data Evaluation Reports (DERs).

The FIFRA Scientific Advisory Panel (October, 2000) has recommended that non-target testing be focused on species exposed to the crop being registered. The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic corn fields were beneficial insects feeding on corn pollen and nectar, and soil invertebrates, particularly Coleoptera ssp. In lieu of extensive and difficult single species soil coleopteran toxicity testing followed by an extrapolation from the results to a community risk assessment, direct field data on coleopteran insect effects and abundance were requested, received and evaluated. Toxicity tests on representative beneficials from other taxa were also performed. The toxicity of the Cry3Bb1 protein has been evaluated following challenge of several species of invertebrates, including: adult and larval honeybees, a parasitic hymenopteran (*Nasonia*), green lacewings, lady beetles, collembola, monarch butterfly and earthworms. Reproductive and developmental effects evaluations were based on the collembola and the honey bee and lady beetle larva

maturation studies. Based on worst-case soil concentration, soil degradation studies show that Cry Bb1 protein in corn tissue is no longer detectable in agricultural field soil in 22 to 28 days.

1. Summary of Non-Target Organism Toxicity Testing

Generally the adult insect studies were of 30 days duration or until the negative control mortality reached 20%. Larval studies went through pupation and adult emergence.

Guideline No	Study	Results	MRID NO
885.4380	Honey Bee Larva Testing	The NOEC for honey bee larvae maturation to adult bees was determined to be >1,790 ppm Cry3Bb1 protein, (100X the concentration in pollen)	449043-10
885.4380	Honey Bee Adult Testing	An adult honey bee feeding study showed the NOEC of the Cry3Bb1 protein to be >360µg/mL. (20X the concentration found in pollen). Therefore, no risk from the Cry3Bb1 protein to honey bees is expected.	449043-11
885.4340	Parasitic Hymenoptera Larva Testing	The NOEC for parasitic Hymenoptera was determined to be >400 ppm Cry3Bb1 protein. Although 400 ppm Cry3Bb1 protein is only 1X field concentration in plants rather than 10X, parasitic Hymenoptera are not expected to feed directly on corn plant tissue. Therefore, minimal exposure and no hazard to parasitic Hymenoptera from Cry3Bb1 protein is expected.	449043-13
885.4340	A Dietary Toxicity Study with Green Lacewing Larvae	The NOEC for green lacewing larvae was determined to be >8,000 ppm Cry3Bb1 protein (20X field exposure). Based on these results it can be concluded that green lacewing will not be adversely affected when exposed to Cry3Bb1 in the field.	449043-12
885.4340	Effects of Bt Protein 11231 on Lady Beetles	This study showed that the NOEC for Cry3Bb1when fed to adult *H. convergens* is >8,000 µg Bt protein/mL diet., equivalent to 20X the maximum Bt protein concentration in plant tissue. A follow-up pollen feeding study was requested.	449043-14
885.4340	Lady Beetle Larval Pollen Feeding Study	The NOEC for Cry3Bb1 expressed in pollen is >93 µg/g fresh pollen weight. The larvae were observed through pupation to adult emergence. It can be concluded from this study that *Coleomegilla maculata* lady beetle larvae will not be adversely affected by Cry3Bb1 field corn pollen.	455382-04
885.4340	Ladybird Beetle, *Coleomegilla maculata* Pollen feeding study	No significant adverse effects were noted in a 30day 50% pollen feeding study. Based on these results , no hazard to *Coleomegilla maculata* is expected when feeding on MON 863 corn pollen in the field.	453613-01
885.4340	Ladybird Beetle, *Hippodamia convergens* Pollen feeding study	No significant adverse effects were noted in a 15 day 50% pollen in honey water feeding study. Based on these results , no hazard to *Hippodamia convergens* is expected when feeding on MON 863 corn pollen in the field.	453613-02

