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

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM MAY 29 2008

SUBJECT: Environmental Risk Assessment for *Bacillus thuringiensis* (*Bt*) Cry1A.1015 and Cry2Ab2 insect control proteins as expressed in MON89034 corn and its associated breeding stack, MON89034 x MON88017 corn, containing *Bt* Cry3Bb1 insect control protein; submitted by Monsanto Company.

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Zigfridas Vaituzis, 5/29/08

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ACTION REQUEST: To review the environmental fate and effects data for *Bt* Cry1A.1015 and Cry2Ab2 proteins as expressed in MON89034 corn and its associated breeding stack, MON89034 x MON88017 corn.

CONCLUSION:
Monsanto is applying for Sec. 3 registration for transgenic corn (*Zea mays* ssp. *mays*) plant lines: Event MON89034 (which expresses *Bt* Cry1A.1015 and Cry2Ab2 proteins) and Event MON89034 x MON88017 (which also expresses Cry3Bb1), its associated breeding stack. At present, the Agency has not identified any significant adverse effects of Cry1A.1015, Cry2Ab2, and Cry3Bb1 proteins on the abundance of non-target organisms (NTOs) in any field population. It is unlikely that direct or indirect harmful effects to NTOs, including federally-listed threatened or endangered species, would result from exposure to the insecticidal proteins of Cry1A.1025 and Cry2Ab2 in MON89034 and when combined with MON88017 corn, containing Cry3Bb1 via traditional cross breeding, as a result of the proposed Sec. 3 registration. The Agency anticipates that for full commercial cultivation, no hazard will result to the environment.

A full review of the non-target test data and an Environmental Risk Assessment are found on pages 2-31 of this document.

Background

Monsanto has requested a registration for *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 proteins and the genetic material necessary for their production in all corn lines and varieties. The nptII selectable marker gene was used in the transformation process, but was isolated and removed from transformed plants via traditional breeding. The result is marker-free MON 89034 corn. The Cry proteins expressed in this event are intended to control the lepidopteran pests European corn borer (ECB, *Ostrinia nubilalis*), corn ear worm (CEW, *Helicoverpa zea*), fall army worm (FAW, *Spodoptera frugiperda*), and black cutworm (BCW, *Agrotis ipsilon*) which are primary pests of corn in the United States. These pests feed on the base of seedlings and on the stalk, leaf, and ear tissue of corn plants, thereby destroying the entire plant, weakening the stalk, and/or damaging the ear. In areas where one or more of these pests is prevalent (*e.g.* corn belt), significant financial losses are realized from decreased corn yields and increased expenditures on chemical pest control agents, including organophosphate, carbamate and pyrethroid insecticides.

The EPA has conducted an environmental risk assessment of Cry1A.105 and MON 89034 when expressed in corn. General topics covered in this assessment include effects on wildlife, gene flow to related wild plants and its potential effects, and fate of these Cry proteins in the environment. This assessment is based on data submitted to EPA during the development of Event MON 89034 corn lines, additional data submitted for registration, Federal Insecticide Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) recommendations, consultations with scientific experts, and public comments on Plant-Incorporated Protectant (PIP) regulation.

A. MON 89034 (lepidopteran active) Environmental Risk Assessment

I. Tiered Testing Hazard and Risk Assessment Process

To minimize data requirements and avoid unnecessary tests, risk assessments are structured such that risk is determined first from estimates of hazard under "worst-case" exposure conditions. A lack of adverse effects under these conditions would provide enough confidence that there is no risk and no further data would be needed. Hence, such screening tests conducted early in an investigation tend to be broad in scope but relatively simple in design, and can be used to demonstrate acceptable risk under most conceivable conditions. When screening studies suggest potentially unacceptable risk additional studies are designed to assess risk under more realistic field exposure conditions. These later tests are more complex than earlier screening studies. Use of this "tiered" testing framework saves valuable time and resources by organizing the studies in a cohesive and coherent manner and eliminating unnecessary lines of investigation. Lower tier, high dose screening studies also allow tighter control over experimental variables and exposure conditions, resulting in a greater ability to produce statistically reliable results at relatively low cost[1].

[1] Non-target invertebrate hazard tests often are conducted at exposure concentrations several times higher than the maximum concentrations expected to occur under realistic exposure scenarios. This has customarily allowed an

Tiered tests are designed to first represent unrealistic worst case scenarios and ONLY progress to real world field scenarios if the earlier tiered tests fail to indicate adequate certainty of acceptable risk. Screening (Tier I) non-target organism hazard tests are conducted at exposure concentrations several times higher than the highest concentrations expected to occur under realistic field exposure scenarios. This has allowed an endpoint of 50% mortality to be used as a trigger for additional higher-tier testing. Less than 50% mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic field exposure scenarios.

The EPA uses a tiered (Tiers I-IV) testing system to assess the toxicity of a PIP to representative non-target organisms that could be exposed to the toxin in the field environment. Tier I high dose studies reflect a screening approach to testing designed to maximize any toxic effects of the test substance on the test (non-target) organism. The screening tests evaluate single species in a laboratory setting with mortality as the end point. Tiers II – IV generally encompass definitive hazard level determinations, longer term greenhouse or field testing, and are implemented when unacceptable effects are seen at the Tier I screening level.

Testing methods which utilize the tiered approach were last published by the EPA as Harmonized OPPTS Testing Guidelines, Series 850 and 885 (EPA 712-C-96-280, February 1996)[2]. These guidelines, as defined in 40 CFR 152.20, apply to microbes and microbial toxins when used as pesticides, including those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation. Therefore PIPs containing microbial toxins are also covered by these testing guidelines.

The Tier I screening maximum hazard dose (MHD) approach to environmental hazard assessment is based on some factor (whenever possible >10) times the maximum amount of active ingredient expected to be available to terrestrial and aquatic non-target organisms in the environment (EEC)[3]. Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels which increase the statistical power to test the hypotheses. Elevated test doses, therefore, add certainty to the assessment, and such tests can be well standardized. The Guidelines call for initial screening testing of a single group or several groups

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endpoint of 50% mortality to be used as a trigger for additional higher-tier testing. Lower levels of mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic exposure scenarios. Thus, it follows that the observed proportion of responding individuals can be compared to a 50% effect to determine if the observed proportion is significantly lower than 50%. For example, using a binomial approach, a sample size of 30 individuals is sufficient to allow a treatment effect of 30% to be differentiated from a 50% effect with 95% confidence using a one-sided Z test. A one-sided test is appropriate because only effects of less than 50% indicate that further experiments are not needed to evaluate risk.

[2] http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/885_Microbial_Pesticide_Test_Guidelines/Series/

[3] The dose margin can be less than 10x where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits or other factors. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the test concentration used must exceed 1X EEC.

of test animals at the maximum hazard dose level. The Guidelines call for testing of one treatment group of at least 30 animals or three groups of 10 test animals at the screening test concentration. The Guidelines further state that the duration of all Tier I tests should be approximately 30 days. Some test species, notably non-target insects, may be difficult to culture and the suggested test duration has been adjusted accordingly. Control and treated insects should be observed for at least 30 days, or in cases where an insect species cannot be cultured for 30 days, until negative control mortality rises above 20 percent.

Failing the Tier I (10 X EEC) screening does not necessarily indicate the presence of an unacceptable risk in the field but it triggers the need for additional testing.[4] A less than 50% mortality effect at the MHD is taken to indicate minimal risk. However, greater than 50% mortality does not necessarily indicate the existence of unacceptable risk in the field, but it does trigger the need to collect additional dose-response information and a refinement of the exposure estimation before deciding if the risk is acceptable or unacceptable. Where potential hazards are detected in Tier I testing (i.e. mortality is greater than 50%), additional information at lower test doses is required which can serve to confirm whether any effect might still be detected at more realistic field [1X EEC] concentrations and routes of exposure[5].

When screening tests indicate a need for additional data, the OPPTS Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} and to quantify the hazard. In the definitive testing, the number of doses and test organisms evaluated must be sufficient to determine an LD_{50} value and, when necessary, the Lowest Observed Effect Concentration (LOEC), No Observed Adverse Effect Level (NOAEL) , or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. In the final analysis, a risk assessment is made by comparing the LOAEC to the EEC; when the EEC is lower than the LOAEC, a no risk conclusion is made. These tests offer greater environmental realism, but they may have lower statistical power. Appropriate statistical methods, and appropriate statistical power, must be employed to evaluate the data from the definitive tests. Higher levels of replication, test species numbers or repetition are needed to enhance statistical power in these circumstances.

Data that shows less than 50 % mortality at the maximum hazard dosage level – (i.e. LC_{50}, ED_{50}, or LD_{50} >10 X EEC) is sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. It is also notable that the recommended >10X EEC maximum hazard dose level is a highly conservative factor. The published EPA Level of Concern [LOC] is 50% mortality at 5X EEC [6].