Guideline No	Study	Results	MRID NO
885.4340	Chronic Collembola Toxicity Study	The NOEC of the Cry3Bb1 protein for Collembola was found to be >872.5µg (50% corn leaf tissue in the diet). No adverse reproductive effects were noted. It can be concluded from this test that Cry3Bb1protein does not pose a hazard to Collembola, a representative soil inhabiting species.	449043-17
885.4340	Monarch Butterfly Larval Pollen Feeding Study	Neonate monarch survival, development, or weight gain were not affected after feeding on milkweed dusted with event MON 863 corn pollen up to 3200 grains/cm^2 for 2, 4 or 10 days of pollen exposure. Pollen densities in the field average 150 grains/cm^2. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in acute toxic or developmental effects to monarch larvae.	455382-05
154-35	Field evaluation of Cry3Bb1corn exposure on non-target organisms	Preliminary results indicate that there are no valid MON 863 corn related data to show an adverse effect on non-target and beneficial invertebrate abundance in the field. Planting MON 863 corn may result in increased numbers of natural enemies in CRW protected Bt corn.	455382-06 456530-03
850.6200	Earthworm Testing	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. The NOEC was >57.0 mg Cry3Bb1 protein/kg dry soil. The data show that no adverse effects to earthworms are expected from exposure to Cry3Bb1 protein producing corn plants.	449043-16
885.5200	Aerobic Soil Degradation of the *B.t.* Protein 11098	Finely ground corn leaf tissue in sandy loam field soil degradation data at worts-case field concentrations show that the Cry3Bb1 protein DT_{50} based on insect bioassays and ELISA were 2.37 and 2.76 days respectively. The DT_{90} estimates for the insect bioassays and ELISA were 7.87 and 9.16 days respectively. At #28 days the CryBb1 protein was below the detection level These results verify that the Cry3Bb1 protein degrades rapidly and does not accumulate in the soil. Additional testing in different soil types is requested.	451568-04
None	Endangered Species Impact Assessment	Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera. EPA performed an independent assessment and determined that no adverse affects from Cry3Bb1event MON 863 are expected to endangered/threatened Coleoptera species listed by the USFWS.	455770-03

2. Non-Target Arthropod Invertebrate Testing

a) Honey Bee Larvae

An acceptable study was conducted based on OPPTS Series 885-4380, Honey bee testing Tier I. 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 affect the integrity of the test.

Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (purity 96%; 1.79 mg active protein/mL water; current nomenclature refers to this protein as Cry3Bb1.) inoculated directly into larval brood cells prior to capping. Within 18 days after treatments were administered, all larvae emerged from capped brood cells. 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. 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 potassium arsenate which indicated that bees were exposed to the treatments. The NOEC for honey bee larvae was determined to be >1,790 ppm Cry3Bb1 protein.

According to the guidelines, non-target 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.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for honey bee larvae is >1,790 ppm. Therefore, no hazard to honey bee larvae and their development is expected from exposure to the Cry3Bb1 protein in corn pollen.

b) Adult Honey Bee Testing

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 affect the integrity of the test. This study was conducted based on OPPTS Series 885-4380, Honey bee testing Tier I.

The testing 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 study concluded that 360 µg/mL Cry3Bb1 protein did not affect survival or behavior of adult honey bees. The >360 µg/mL NOEC is 20X the concentration found in pollen. Therefore, no hazard to adult honey bees is expected from exposure to the Cry3Bb1 protein in corn pollen.

c) Parasitic Hymenoptera Testing

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 affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Non-target Insect Testing, Tier I.

A dietary toxicity study with the parasitic Hymenoptera (*Nasonia vitripennis*) was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (purity 96%; 34.5 mg active protein/mL water). 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. The NOEC for parasitic Hymenoptera was determined to be >400 ppm Cry3Bb1 protein. When 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.

Based on this test, the NOEC for adult parasitic Hymenoptera exposed to Cry3Bb1 in diet is >400 ppm and the LC_{50} is 8,000 ppm. The risk assessment is based on 400 ppm Cry3Bb1 protein which is 1X rather than 10X the field concentration in plants because parasitic Hymenoptera do not feed directly on corn plant tissues. Therefore, minimal exposure of parasitic Hymenoptera to Cry3Bb1 protein is expected. As a result, no hazard to *Nasonia vitripennis* is expected from exposure to MON 863 Cry3Bb1 corn.

d) Green Lacewing Larva Testing

An acceptable 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 affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Non-target Insect Testing, Tier I

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. There was 20% mortality in the negative control group on Day 10. Compared to the negative control, at day 10, 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. At test termination mortality for the 1,000 ppm reference group was 43% (13 of 30) and 100% mortality in the 10,000 ppm reference group (potassium arsenate).

The data show that the NOEC for green lacewing larvae exposed to Cry3Bb1 in diet is >8,000 ppm . Based on these results it is not expected that the green lacewing will be adversely effected when exposed to Cry3Bb1 in the field.

e) Lady Beetle Testing

EPA routinely requests studies on the effects of Bt proteins on beneficial non-target insects such as lady beetles (Coccinellidae), green lacewings (*Chrysoperla carnea*), parasitic Hymenoptera and honey bees (*Apis mellifera*). Since the Cry3Bb1 protein specifically targets coleopteran (beetle) insects, particular attention is warranted regarding potential effects of MON 863 on lady beetles. In a memorandum from Robyn Rose to Mike Mendelsohn dated March 10, 2000, in addition to a dietary exposure to the purified Cry protein (44903-14), the Agency requested a test demonstrating the effect of lady beetles feeding on corn pollen containing Cry3Bb1. Monsanto conducted three laboratory studies (MRID Nos 453613-01, 453613-02 and 455382-04) on two different lady beetle species (*Coleomegilla maculata* and *Hippodamia convergens*) in response to this request.

Adult protein dietary study

A diet containing purified Cry protein and honey was fed to the adult lady beetle (*H. convergens)* at rates one and 20 times the maximum protein concentration found in corn leaf tissue (MRID NO.:449043-14) . When the negative control group reached 20% mortality (10 days), the results showed no significant differences in mortality the rate between lady beetles fed 400 and 8,000 µg Cry3Bb1/mL of diet. Results from this study showed that the no-observed-effect-concentration NOEC for Cry3Bb1 when incorporated in diet and fed to *H. convergens* is >8,000 µg Bt protein/mL diet. Mortality for the 1,000 and 10,000 µg potassium arsenate/mL diet groups were 55% and 95% respectively at day 10. This demonstrates that toxicity can be measured by mixing a test substance in the lady beetle diet. Lady beetles do not feed on corn plant tissue. They do, however, prey on pest insects that may feed on corn tissue and contain Cry3Bb1 in their gut, thus exposing lady beetles to the Bt protein. There is approximately 390 µg Cry3Bb1/g fresh weight corn tissue. Lady beetle exposure is expected to be significantly lower than this since the corn tissue would be metabolized, eliminated, or otherwise degraded within the prey species. Since the NOEC was found to be 8,000 µg Cry3Bb1/mL diet which is 20 times higher than maximum expected exposure levels, no risk from Cry3Bb1 in corn plants to adult lady beetles is anticipated.

Larval pollen feeding study

Since corn pollen may comprise up to 50% of lady beetle larvae's diet, the effects of corn pollen containing event MON 863 Cry3Bb1 protein on lady beetle larvae (*Coleomegilla maculata*) was evaluated (MRID NO: 455382-04). 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 (93 µg/g fresh pollen weight) since this is the potential level of field exposure and an equal amount of the tephritid fruit fly diet. First instar lady beetle larvae were individually placed in test arenas to avoid cannibalism. There was not a statistically significant difference between developmental time of larvae to pupae and/or adults; nor was there a difference in adult weight survival between larvae fed bee pollen or corn pollen nor was there a difference 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. 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. This study demonstrates that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

Coleomegilla maculata* and *Hippodamia convergens adult pollen feeding studies

Coleomegilla maculata lady beetle adults were fed diets of transgenic corn pollen mixed with fruit fly eggs to determine potential effects of transgenic pollen to beetles (MRID NO: 453613-01). The corn (MON 863) test pollen contained the Cry3Bb1 protein at a concentration of 37.4 µg/g pollen. After 30 days of diet exposure, 83.3 and 80.0% of adult *C. maculata* survived in the test and control pollen groups, respectively. While these survival rates were significantly less than that in the assay control group (bee pollen which exhibited 100% survival), there were no significant differences between the test and control pollen groups. All adults in the positive control (arsenate treated corn pollen) died in less than 8 days. Results indicated that transgenic Bt corn pollen expressing the variant Cry3Bb1 protein has no negative effects on the survival of *Coleomegilla maculata* adults.