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[4] It is notable that that the 10 X EEC MHD testing approach is not equivalent to what is commonly known as "testing at a 10X SAFETY FACTOR" where any adverse effect is considered significant. Tier I screen testing is not 'safety factor testing'. In a "10X safety factor" test any adverse effect noted is a "level of concern", whereas in the EPA environmental risk assessment scenario any adverse effect is viewed as a concern only at 1X the field exposure.

[5] The 1X EEC test dose is based on plant tissue content and is considered a high worst case dose (sometimes referred to as HEEC). This 1X EEC is still much greater than any amount which any given non-target organism may be ingesting in the field because most non-target organisms do not ingest plant tissue.

[6] Environmental Protection Agency (USEPA) (1998). "Guidelines for Ecological Risk Assessment." EPA 630/R-

Validation: The tiered hazard assessment approach was developed for the EPA by the American Institute of Biological Sciences (AIBS) and confirmed, in 1996, as an acceptable method of environmental hazard assessment by a FIFRA Scientific Advisory Panel (SAP) on microbial pesticides and microbial toxins. The December 9, 1999 SAP agreed that the Tiered approach was suitable for use with Plant Incorporated Protectants (PIP); however, this panel recommended that, for PIPs with insecticidal properties, additional testing of beneficial invertebrates closely related to target species and/or likely to be present in GM crop fields should be conducted. Testing of Bt Cry proteins on species not closely related to the target insect pest was not recommended, although it is still performed to fulfill the published EPA non-target species data requirements. In October 2000, another SAP also recommended that field testing should be used to evaluate population-level effects on non-target organisms. The August 2002 SAP, and some public comments, generally agreed with this approach, with the additional recommendation that indicator organisms should be selected on the basis of potential for field exposure to the subject protein.[7]

Chronic studies: Since delayed adverse effects and/or accumulation of toxins through the food chain are not expected to result from exposure to proteins, protein toxins are not routinely tested for chronic effects on non-target organisms. The 30 day test duration requirement does, however, amount to subchronic testing when performed at field exposure test doses. Proteins do not bioaccumulate. The biological nature of proteins makes them readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports that some proteins (Cry proteins) bind to soil particles, it has also been shown that these proteins are degraded rapidly by soil microbial flora upon elution from soil particles.

Conclusion The tiered approach to test guidelines ensures, to the greatest extent possible, that the Agency requires the minimum amount of data needed to make scientifically sound regulatory decisions. The EPA believes that maximum hazard dose Tier I screening testing presents a

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95-002F. Washington, DC, USA. [Federal Register, May 14, 1998. 63(93): 26846-26924.] The established peer and EPA Science Board reviewed guidance on screening test levels of concern is 50% mortality at 5X environmental concentration. The appropriate endpoints in high dose limit/screening testing are based on mortality of the treated, as compared to the untreated (control) non-target organisms. A single group of 30 test animals may be tested at the maximum hazard dose

[7] EPA-SAP. February 4, 2000. Characterization and non-target organism data requirements for protein plant-pesticides. SAP report No. 99-06A for FIFRA Scientific Advisory Panel Meeting held December 8, 1999, held at the Sheraton Crystal City Hotel, Arlington, VA.

EPA-SAP. March 12, 2001. Bt plant-pesticides risk benefit assessments. SAP report No. 2000-07 for FIFRA Scientific Advisory Panel Meeting held October 18-20, 2000 at the Marriott Crystal City Hotel, Arlington, VA.

EPA-SAP. November 6, 2002. Corn rootworm plant-incorporated protectant insect resistance management and non-target insect issues. Transmittal of meeting minutes of the FIFRA Scientific Advisory Panel Meeting held August 27-29 at the Marriott Crystal City Hotel, Arlington, VA.

EPA-SAP. August 19, 2004. Product characterization, human health risk, ecological risk, and insect resistance management for *Bacillus thuringiensis* (Bt) cotton products. Transmittal of meeting minutes of the FIFRA Scientific Advisory Panel Meeting held June 8-10 at the Holiday Inn Ballston, Arlington, VA.

reasonable approach for evaluating hazards related to the use of biological pesticides and for identifying negative results with a high degree of confidence. The Agency expects that Tier 1 testing for short-term hazard assessment will be sufficient for most studies submitted in support of PIP registrations. However, if long range adverse effects must be ascertained, then higher-tier longer-term field testing will be required. The Agency has been frequently asking the registrants to conduct post-registration long term invertebrate population/community and Cry protein accumulation in soils studies as a condition of registration. As noted above, the October 2000 SAP and the National Academy of Sciences[8] (NAS 2000) recommended testing non-target organisms directly in the field. This approach, with an emphasis on testing invertebrates found in crop fields, was also recommended by the August 2002 SAP and was supported by several public comments. The issue of long range effects of cultivation of currently registered Cry proteins on the invertebrate community structure in Bt crop fields has since been adequately addressed by a meta analysis of field studies performed during the last 10 years. No unexpected adverse effects on invertebrate community structure were reported.[9]. The meta analysis of short term and long term field study effects on invertebrate populations in *Bt* corn and cotton fields indicate that no unreasonable adverse effects are taking place as a result of wide scale Bt crop cultivation. The Agency is in agreement with these conclusions. Slight reductions in some invertebrate predator populations are an inevitable result of all pest management practices which result in reductions in the abundance of the pests as prey. Based on these considerations, regulatory testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

II. Environmental Exposure Assessment

The EPA risk assessment is centered only on adverse effects at the field exposure rates (1X EEC), and not on adverse effects at greater concentrations. The dose margin can be less than 10x where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the concentration used must exceed 1X EEC. It is important to note that Tier I screen testing is not "safety factor testing". In a traditional "10X safety factor" test any adverse effect noted is a "level of concern", whereas in the EPA environmental risk assessment scenario any adverse effect is viewed as a concern only at 1X the field exposure.

For the purposes of the nontarget organism (NTO) studies submitted in support of the MON 89034 registration, test material dose levels were based on the estimated concentration of

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[8] *Environmental Effects of Transgenic Plants: The Scope and Adequacy of Regulation* is available from the National Academy Press, 2101 Constitution Avenue, N.W., Lockbox 285, Washington, DC 20055; (800) 624-6242 or (202) 334-3313 (in the Washington metropolitan area); http://www.nap.edu.

[9] Marvier, M., McCreedy, C., Regetz, J. & Kareiva, P. A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316, 1475–1477 (2007).
Sanvido,O., Romeis, J., Bigler, F. (2007). Ecological Impacts of Genetically Modified Crops: Ten Years of Field Research and Commercial Cultivation. Adv Biochem Engin/Biotechnol 107: 235–278

Cry1A.105 and/or Cry2Ab2 protein expressed in the tissue(s) that NTO would most likely be exposed to in the environment (see Edelstein, 2007 for protein expression levels). Whenever possible, a targeted margin of exposure (MOE) of greater than 10X the maximum environmental exposure was used in the tests. The primary route of Cry1A.105 and Cry2Ab2 protein exposure for honeybee, ladybird beetle, parasitic wasp, and minute pirate bug is corn pollen. Consequently, test material dose levels were based on the maximum level of measured protein expression in pollen (8.8 ug/g fwt for Cry1A.105 and 0.47 ug/g fwt for Cry2Ab2). The principal route of Cry1A.105 and Cry2Ab2 protein exposure for soil-dwelling organisms, such as Collembola and earthworms, is assumed to be from decomposing plant tissue and plant exudates in soil. Consequently, the test material dose levels were based on the maximum level of estimated protein expression in the soil environment.

III. Non-Target Wildlife Hazard Assessment for MON 89034 corn

Two separate SAP reports (October 2000 and August 2002) recommended that non-target testing of *Bt* Cry proteins should focus on invertebrate species exposed to the crop being registered. Following SAP recommendations, the EPA determined that non-target organisms with the greatest exposure potential to Cry protein in transgenic corn fields are beneficial insects, which feed on corn pollen and nectar, and soil invertebrates, particularly Coleoptera species. Therefore, maximum hazard dose toxicity testing on representative beneficial organisms from several taxa was performed in support of this Section 3 FIFRA registration. The toxicity of the Cry1A.105 and Cry2Ab2 proteins has been evaluated on several species of invertebrates including the lady beetle, minute pirate bug, parasitic hymenoptera, collembolan, daphnia, honey bee, and earthworm. Reproductive and developmental observations were also made in the lady beetle, minute pirate bug and honeybee studies. Observations of possible reproductive effects were also made in the collembola studies.

Although the Cry1A.105 and Cry2Ab2 proteins in MON 89034 are known to be very host specific, conferring toxic effects on ECB, CEW, FAW, BCW and closely related species, and despite the October 2000 and August 2002 SAP's recommendations against testing of non-target species not related to susceptible target pests, EPA has done a risk assessment on a range of non-target wildlife to comply with the Agency's published non-target data requirements (in the absence of PIP-specific risk assessment guidance, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins). These requirements include birds, mammals, plants and aquatic species. In addition, an earthworm study was voluntarily submitted to the Agency to ascertain the potential effects of the Cry1A.105 and Cry2Ab2 proteins on beneficial decomposer species.

Test substances used for studies submitted in support of the MON 89034 registration included bacterially-produced purified Cry1A.105 and Cry2Ab2 proteins and MON 89034 corn grain. The October 2000 SAP recommended that while actual plant material is the preferred test material, bacterially-derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume corn plant tissue and where large amounts of Cry protein (Cry protein concentrations that exceed levels present in plant tissue) are needed for maximum hazard dose testing. An insect feeding study, which compared the relative potency of plant produced

Cry1A.105 and Cry2Ab2 proteins to the microbe produced proteins, indicated that plant produced protein was similar in toxicity to the microbe produced protein (Edelstein Memo, November 7, 2007)

Potential Interaction between the Cry1A.105 and Cry2b2 proteins was addressed in a memorandum for the MON 89034 Experimental Use Permit accompanying the Agency's review of "Evaluation of the Potential for Interactions Between the *Bacillus thuringiensis* Proteins Cru1A.105 and Cry2Ab2", (Hunter, M. July 6, 2006.). The purpose of this study was to characterize the potential for interaction between the lepidopteran-active proteins Cry1A.105 and Cry2Ab2. The study provides evidence that the proteins do not interact in an antagonistic, additive, or synergistic manner and that there will not be any unexpected interaction with regard to target and non-target insects. New data on the potential synergistic interaction between Cry1A.105, Cry2b2 and Cry3Bb1 proteins were requested and were developed to confirm this hypothesis (MRID 469513-05 & 469513-06).

The results of ecological effects studies submitted in support of the MON 89034 Section 3 FIFRA registration are summarized in Table 1 and presented in a more descriptive format in subsequent sections of this risk assessment document. Full reviews of each study can be found in the individual Data Evaluation Reports (DERs/MRID#s) and accompanying memos.

Table 1. Summary of environmental effects studies and waiver justifications submitted to comply with data requirements published in 40 CFR § 158.2150(d).

Data Requirement	Guideline	Classification	Test Substance	Results Summary	MRID #
Avian oral	885.4050* 154-16**	Acceptable	MON 89034 corn grain†	A 42-day dietary study showed that Event MON 89034 did not adversely affect broiler chickens.	469514-12
Avian injection	885.4100 154-17	Acceptable waiver rationale	N/A	N/A	N/A
Avian acute oral	850.2100	Acceptable	MON 89034 corn grain	An eight-day dietary study showed that the LC_{50} for MON 89034 is >500,000 ppm in the diet northern bobwhite quail.	469514-27
Wild mammal	885.4150 154-18	Acceptable waiver rationale	N/A	N/A	N/A
Freshwater fish	885.4200 154-19	Acceptable waiver rationale	N/A	Freshwater fish studies were not required because of the low potential that fish will be exposed to high levels of the Cry1A.105 and Cry2Ab2 proteins	N/A
Freshwater aquatic invertebrate *Daphnia magna*	885.4240 154-20	Unacceptable [The 885 Series Guidelines call for a 21 day study. The submitted 48 hour acute study is inadequate.]	MON 89034 corn pollen	A 48-hour static renewal limit bioassay resulted in 17% mortality compared with 0% mortality in the control groups (120 mg/L). A 48-hour static renewal dose-response bioassay was conducted and no mortality or adverse effects were observed at any concentration (6.3-120 mg/L). The acute EC50 was	469514-17

Data Requirement	Guideline	Classification	Test Substance	Results Summary	MRID #
				estimated to be >120 mg/L and the NOEC was 100 mg/L.	
Estuarine and marine animal	885.4280 154-21	Acceptable waiver rationale	N/A	N/A	N/A
Non-target plant	885.4300 154-22	Acceptable waiver rationale	N/A	N/A	N/A
Non-target insect testing, minute pirate/insidious flower bug *Orius insidiosus*	885.4340 154-23	Acceptable	Cry2Ab2 protein (Lot No. 20-100071)	*Orius* nymphs were fed a pollen diet containing 100 µg Cry2Ab2 protein/diet for 14 days. No adverse effects were observed.	469514-24
Non-target insect testing, minute pirate/insidious flower bug *Orius insidiosus*	885.4340 154-23	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	*Orius* nymphs were fed a diet containing 30 to 240 µg Cry1A.105/g diet for 14 days. In an initial maximum dose test (240 µg) the survival rate was 47% compared to 88% in the control groups. In the three subsequent dose-response tests, the mean survival rate of the 240 µg group was 55% compared to 91% and 89% in the control groups. No statistically significant effects on survival or development were seen at concentrations greater than or equal to 120 µg Cry1A.105/g diet.	269514-23
Non-target insect testing, parasitic wasp, *Ichneumon promissorius*	885.4340 154-23	Acceptable	Cry2Ab2 protein (Lot No. 20-100071)	Adult female wasps were fed a sucrose solution containing 100 µg Cry2Ab2 protein/mL for 21 days. Mortality in the Cry2Ab2 group was 3% and the LC50 was determined to be >100 µg/L.	469514-26
Non-target insect testing, parasitic wasp *Ichneumon promissorius*	885.4340 154-23	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Adult female wasps were fed a sucrose solution containing 240 µg Cry1A.105 protein/mL for 21 days. Mortality in the Cry1A.105 group was 7% and the LC50 was determined to be >240 µg/L.	469514-25
Non-target insect testing, ladybird beetle *Coleomegilla maculata*	885.4340 154-23	Acceptable	Cry2Ab2 protein (Lot No. 20-100071)	*C. maculata* larvae were fed a diet containing 120 µg Cry2Ab2 protein/g diet for 17-20 days. No statistically significant difference in survival or development to adult was found between the test and control groups. A slight (~5%) statistical decrease in mean adult body weight was found between the test and buffer control groups; however, this difference was not observed between the test and assay control group.	469514-22
Non-target insect testing, ladybird beetle	885.4340 154-23	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Ladybird beetle larvae were fed a diet containing 240 µg Cru1A.105 protein/g diet for 14	469514-21

Data Requirement	Guideline	Classification	Test Substance	Results Summary	MRID #
Coleomegilla maculata				days. No statistically significant differences in survival, development, or adult beetle weight were found between the test and control groups.	
Non-target insect testing, collembolan *Folsomia candida*	885.4340 154-23	Acceptable	MON 89034 Leaf Tissue (80 µg Cry1A.105 and 70 µg Cry2Ab2/g diet)	Collembola were fed a diet containing 50% Brewer's yeast and 50% lyophilized leaf tissue for 28 days. No statistically significant effects on survival or reproduction were found among the test and negative control groups.	469514-16
Honeybee testing, Adult Honeybee, *Apis mellifera*	885.4380 154-24	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Adult honeybees were fed a 30% sucrose solution containing 550 µg Cry1A.105 protein/mL for 19 days. No statistically significant differences in mortality were observed between the test group and negative controls. The NOEC was determined to be at least 550 µg Cry1A.105 protein/mL.	469514-20
Honeybee testing, Honeybee larvae, *Apis mellifera*	885.4380 154-24	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Two-to-three day old honeybee larvae in brood frames were administered a single 10 µg dose of Cry1A.105 protein per brood cell (equivalent to 12 µg total protein/cell). On day 18 after dosing mean survival of the test group was 95%. The NOEC was determined to be at least 12 µg Cry1A.105 protein per brood cell	469514-20
Earthworm subchronic toxicity, *Eisenia fetida*	850.620	Acceptable	Cry1A.105 protein (Lot No .20-100073)	Adult earthworms were exposed to artificial soil containing 178 mg Cry1A.105 protein/kg dry soil for 14 days. No mortality was observed in the test group. The LC50 was determined to be >178 mg Cry1A.105/kg dry soil and the NOEC was 178 mg Cry1A.105 mg/kg dry soil.	469514-18
Soil fate	885.5200	Acceptable	Purified Cry1A.105 (Lot No. 20-100073) and Cry2Ab2 protein (Lot No. 20-100071)	Results of this degradation study indicate that Cry1A.105 and Cry2Ab2 proteins do not persist in soil beyond approximately three weeks.	469514-28

Note: Earthworm and honey bee studies for Cry2Ab2 protein were submitted and reviewed with previously registered products. The interaction study between Cry1A.105 and Cry2Ab2 was reviewed for the MON 89034 Experimental Use Permit

*OPPTS Microbial pesticide test guidelines

**Microbial pesticide test guidelines identified in the 40 CFR data tables.

† Cry1A.105 and Cry2Ab2 are the active ingredients (a.i.) in MON 89034 corn.