Hippodamia convergens adults were fed diets of transgenic corn pollen in honey to determine potential effects of transgenic pollen to beetles (MRID NO: 453613-02). The corn (MON 863) test pollen contained the Cry3Bb1 protein at a concentration of 37.4 µg/g pollen. After 15 days of diet exposure, 84% and 81% of adult *Hippodamia convergens* survived in the test pollen and control pollen groups, respectively. There were no significant differences in survival among the test pollen, control pollen and the assay control (honey only) treatment groups. Only 5% of beetles exposed to the positive control (arsenate treated corn pollen) survived. Results demonstrate that transgenic Bt corn pollen expressing the variant Cry3Bb1 protein had no negative effects on the survival of *Hippodamia convergens* adults from dietary exposure.

No adverse effects were detected when *Coleomegilla maculata* and *Hippodamia convergens* were fed MON 863 pollen in diet in the laboratory. Pollen levels fed on by the lady beetles in this study exceeded concentrations that are expected to be encountered in the field. Therefore, it can be concluded the MON 863 will not pose a risk to lady beetle adults in the field.

f) Collembola Feeding study

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 based on OPPTS Series 885-4340 Non-target Insect Testing, Tier I

Collembola were fed diets consisting 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 Cry protein- per gram diet respectively. Corn leaf tissue contained 1,745 µg Cry3Bb1 protein/g dried leaf tissue. These results show a NOEC of Cry3Bb1 protein in diet for Collembola to be > 872.5 µg/g diet. The study demonstrated that diet containing 50% corn leaf tissue expressing the Cry3Bb1 Bt protein did not adversely affect survival or reproduction of Collembola. This test was conducted

at concentration levels much greater than Collembola are expected to be exposed to in the field. 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.

This study adequately addresses potential concerns for Cry3Bb1 protein expressed in transgenic corn to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry3Bb1 proteins found in transgenic corn pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial non-coleopteran soil insects.

g) Monarch Butterfly Larval Pollen Feeding Study

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 lepidopteran-active Bt corn pollen has shown a lack of concern for subchronic 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 subchronic toxic or developmental effects to monarch larvae. Neonate monarch survival was not affected after feeding on milkweed dusted with up to 3200 pollen grains/cm^2. expressing Cry3Bb1 for 2, 4 or 10 days of pollen exposure. Larval development, weight gain and milkweed leaf consumption were also not affected by feeding on Bt pollen 96 hours and 10 days after exposure.

Pollen densities in the field are not expected to be as great as 3200 grains/cm^2. Pollen densities in the field average 150 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 larvae (at the most sensitive stage) will not be adversely affected by exposure to Bt corn pollen expressing Cry3Bb1 in the field.

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.

h) Field Evaluation of Cry3Bb1 Corn Exposure on non-target Invertebrates

These studies (MRID No 455382-06, 456530-03) are being conducted in Kansas, Nebraska, Illinois, Virginia, South Dakota and New York 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. Although these studies are not being conducted according to FIFRA GLP, they are being performed according to scientifically valid, peer reviewed protocols that are acceptable. The data suggest that planting event MON 863 CRW protected corn will not have an

unreasonable adverse affect non-target and beneficial invertebrate abundance in the field. On the contrary, a reduction in pesticide use that would result from planting MON 863 corn will probably result in increased numbers of natural enemies in CRW protected Bt corn.