1. Non-target Wildlife Study Summaries

a. Avian species

Published data and studies on file at EPA show that consumption of *Bt* corn has no measurable deleterious effects on avian species. However, to comply with published data requirements, the following studies were submitted to EPA in support of the MON 89034 product registration. These studies were GLP compliant and, when considered together, meet EPA data requirements for avian species.

i. Broiler (MRID 469514-12)

For the first 42 days of life, commercial broiler chickens (*Gallus domesticus*) were fed a corn and soybean diet that contained up to 59% ground corn grain. Treatments consisted of soybean meal with MON 89034, a similar isoline (negative control), or one of four different commercial hybrid corn varieties. At test end, chickens were processed in order to obtain performance and carcass yield data. Breast and thigh meat were also analyzed for moisture, protein, and fat content. Among treatments, there were no biologically significant differences in broiler performance, carcass, or meat quality.

ii. Northern Bobwhite Quail (MRID 469514-27)

In this eight-day dietary study, 10-day-old northern bobwhites (Colinus virginianus) were fed a corn and game bird ration containing 50% ground corn grain. Treatments consisted of game bird ration with MON 89034, a similar isoline (negative control), or one of three different commercial hybrid corn varieties. At test end, no mortality was seen in the MON 89034 treatment group, all birds appeared normal for test duration, and feed consumption was comparable to that of the control group. The dietary LC_{50} was determined to be >500,000 ppm Cry protein in the diet.

b. Wild mammalian species

Mammalian wildlife exposure to Cry1A.105 and Cry2Ab2 proteins is considered likely; however, mammalian toxicology information gathered to date on *Bt* Cry proteins does not show a hazard to wild mammals. In addition, acute oral toxicity studies submitted to EPA in support of the MON 89034 registration indicated that no significant toxicity was seen when rodents were exposed to Cry1A.105 or Cry2Ab2 at the maximum hazard dose level. Therefore, no hazard to mammalian wildlife is anticipated and data on wild mammal testing is not required for this registration.

c. Aquatic species

There is no evidence for sensitivity of aquatic species to anti-coleopteran Cry proteins. A published laboratory study with lepidopteran-active Cry proteins has revealed that the leaf shredding (caddis fly) trichopteran, *Lepidostoma liba*, had 50% lower growth rate when fed Bt

corn litter (Rosi-Marshall, et al. 2007). Two previous field study reports by the same authors did not find adverse effects on head stream invertebrates. The Agency's position on this matter is that until Tier III and Tier IV field studies are performed, there is not enough information to assert that sufficient corn plant litter enters streams to cause unreasonable adverse effects on stream invertebrate populations or communities (See Section A.I. above - Tiered Hazard and Risk Assessment Process). Two years ago the Iowa State University and the University of Maryland received Research grants to study the effects of Bt corn cultivation on streams and to develop methods for aquatic hazard assessment. The results of these studies are pending. When the study reports are reviewed the Agency will respond with action commensurate with the outcome of the studies.

The Agency's current position is that there is no evidence to conclude that there is sufficient aquatic exposure to Cry proteins in corn plant litter to result in adverse effects on stream invertebrate populations or communities. Aquatic animal exposure to Bt crops is extremely small.

i. Freshwater fish-*Waiver granted*

Freshwater fish studies were not required for this product, because of the low potential that aquatic systems will be exposed to the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 corn plant tissues.

ii. Freshwater aquatic invertebrates (MRID 469514-17)

The objective of this study was to determine the potential for acute effects to the aquatic organism, *Daphnia magna*, during a static renewal exposure to MON 89034 corn pollen. The test was initially conducted as a limit test using one test concentration. However, slight effects were noted at the limit concentration. In response, a dose-response test was conducted. The the test substance, MON 89034 pollen, was tested for the presence and levels of Cry1A.105 and Cry2Ab2 proteins. Test organisms were produced by adult daphnia maintained by the study author.

Limit Test

In the initial test, daphnids were exposed to a single nominal test concentration of 120 mg pollen/L for 48 hours with renewal of the test solution at approximately 24 hours. Two control groups were included: a group in well water exposed to pollen (120 mg/L) from conventional corn with a genetic background similar to MON 89034, and an assay control group exposed to well water only. Each treatment was replicated three times and each replicate contained 10 neonate daphnids. Test chambers consisted of 600-mL glass beakers containing 300 mL of the appropriate treatment solution. Observations of mortality, immobility and other clinical signs were made at approximately 3.5, 24 and 48 hours after test initiation. At test termination there was 17% immobility in the 120 mg/L treatment group, with two daphnids exhibiting lethargy. All daphnids in the assay control group and 120 mg pollen/L control group appeared normal throughout the testing period.

Dose-Response Test

In the dose-response test, daphnids were exposed to six concentrations of MON 89034 pollen for 48 hours. The concentrations tested were 6.3, 13, 25, 50, 100, and 120 mg pollen/L. Two control groups were included: a group in well water exposed to pollen (120 mg pollen/L) from conventional corn with a genetic background similar to MON 89034, and an assay control group exposed to well water only. The test and control solutions were renewed at approximately 24 hours. Each treatment was replicated two times and each replicate contained 10 neonate daphnids. Test chambers consisted of 600-mL glass beakers containing 300 mL of the appropriate treatment solution. Observations of mortality, immobility and other clinical signs were made at approximately 5, 24 and 48 hours after test initiation. The NOEC was estimated by visual interpretation of the mortality, immobility and observation data. At test termination there were no mortalities, immobile daphnids or signs of toxicity noted in any control or test substance group during the 48 hour exposure period.

Conclusions/Recommendations: Based on the results of the dose-response test the 48-hour EC_{50} was estimated to be greater than 120 mg MON 89034 pollen/L. Based on the results of both studies, the 48-hour NOEC was 100 mg MON 89034 pollen/L. This study is unacceptable because it is an 850 Series OPPTS Guideline study. The 48 hour test duration is not sufficient to show mortality for Bt toxins. It takes more than 48 hours for the target pests to succumb to the Cry proteins therefore 48 hours is also not expected to show mortality or reproductive effects on *Daphnia.* A 7 to 14 day *Daphnia* study as per the 885 Series OPPTS Guidelines needs to be performed. The study may be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the *Daphnia* study.

iii. Estuarine and marine animals-*Waiver granted*

Estuarine and marine animal studies were not required for this product, because of the low probability that estuarine or marine systems will be exposed to the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 corn plant tissues.

d. Terrestrial and aquatic plant species-*Waiver granted*

Plant toxicity studies were not required for this product because the active ingredient is an insect toxin (*Bt* endotoxin) that has never shown any toxicity to plants.

e. Invertebrate species

The Cry1A.105 and Cry2Ab2 proteins are meant to target species within the order Lepidoptera (moths and butterflies). *Bt* toxins are known to have a limited host range, however, to address any unforeseen change in activity spectrum as a result of laboratory Cry protein synthesis and to fulfill the published registration data requirements EPA requires that test species used for non-target insect evaluations should include several species that are not related to the target pests. Earthworm studies are also recommended.

i. Ladybird beetle

MRID 469514-21

The purpose of this study was to determine the potential dietary effects of the Cry1A.105 protein on the mortality and development of the ladybird beetle, *Coleomegilla maculata*. The test material, Cry1A.105 protein, was produced by a recombinant *E. coli* fermentation system. The test substance was incorporated at 240 µg Cry1A.105 protein/g of diet. The diet consisted of an artificial agar-based diet. Three control treatments were included in the experiment. The buffer control contained 25 mM CAPS buffer, which was the buffer used for storage of the test material. The assay control (purified water) was used to generate a diet-only treatment and a positive control treatment was also tested, containing potassium arsenate. Ladybird beetle larvae were less than 48 hours old at test initiation. The larvae were contained in individual test arenas (inverted 60 x 15 mm Petri dishes) and were allowed to feed *ad libitum* on the appropriate test diet. Each treatment was replicated six times and each replicate contained 15 or 16 larvae. All six replicates met the acceptance criteria; the mortality of larvae ranged from 0 to 18.8% in the assay control treatment groups. The diet treatments were replaced with fresh diet approximately every 48 to 72 hours. The larvae were monitored every 24 to 72 hours for survival and development to the adult stage. Adults were weighed within 30 hours of eclosion and each adult was dissected and sexed. Any abnormal behavior or development was noted during feedings and observational evaluations. The study duration ranged from 17 to 20 days depending on adult emergence. Samples were taken to test the biological activity, homogeneity, and stability of the Cry1A.105 protein. Results showed that there were no differences in the mean survival percentage of *C. maculata* between the Cry1A.105 protein, buffer control, and assay control treatments (88.5%, 87.5%, 91.6%). The survival rate was 2.08% for the positive control treatment. There were no significant differences in the mean percent of *C. maculata* that developed to adults when comparing the Cry1A.105, buffer control, and assay control treatments (88.5%, 85.4%, 90.6%). None of the larvae developed to adults in the positive control treatment. Further, there were no significant differences in the mean weight of *C. maculata* adults between the Cry1A.105, buffer control, and assay control. No significant interaction was found between insect sex and treatment. In the bioactivity confirmation assay, results indicated equivalent biological activity among the Cry1A.105 used in the test diets and the reference standard. In addition, the homogeneity and stability study confirmed that Cry1A.105 was homogeneous in the test substance diet and was stable under the storage conditions employed in the study.