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 these studies 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 (imidacloprid) prior to planting; 3. The granular insecticide Force 3G (teflathtin) applied and incorporated in furrows at planting; and 4. A foliar insecticide, Pounce 3.2 EC (permethrin), 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. Surface-dwelling invertebrate were sampled in the field with pitfall traps. Foliage-dwelling invertebrate were monitored by yellow sticky traps (Pherocon AM™) set in the field at canopy level and adjusted as the season progressed. Sampling for lady beetles was also done using a drop cloth technique.

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 (spiders) were less in the non-Bt plots treated with soil insecticide and the number of Oligochaeta (earthworms) and Japygidae (diplurans) were very variable but with no trends observed. On the other hand, the number of Chilopods (centipedes) and Staphaylinds (rove beetles) 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 (ground beetles), 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 affect the number of Carabids or crickets but did affect spider abundance. There were 30% fewer spiders found in soil treated plots and 50% fewer in foliar treated plots than the seed and no insecticide treated plots.

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 were 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 abundance was not significantly effected by the chemical 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. 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.

The second year preliminary report shows some possible effects of MON 863 on corn field insects. Preliminary data are being analyzed which suggesting an increase in Coccinellid larvae, an increase in abundance of mites on MON 863 residues, a laboratory study showing significantly reduced root population and egg production of the plant pathogenic nematode *M. incognita*, a reduction in the population of the non-pathogenic *E. elegans* in root extracts (no effects in soil leachate), and no effects were noted on the non-pathogenic nematode *S. carpocapsae*. It was also reported that of 14 species of Collembola, a decrease in one species was noted, although no effect was seen on total Collembola populations. One inconclusive study found a decrease in Carabids, but the data are suspect because of limitations in movement of ground dwelling insects due to plot layout problems. These studies are being repeated in more adequately rearranged and randomized plots.

Conclusions:
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 invertebrates. Data also indicated that planting event MON 863 results in less impact on non-target invertebrate than conventional pest management practices. Preliminary data from the second year studies noted increases in mite populations and Coccinellid larvae, a possible shift in Collembola diversity but not abundance, an apparently detrimental effect on a plant pathogenic nematode, and a variable effect on two non-pathogenic nematodes. Further analysis of the data already collected, as well as additional results from the ongoing trials are needed before a final assessment can be fully developed. However, the preliminary data do not point to any unreasonable adverse effects on soil invertebrate populations.

3. Nontarget Invertebrate - Earthworm Testing

The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Parts 160 and 792; Organization for Economic Development (OECD) Principles of Good Laboratory Practice; and Japan Ministries of Agricultural Forestry and Fisheries (MAFF), with certain exceptions that did not affect the integrity of the test. The testing was conducted based on OPPTS Series 850.6200 Earthworm Subchronic Toxicity Test and OECD Guideline 207.
This study meets current testing requirements for assessing subchronic risks to earthworms from plant-incorporated protectants derived from *Bacillus thuringiensis*.

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 inconclusive effects (due to procedural error) observed among worms in the 570 mg Cry3b1 protein/kg dry soil treated group, the NOEC was determined to be >57.0 mg Cry3Bb1 protein/kg dry soil. 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.

The study was procedurally sound and the data show that no adverse effects to earthworms are expected from the growing of Cry3Bb1 protein containing corn plants. Field study data (MRID NO.: 455382-06) confirm this assessment.

4. Soil Degradation Studies

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 two exceptions that did not affect the integrity of the test.

An insect bioassay and an ELISA was conducted to measure the level of functional and non-functional Cry3Bb1 protein present in field soil samples. The amount of lyophilized corn tissue added to the field soil in this study was based on the amount of plant tissue that could potentially be incorporated into the top six inches of soil under field conditions. Since field incorporation of plant tissue will not take place until the Fall, this amount of Cry3Bb1 protein represents the worst-case scenario during the growing season. Based on this calculation 0.03 g (rounded up from 0.028) dry weight plant tissue was added to each gram of dry sandy loam field soil (from Fayette County of Lexington, KY.); therefore, 3% of the dry weight of soil was dry weight of lyophilized plant tissue. An additional test was conducted with 10% of the soil containing lyophilized dry weight plant tissue (0.10 g leaf tissue/g soil). Insect bioassays included a mixture of test and control substances with an agar-based insect diet added to wells of bioassay trays. Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) larvae were added one larva/well and each treatment bioassay was replicated twice for a total of 16 CPB/replicate. CPB are more sensitive to the Cry3Bb1 protein then CRW and were, therefore, expected to result in a more measurable response then CRW, the target species. In addition to an insect bioassay, an ELISA was conducted to measure the level of Cry3Bb1 protein present in samples. However, the ELISA test will only show extractable protein and does not distinguish between functional and non-functional proteins.