Conclusions/Recommendations: This study is acceptable. The results indicate that Cry1A.105 protein had no adverse effect on the survival, development, and growth of the ladybird beetles at a dietary concentration of 240 µg/g of diet.

MRID 469514-22

The objective of this study was to determine the potential dietary effects of Cry2Ab2 protein on the mortality and development of the ladybird beetle, *Coleomegilla maculata*. The test substance, Cry2Ab2, was produced by recombinant *E. coli* fermentation system. The endpoints evaluated were survival and development through 20 days (some replicates were completed before 20 days if all insects had developed to adults). The Cry2Ab2 protein was incorporated in to an agar-based artificial diet at a concentration of 120 µg Cry2Ab2/g diet. Three control treatments were included in the study: 1) buffer control, 2) assay control (purified water), and 3)

positive control (potassium arsenate). The ladybird beetle larvae were less than 48 hours old when testing began and the larvae were allowed to feed *ad libitum*. The diet treatments were replaced approximately every 48 to 72 hours. Each treatment was replicated six times and each replicate included 14 to 16 ladybird beetle larvae. Each larva was contained in its own test arena which consisted of an inverted 60 x 15 mm Petri dish. The larvae were monitored every 24 to 72 hours for survival and development to the adult stage. Adults were weighed within 30 hours of eclosion and adults were sexed. The biological activity, homogeneity, and stability of the Cry2Ab2 protein in the diet were confirmed in a separate bioassay using *Helicoverpa zea*. The mean survival for *C. maculata* was 94.7% for the Cry2Ab2 treatment, 88.8% for the buffer control treatment, 91.6% for the assay control, and 2.08% for the positive control. The mean percent of larvae that developed to adults was 92.6% in the Cry2Ab2 treatment, 85.3% for the buffer control treatment, and 90.6% for the assay control. None of the larvae developed to adults in the positive control treatment. The mean adult weights for the test material and groups were about 5% lower buffer control than those of the assay control group, which was a statistically significant difference. However, there was no significant difference in adult weight of the test material and buffer control groups.

Conclusions/Recommendations: This study is acceptable. No adverse effects were seen in *C. maculata* fed 120 µg Cry2Ab2 protein/g diet. Although the mean adult weight of the Cry2Ab2 protein treatment group was statistically significantly lower than that of the assay control group, the difference was slight (~5%) and there was no significant difference between the weight of the Cry2Ab2 protein group and buffer control groups.

ii. Minute pirate bug

MRID 469514-23

The purpose of this study was to determine the potential dietary effects of Cry1A.105 protein on mortality and development of *Orius insidiosus*, the minute pirate bug or insidious flower bug. The Cry1A.105 protein (*E. coli*-produced) was incorporated into a pollen-based diet for treatment of the test group. Both a buffer control diet and an assay control diet (pollen diet only) were included in the study. A positive control group was fed a diet treated with potassium arsenate. The initial test involved dosing the insects with a single maximum dose level (240 µg Cry1A.105/g diet) for 14 days, resulting in 47% survival. The assay and buffer control resulted in 88% survival. A total of 75 *Orius* were tested in each treatment. Based on the results of the maximum hazard dose assay, three 14-day dose-response tests were conducted with test substance exposure levels of 30, 60, 120 and 240 µg Cry1A.105/g diet. Again, a buffer control, assay control, and positive control were included in each of the three tests. Each exposure test was conducted independently at a different time using separate groups of *Orius*. During the test, *Orius* were supplied with a capsule (50 µL) of the appropriate diet and the capsules were replaced every other day. The test arenas consisted of 1-ounce plastic cups with plastic covers and each cup contained one *Orius*. For each dose-response exposure, 25 test arenas were included for each diet treatment. Observations and feeding behavior were recorded each deeding day for each test arena. Results of the first replicate resulted in percent survival in the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet was 88, 84, 88 and 56%, respectively. Percent survival in the assay, buffer, and positive control was 92, 88, and 36%, respectively. The percent of

nymphs developing to adults for the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet, assay control, buffer control and potassium arsenate control organisms was 92, 100, 96, 92, 96, 96 and 40%, respectively. No statistically significant differences were detected between the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet. Percent survival in the second replicate in the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet was 88, 88, 92, and 52%, respectively. Percent survival in the assay and buffer controls was 88% and percent survival in the positive control was 32%. The percent of nymphs developing to adults for the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet, assay control, buffer control and positive control organisms was 100, 92, 96, 92, 96, 92 and 96%, respectively. The mean number of days to development for all treatments was 6.0 days. Percent survival in the third replicate in the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet treatments was 92, 80, 80, and 56%, respectively. Percent survival in the assay, buffer and positive controls was 92 and 28%, respectively. The percent of nymphs developing to adults for the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet, assay control, buffer control and positive control organisms was 100, 96, 100, 92, 100, 100 and 80% respectively. The mean number of days to development for all treatments was 6.0 days. Throughout the study samples of the test and control substances were taken to be used in a bioassay with *Helicoverpa zea* to confirm the presence of the test substance, homogeneity of the test substance in the diet, diet stability and bioactivity of the test material. The bioassay confirmed that the test substance was stable throughout the study, was biologically active at anticipated levels and was appropriately mixed in the test diet.

Conclusions/Recommendations: This study is acceptable. *Orius insidiosus* were exposed for 14 days to a range of dietary concentrations of Cry1A.105. For the three dose-response replicates the mean survival for the 240 µg Cry1A.105/g diet treatments was 55%. Therefore, the LC_{50} value was empirically determined to be greater than 240 µg Cry1A.105/g diet. No adverse effects were observed at concentrations less than or equal to 120 µg Cry1A.105/g diet.

MRID 469514-24

The purpose of this study was to determine the potential dietary effects of Cry2Ab2 protein on mortality and development of *Orius insidiosus*. The Cry2Ab2 protein was incorporated into a pollen-based diet at a concentration of 100 µg Cry2Ab2 protein/g diet. The protein was produced by a recombinant *E. coli* fermentation system. Both a buffer control treatment and an assay control (pollen-based diet only) were included in the study. In addition, a positive control was included which consisted of the pollen-diet treated with potassium arsenate. The duration of the experiment was 14 days which was long enough to observe the *Orius* develop from nymph to adult. The test insects were approximately 3 days old at test initiation. Each treatment contained seventy-five insects and each insect was contained in its own test arena. During the exposure period, one capsule of approximately 50 µL of the appropriate test diet was provided in each test arena on Day 0 and every other day thereafter for the duration of the test. Observations and feeding behavior were recorded each day for each test arena. The biological activity, homogeneity, and stability of Cry2Ab2 protein in the test diet were tested and confirmed in a separate bioassay using *Helicoverpa zea*. The percent survival of insects exposed to the 100 µg Cry2Ab2 protein/g diet treatment was 91%, which was similar to the percent survival of the insects in the buffer and assay control groups. The percent of nymphs developing to adults in the 100 µg Cry2Ab2 protein/g diet treatment, assay control, buffer control and positive control was

93, 95, 91, and 73%, respectively. The mean number of days to develop to adult for insects exposed to the 100 µg Cry2Ab2 protein/g diet, assay control, buffer control and positive control treatments was 6.1, 7.1, 8.0 and 6.0 days, respectively.

Conclusions/Recommendations: This study is acceptable. No adverse effects were observed for *Orius insidiosus* at the concentration level of 100 µg Cry2Ab2 protein/g diet. Therefore, the LC_{50} is greater than 100 µg Cry2Ab2 protein/g diet.

iii. Parasitic hymenoptera

MRID 469514-25
This study was conducted to evaluate the potential effects of acute exposure of Cry1A.105 protein to the parasitic wasp, *Ichneumon promissorius*. The Cry1A.105 protein was administered to the wasps at a concentration of 240 µg/mL in a 30% sucrose solution. The protein was produced by a recombinant *E. coli* fermentation system. Three control treatments were included in the experiment: 1) buffer control, 2) assay control (sucrose solution only), and 3) positive control (potassium arsenate). The positive control substance was tested at two concentrations (100 and 400 ppm). There were three replications per treatment and each replication contained 10 female wasps. The wasps were 3 to 6 days old at the time of test initiation. The test chambers were disposable 64 ounce containers. Observations of mortality and clinical signs were conducted once within the hour of test initiation and then continued daily until Day 21 of the test. Samples of the assay control, control substance and protein group diets were collected for analysis by bioassay to test for bioactivity and stability of the Cry1A.105 protein. Mortality in the assay control, buffer control, and test material treatments was 10%, 8%, and 7% respectively. All surviving wasps in those groups appeared normal in appearance and behavior. There was no statistically significant difference in mean mortality in the Cry1A.105 treatment and buffer control treatments. The biological activity and stability of the Cry1A.105 protein was confirmed in a seven-day bioassay using the corn earworm (*Helicoverpa zea*).

Conclusions/Recommendations: This study is acceptable. The LC_{50} for *Ichneumon promissorius* was greater than 240 µg Cry1A.105 protein/mL and the NOEC was at least 240 µg Cry1A.105 protein/mL.

MRID 469514-26
A laboratory bioassay was conducted to evaluate the potential effects of acute exposure to Cry2Ab2 protein to the parasitic wasp *Ichneumon promissorius*. The Cry2Ab2 protein used was produced by an *E. coli* fermentation system. Wasps were exposed to the Cry2Ab2 at a concentration of 100 µg/L. Three control groups were utilized, including: 1) buffer control, 2) negative assay control group (sucrose solution only) and 3) two positive controls using two concentrations of potassium arsenate. The test diets were prepared by diluting 60% (w:v) sucrose solution with equal amounts of solutions containing test and control substances in deionized water to obtain diets with approximately 30% sucrose. Test diet containing Cry2Ab2 protein was prepared weekly. The wasps were given fresh diet daily. Three replicate test chambers were used for each treatment and control group and 10 female wasps were contained in each test chamber. The test chambers consisted of disposable 64 oz. polypropylene containers.

The wasps were approximately 3 to 6 days old at test initiation. Observations were made once during the hour of test initiation and once daily until Day 21 of the test. Samples of the assay control, control substance and protein group diets were collected for analysis by bioassay. Dose confirmation analyses verified that the Cry2Ab2 protein diet was biologically active against a susceptible insect, *Helicoverpa zea*. At test termination (Day 21), mortality in the assay control, buffer substance, and test substance groups was 10%, 3%, and 3%, respectively. All surviving wasps were normal in appearance and behavior. No statistically significant differences in mean mortality were found between the Cry2Ab2 treatment group and the negative control group.

Conclusions/Recommendations: This study is acceptable. The LC_{50} for *Ichneumon promissorius* was determined to be >100 µg/L Cry2Ab2 protein/mL.

iv. Collembola

MRID 469514-16

The objective of this study was to determine the potential effect of chronic exposure of lyophilized corn leaf tissue from MON 89034 maize on the survival and reproduction of *Folsomia candida*. The study sponsor verified the identity and the concentrations of Cry1A.105 and Cry2Ab2 in the lyophilized leaf material. The concentration of Cry1A.105 was 160 µg/g lyophilized leaf dry weight and the concentration of Cry2Ab2 was 140 µg/g lyophilized leaf dry weight. The lyophilized test material was incorporated in to a diet containing 50% Brewer's yeast and 50% test material (0.500g leaf tissue with 0.500 g yeast). Therefore, the test diet contained Cry1A.105 at a nominal concentration of 80 µg/g lyophilized leaf dry weight and Cry2Ab2 at a nominal concentration of 70 µg/g lyophilized leaf dry weight. A control diet was prepared by mixing 50% control leaf tissue, by weight, with 50% Brewer's yeast. An additional control treatment consisted of a test diet containing only Brewer's yeast. Three positive control treatments were included. The three treatments included three treatments of thiodicarb, representing nominal concentrations of 1.0, 10 and 100 mg a.i./kg. Collembola were provided enough food such that an excess was always available. Each treatment contained four replicates and each replicate initially contained 10 juvenile Collembola (12 days old). Each replicate was contained in a glass jars containing a water saturated substrate consisting of plaster of Paris and charcoal at a ration of 8:1 by weight. To confirm dose and biological activity of the test substance, a bioassay was conducted using *Helicoverpa zea*. The bioassay confirmed that the test material was biologically active against CEW and the level of activity was not significantly different from that of the reference standard. Mortality and observations of sublethal effects of the surviving Collembola were recorded on day 28 (test termination). Collembola were removed from the test arenas and the number of adult and young Collembola were counted. Among the yeast-only diet control organisms, mean survival was 98% and mean reproduction was 170 offspring per arena. Mean survival of Collembola exposed to the control diet (control leaf tissue) and MON 89034 diet was 100 and 98%, respectively. The mean number of offspring produced in the control substance diet (control leaf tissue) and the MON 89034 diet was 260 and 257 offspring per arena, respectively. Mean survival in the positive control substance treatments 1.0, 10 and 100 mg thiodicarb/kg diet was 95, 63 and 35% respectively. The mean number of offspring produced in the 1.0, 10 and 100 mg thiodicarb/kg diet treatments was 136, 93, and 16 offspring per arena, respectively. Statistical analysis demonstrated no significant reduction in

survival or reproduction among Collembola exposed to the MON 89034 diet when compared to either negative control.

Conclusions/Recommendations: The study is acceptable. The NOEC for *Folsomia candida* is at least 80 µg Cry1A.105 and 70 µg Cry2Ab2 per gram of diet.

v. Honeybee

MRID 469514-19

The objective of this study was to evaluate potential dietary effects of Cry1A.105 protein when administered to honeybee larvae (*Apis mellifera*). Honeybees were approximately 2 to 3 days old during the experiment. The test substance was Cry1A.105 protein produced by an *E. coli* fermentation system. The protein was used to prepare a test solution at a concentration of 1200 µg/mL. This concentration is equivalent to 12 µg total protein per cell. Additional treatment groups included an assay control, buffer control, and two reference substance concentrations (potassium arsenate at low and high doses). Each treatment included four replications of 20 bees for a total of 80 bees per treatment. The larvae were exposed to a single dose (10 mL) of the appropriate dosing solution at initiation and observed during larval and pupal development. Survival of larvae was assessed at study completion (Day 18) by observing adult emergence. The Cry1A.105 treatment group resulted in 95% survival. Survival in the assay control and buffer control was 92.5%. Adult emergence in the low and high dose potassium arsenate treatments was 26.5% and 5.0%, respectively. To verify test concentration and bioactivity of the Cry1A.105 protein, samples of the test material were taken at test initiation. A bioassay using *Helicoverpa zea* was conducted to test for bioactivity and no significant difference was observed between the test substance and the Cry1A.105 reference standard.

Conclusions/Recommendations: This study is acceptable. The NOEC for Cry1A.105 protein to honey bee larvae was determined to be at least 12 µg Cry1A.105 protein/cell.

MRID 469514-20

An acute bioassay was conducted to determine the effects of Cry1A.105 protein on adult honeybees (*Apis mellifera*). The study was initiated a total of five times, with the first four attempts resulting in early termination (high control mortality). Adult bees were approximately five days old at the start of the bioassay. The test material was *E. coli*-produced Cry1A.105 protein supplied by the study sponsor. The protein was used to prepare a 30% sucrose solution containing 550 µg Cry1A.105 protein/mL. A buffer control diet was prepared by combining the buffer solution with stock sucrose solution producing a 12.5 mM buffer in a 30% sucrose solution. An assay control diet was also prepared and consisted of only the 30% sucrose solution. A positive control diet was prepared and contained 100 µg/mL potassium arsenate in a solution of 30% sucrose. Honeybees were maintained in cages that were approximately 12.7 cm on each side. To induce clustering, a small cone of beeswax was attached to the cage cover and extended down into the cage. The diet was provided via an inverted 12 mL glass vial fitted with a plastic screw cap containing two ~1.0 mm holes. Each treatment group included 270 adult honeybees in six replicates of 45 adult honeybees per replicate. The number of dead bees in each

cage was assessed on a daily basis. The study acceptance criteria stipulated that the assay be terminated at 30 days or when the adult control mortality reached 30%. The 30% criterion was met between Day 18 and 19 and the study was terminated on Day 20. On Day 18, the buffer control treatment produced significantly higher mortality (37.41%) than either the sucrose (20.00%) or the Cry1A.105 treatments (20.37%). Mortality in the Cry1A.105 treatment was not statistically different than the sucrose treatment on Day 18. On Day 19, no significant differences were detected among the three treatment groups (buffer, sucrose, and Cry1A.105 protein) with mortalities of 52.22%, 51.48%, and 47.04%, respectively. The potassium arsenate treatment resulted in 100% mortality by Day 2 of the study. A diet incorporation assay using the Cry1A.105 test diet was conducted to confirm the bioactivity of the protein. The biological activity of the test substance was evaluated using *Helicoverpa zea* and compared with the biological activity of a Cry1A.105 reference standard. In addition, the control substance and the assay control substance were evaluated in the diet incorporation assay. The bioassay confirmed the test diet contained the expected level of Cry1A.105 activity.