Results from this study show the DT_{50} and DT_{90} for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. The 21 day ELISA sample was the last to show traces of Cry protein. At 28 days the CryBb1 protein was below the detection level. However, the value of these results need to be considered with regard to biological activity because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT_{50} and DT_{90} determined by insect bioassays with CPB were 2.37 and 7.87 days respectively. The no-detection level was in the range of the results obtained by ELISA.

Based on these results Cry3Bb1 protein degrades rapidly and does not accumulate in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silt loam soils in various regions of the U.S. Testing clay soils would, therefore, be considered a "worst case scenario." In addition, this test does not account for all plant tissue such as roots, or root exudation of the Cry protein in the field. It is possible that root tissue is degrading slower then leaf tissue in the soil which may result in a longer duration of degradation time of the Cry3Bb1 protein. Therefore, field testing should be continued in a variety of soil types including clay and humic acid soils over a three year period of time to determine the long-term degradation rate and accumulation/persistence of Cry3Bb1 in soil.

B. Endangered Species Considerations

Exposure Assessment for Endangered Coleoptera

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 known to be sensitive to Cry3Bb1. Therefore, no adverse affects from Cry3Bb1 event MON 863 are expected against endangered species. Nevertheless, all endangered/threatened beetle species found in the counties where corn is grown (including the recently listed Stephan's riffle beetle *Heterelmis stephani*) were checked to determine possible exposure to corn pollen. Their habitat (and breeding grounds) were found not to overlap with corn fields. Therefore no endangered beetles will be exposed to potentially harmful levels of corn tissue or pollen containing Cry3Bb1 protein.

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 has been determined to be 93 µg/g fresh weight pollen. 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 expected to be negligible, pollen drift was considered the primary source of potential risk to endangered aquatic Coleoptera. According to estimates 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.. This is a few orders below the toxic level to any insect.

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 on which their larvae feed. Carrion regurgitated by adults is fed to larvae until they are able to feed directly on a carcass.

In addition, Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera (MRID NO: 455770-03). This endangered species assessment was based on the Hazard Evaluation Division, Standard Evaluation Procedure, Ecological Risk Assessment (U.S. EPA, 1986). The Agency reviewed this assessment and found it acceptable.

The reviewed non-target data confirm the expectation that CryBb1 corn will have no adverse effect on endangered and/or threatened species listed by the US Fish and Wildlife Service, including mammals, birds, plants, terrestrial and aquatic invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

C. Summary

In evaluating the risks from use of a pesticide, EPA considers the toxicity and the exposure. Where there is no observable adverse effect (especially when the test dose is several times that found in the environment), EPA concludes that there are no unreasonable adverse effects from the use of the pesticide. When a toxic endpoint is found, then the level of field exposure is factored in to arrive at a risk at field exposures. From all of the submitted indicator species test data on Cry3Bb1 corn, including the preliminary field data, EPA concludes that the levels of Cry3Bb1 protein in corn will not pose unreasonable adverse effects to invertebrate corn field fauna. Available data also indicate that there should be no accumulation of Cry3Bb1 protein in agricultural soil. In addition, no adverse effect on listed endangered/threatened species is expected from the proposed Cry3Bb1 CRW resistant corn registration. However, continued field study data are needed to confirm that there will be no unreasonable adverse environmental effects from long term use of this product. EPA does not believe that this data requirement was reasonably foreseeable, or practically achievable, by the applicant at the time of application.