Conclusions/Recommendations: This study is acceptable. The NOEC for Cry1A.105 protein fed to adult honey bees is at least 550 µg/mL.

vi. Earthworm

MRID 46954-18

The objective of this study was to evaluate the potential effects of acute exposure of Cry1A.105 protein administered to earthworms (*Eisenia fetida*) during a 14-day exposure period. In the test, earthworms were exposed to a single concentration of Cry1A.105 protein that was incorporated into an artificial soil substrate. The Cry1A.105 protein used in this study was *E. coli* produced. The concentration of the test substance was 178 mg Cry1A.105 protein/kg soil dry weight. A total of four control treatments were included in the study, including: 1) buffer solution control, 2) assay control group containing neither test substance or buffer solution, 3) positive control group exposed to 15 mg chloroacetamide/kg dry soil and 4) additional positive control group exposed to 30 mg chloroacetamide/kg dry soil. Each treatment was replicated four times and each replicate contained 10 earthworms. Test chambers consisted of one-liter glass beakers covered with plastic wrap what was perforated for air exchange. The artificial soil was prepared in bulk by blending 70% sand, 20% kaolin clay and 10% sphagnum peat. Each test container contained 750 grams of prepared soil. The worms were not provided food during the test period. At test initiation (Day 0), the worms were placed on the surface of the soil and observed for 30 minutes to assess burrowing behavior. On Days 7 and 14, the contents of each test chamber were removed to determine the number of surviving worms. On Day 7, following observations, the test soil was returned to the test chambers and the worms were placed on the soil surface in order to observe burrowing behavior. On Day 14, following observations and body weight determinations, surviving earthworms were euthanized. Samples of soil were collected from each treatment (except positive controls) and saved to verify the presence or absence of biological activity. This was done by conducting a bioassay with *Helicoverpa zea*. The bioactivity of the Cry1A.105 treated soil was also compared against a reference standard of Cry1A.105 provided by the study sponsor. There were no mortalities in the assay control group, buffer control group, or Cry1A.105 protein group. In the 15 mg chloroacetamide/kg reference

group there was 48% mortality and in the 30 mg chloroacetamide/kg reference group there was 100% mortality at test termination. A slight loss in average individual body weight from test initiation to test termination was noted in all test groups and was expected since the worms were not fed during the 14-day test. Losses in body weight in the Cry1A.105 protein test substance group were not statistically significant when compared to the control substance group. Analysis of the test soil showed that Cry1A.105 was present in the soil and was biologically active against *Helicoverpa zea*.

Conclusions/Recommendations: This study is acceptable. The 14-day LC_{50} for earthworms was determined to be greater than 178 mg Cry1A.105 protein/kg dry soil. The NOEC was determined to be greater than 178 mg Cry1A.105 protein/kg dry soil.

2. Soil Fate (MRID 469514-28)

Soil organisms may be exposed to Cry1A.105 and Cry2Ab2 protein through contact with corn plant roots (by direct feeding), corn plant root exudates, incorporation of above-ground plant tissues into soil following harvest, or by soil-deposited pollen. Some evidence suggests that soils which are high in clays and humic acids are more likely to bind Cry protein. However, neutral pH soils (above pH 5.6), that are typical of corn production sites, tend to have high microbial activity and microbes contribute to Cry protein degradation. Despite evidence that soils high in clay and humic acids may bind Cry proteins, and thus interfere with the microbial degradation processes, the weight of evidence indicates that Cry proteins do not accumulate in soil to arthropod-toxic levels. Nonetheless, the Agency required the following soil fate evaluations to support the MON 89034 *Bt* corn registration.

A study of Cry protein degradation in soil evaluated clay, silt loam, and loamy sand soils that were spiked with Cry2Ab2 (0.60 µg/g) or Cry1A.105 (0.062 µg/g) protein and incubated under controlled conditions for four months. The soils were dosed with an approximately 500-fold excess of the maximum calculated protein concentrations in the field. Samples of the treated soils were collected eleven times during the incubation period and analyzed for protein content using western blot analysis (Cry2Ab2 only) and a corn earworm bioassay. Results indicated that Cry2Ab2 protein concentration decreased by 50% in about 1 to 6 days, and by 90% in about 3 to 14 days in the three soils. The amount of Cry1A.105 protein decreased by 50% in about 2 to 7 days and by 90% in about 7 to 19 days in the same soils.

This study utilized field soil spiked with purified insecticidal protein. This approach is useful because dose responses can be easily quantified. However, the degradation and accumulation of Cry proteins found within decaying plant tissue may behave differently than proteins in artificially spiked soil. Thus the presence of low levels of Cry protein in the soil (at or below the level of detection) is anticipated until all plant tissue is 'mineralized'. The data reviewed here do, however, show that Cry proteins will be quickly degraded upon release from decaying plant tissue. More specifically, a study that evaluated Cry1Ab protein accumulation in a field with three years of continuous Cry1Ab field corn production showed that the protein had not accumulated in soil to a level that would elicit a toxic response from ECB larvae, a species that is highly susceptible to Cry1Ab protein (Milofsky, 2006).

As a result of FIFRA Scientific Advisory Panel recommendations and public comments, the Agency has been receiving three year soil fate studies for the currently registered Cry protein producing crops grown in a variety of soils and environmental conditions. The results of these studies show that there is no detectable Cry protein accumulation in agricultural soils during commercial planting of currently registered Cry protein producing crops. Therefore no additional long term soil degradation studies are required for Cry2Ab2 or Cry1A.105 proteins.

3. Effects on Soil Microorganisms

Numerous published studies indicate that exposure to Cry protein produced in *Bt* PIP crop plants does not adversely affect soil microorganisms. Although a minimal transient increase and shift in microbial populations may result from the presence of transgenic plant tissue in soil, no adverse effects have been attributed to the Cry protein. In addition, there are several ongoing U.S. Department of Agriculture and EPA Office of Research and Development funded research projects evaluating the effects of Cry protein crops on soil microbial flora. If adverse effects are seen from this or any other research, the Agency will take appropriate action to mitigate potential risks.

With regard to the impact of genetically engineered crops on soil, it is important to note that agricultural practices themselves cause large changes in soil and soil microbial composition. Furthermore, factors such variations in seasons and weather, plant growth stage, and plant varieties, independent of their being genetically engineered, are also responsible for significant shifts in soil microbial communities. Most studies with genetically engineered crops to date have shown minor or no effects on soil microbes beyond the variation caused by the factors listed above

4. Horizontal Transfer of Transgenes from *Bt* Crops

The EPA has evaluated the potential for horizontal gene transfer (HGT) from *Bt* crops to soil organisms and has considered possible risk implications if such a transfer were to occur. Genes that have been engineered into *Bt* crops are mostly found in, or have their origin in, soil-inhabiting bacteria. Soil is also the habitat of anthrax, tetanus and botulinum toxin-producing bacteria. Transfer of these genes and/or toxins to other microorganisms or plants has not been detected. Further, several published studies conducted to assess the likelihood of HGT have been unable to detect gene transfer under typical environmental conditions. Horizontal gene transfer to soil organisms has only been detected with very promiscuous microbes under laboratory conditions designed to favor transfer.

As a result of these findings, which suggest that HGT is at most an artificial event, and the fact that *Bt* toxins engineered into MON 89034 were derived from soil-inhabiting bacteria, the EPA has concluded that there is a low probability of risk from HGT of transgenes found in Cry1A.105 and Cry2Ab2 producing corn.

5. Gene Flow and Weediness Potential

Movement of transgenes from crop plants into weeds is a significant concern, due to uncertainty regarding the effect that a new pest resistance gene may have on plant populations in the wild. Under FIFRA, the EPA has reviewed the potential for gene capture and expression of *Bt* endotoxins by wild or weedy relatives of corn, cotton, and potatoes in the U.S., its possessions and/or territories. To date, *Bt* plant-incorporated protectants have been registered for use in agronomic plant species that do not have a reasonable possibility of passing their traits to wild native plants. However, due to concern over the possibility that species related to corn (*Zea mays* ssp. *mays*), such as *Tripsacum* species and the teosintes, could be recipients of gene flow from genetically modified *Z. mays,* EPA conducted a thorough review of the scientific literature on what is known about the gene flow potential of *Z. mays* (*Bt* Reassessment Document, 2001). Conclusions gathered from this review process are as follows:

- The potential for pollen-directed gene flow from corn to Eastern Gama Grass is extremely remote (DeWald *et al.*, 1999). This is evidenced by the difficulty with which *Tripsacum dactyloides* x *Z. mays* hybrids are produced in structured breeding programs. Additionally, the genus *Zea* does not represent any species considered as serious or pernicious weeds in the United States or its territories (Holm *et al.*,1979). Any introgression of genes into this species as a result of cross fertilization with genetically-modified corn is not expected to result in a species that is weedy or difficult to control. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the corn chromosomal complement in subsequent generations (Chester DeWald, personal communication, 1999).

- Many of the *Zea* species loosely referred to as "teosintes" will produce viable offspring when crossed with *Zea mays* ssp. *mays*. However, none of these plants are known to harbor weedy characteristics and none of the native teosinte species, subspecies or races are considered to be aggressive weeds in their native or introduced habitats (John Schoper, personal communication, 1999). In fact, many are on the brink of extinction where they are indigenous and will be lost without human intervention (*i.e.* conservation measures). Further, none of the landraces or cultivated lines of *Z. mays* are considered to have weedy potential and are generally considered to be incapable of survival in the wild as a result of breeding practices (*i.e.* selection) during domestication of the crop.

The October 2000 Scientific Advisory Panel agreed that the potential for gene transfer between corn and any receptive plants within the U.S., its possessions and territories was of limited probability and nearly risk free. As a result of these findings, the EPA has determined that there is no significant risk of gene capture and expression of Cry1A.105 and Cry2Ab2 proteins by wild or weedy relatives of corn in the U.S., its possessions or territories.

6. Impacts on Endangered Species

The primary route of exposure to Cry1A.105 and Cry2Ab2 proteins in corn is through ingestion of corn tissue. There are no reports of threatened or endangered species feeding on corn plants,

therefore such species would not be exposed to corn tissue containing Cry protein. Since Cry1A.105 and Cry2Ab2 proteins have not been shown to have toxic effects on mammals, birds, plants, aquatic species, insects and other invertebrate species at the Estimated Environmental Concentration (EEC), a "may affect" situation for endangered land and aquatic species is not anticipated. In addition, EPA does not expect that any threatened or endangered plant species will be affected by outcrossing to wild relatives or by competition with such entities. Hybrid corn does not exist in the wild, nor are there wild plants that can interbreed with corn in the United States.

Because of the selectivity of Cry1A.105 and Cry2Ab2 proteins for lepidopteran species, endangered species concerns are mainly restricted to the order Lepidoptera. Examination of an overlay map showing the county level distribution of endangered/threatened lepidopteran species (currently listed by the U.S. Fish and Wildlife Service) relative to corn production counties in the United States clearly indicated that any potential concern regarding range overlap with corn production was mainly restricted to the Karner blue butterfly (*Lycaeides melissa samuelis*). Research demonstrates that the Cry1A.105 and Cry2Ab2 proteins are selectively toxic to lepidopteran larvae at field concentrations and that the Karner Blue butterfly is the only endangered lepidopteran species that may be exposed to MON 89034 (via pollen). A model developed to assess the risk of *Bt* corn to Monarch butterfly larvae was used to assess the risk of MON 89034 to Karner blue larvae. Based on the LC_{50} value for larvae of the most sensitive known lepidopteran species (ECB) and the maximum estimated level of Cry protein in pollen-contaminated food (9.27 µg/g fresh weight), the margin of safety was calculated to be >10X maximum estimated exposure of Karner blue larvae to corn pollen. These results indicate that cultivation of MON 89034 is not likely to pose a risk to endangered species.

After careful review of available data, the EPA determined that exposure of the Karner blue butterfly to harmful levels of MON 89034 corn plant tissue is not expected. Likewise, a review of the preferred habitats of other lepidopteranan species listed as endangered by the U.S. Fish and Wildlife Service indicated that exposure to harmful levels of Cry1A.105 or Cry2Ab2 protein would not take place. The main reasons for the lack of exposure are geographical and habitat limitations. These species are located in non-corn production areas and/or their habitat does not encompass agricultural areas.

Likewise, other insect species in the orders Diptera, Hemiptera, Lepidoptera, Odonata and Orthoptera that are listed as endangered/threatened species are found in dune, meadow/prairie or open forest habitats and are not closely associated with row crop production, often times due to the specificity of the habitat of their host plants. The reviewed toxicological data shows the relative insensitivity of a range of insects in non-lepidopteran orders to the Cry1A.105 and Cry2Ab2 proteins, indicating that MON 89034 maize hybrids are not likely to have detrimental effects on non-lepidopteran insects included on the endangered/threatened species list.

In light of the above considerations (based on no spatial and temporal overlap), the Agency has determined that registered uses of MON 89034 corn will have No Effects (NE), direct or indirect, on endangered and threatened species or their habitat as listed by the United States Fish

and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds or terrestrial and aquatic plants and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

IV. MON 89034 Corn Environmental Risk Assessment

The EPA uses a Maximum Hazard Dose Tiered system for biopesticide non-target wildlife hazard assessment. When no adverse effects at the maximum hazard screening dose are observed on representative non-target species, the Agency concludes that there are no unreasonable adverse effects on non-target populations from the use of the pesticide.

1. Direct effects

At present, the Agency is aware of no identified significant adverse effects of Cry protein on the abundance of non-target organisms in any population in the aquatic or terrestrial field environment, whether they are animals, plants, pest parasites, pest predators, or pollinators. Further, the EPA believes that cultivation of MON 89034 corn may have fewer adverse impacts on non-target organisms than use of chemical pesticides for corn production, because under normal circumstances, MON 89034 corn requires substantially fewer applications of chemical pesticides, compared to production of non-Bt corn. Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers. In addition, no adverse effect on Federally listed endangered and threatened species is expected from the proposed lepidopteran-resistant corn registration. Further, the EPA has determined that there is no significant risk of gene capture and expression of Cry1A.105 or Cry2b2 proteins by wild or weedy relatives of corn in the U.S., its possessions, or territories (see Section A.III.5 above), available data do not indicate that Cry proteins have any measurable adverse effect on microbial populations in the soil (see Section A.III.3 above), nor has horizontal transfer of genes from transgenic plants to soil bacteria been demonstrated (see Section A.III.4 above). In conclusion, this risk assessment finds no hazard to the environment at the present time from cultivation of MON 89034 corn for a time-limited registration.

2. Indirect effects:

The purpose of using PIP plants is the same as for any other pest management tactic, i.e., to reduce pest populations below economic injury levels. As a result the abundance of pest insects should be significantly reduced and this will have corresponding implications for those organisms that exploit these pests as prey and hosts. Thus, the potential for these indirect ecological effects on biological control organisms should not be regarded as a unique ecological risk associated with the PIP crop. Some reductions, however, should be expected if the pest management strategy is effective. Since PIP crops are often grown in vicinity with conventional crops to prevent resistance build-up by the target pest(s), specialist antagonists can persist in these 'refuges', in other crops and in non-crop habitats and retain the potential for recolonization of the PIP crop area. Based on these considerations, regulatory testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

B. Supplemental data needed to confirm MON 89034 Non-Target Hazard Assessment

The Agency has sufficient information to believe that there is no risk from the proposed uses of MON 89034 corn to non-target terrestrial wildlife, aquatic, and soil organisms. The Agency has been frequently asking the registrants to conduct post-registration long term invertebrate population/community and Cry protein accumulation in soils studies as a condition of registration. The issue of long range effects of cultivation of these Cry proteins on the invertebrate community structure in corn fields has since been adequately addressed by the analysis of field studies performed during the last 10 years (Marvier, et al. 2007; Sanvido, et al. 2007). No unexpected adverse effects on invertebrate community structure were reported. The Agency is in agreement with these conclusions. Likewise, no unexpected accumulation of Cry proteins in agricultural soils was seen in published studies (Icoz and Stotzky 2007; Sanvido, et al. 2007) and in numerous studies submitted directly to the EPA for the currently registered Cry proteins. (Milofsky, 2006; Section A.III.2 above).

However, in light of recently published laboratory studies showing reduced growth in shredding caddis flies exposed to anti-lepidopteran Cry1A protein corn litter (Rosi-Marshall, et al. 2007), additional aquatic invertebrate data are required. The submitted *Daphnia magna* study is unacceptable because it is an 850 Series OPPTS Guideline study. The 48 hour duration of this study is not sufficient to detect mortality due to Bt proteins. It takes more than 48 hours for the target pests to succumb to the Cry proteins, therefore 48 hours is also not expected to show mortality or reproductive effects on *Daphnia.* A 7 to 14 day *Daphnia* study as per the 885 Series OPPTS Guidelines needs to be performed. The study may be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the *Daphnia* study.

Table 2. Supplemental non-target data requirements for MON 89034 corn.

Testing Category	Type of Data
Aquatic invertebrate	A 7 to14 day *Daphnia* study as per the 885 Series OPPTS Guidelines has to be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the *Daphnia* study.

C. MON 88017 (coleopteran active Cry3Bb1) Environmental Risk Assessment

Potential adverse effects to non-target organisms by Cry3Bb1 protein has been reviewed (EPA. 2003). The following is a summary of the Cry3Bb1 environmental risk assessment.

For registration of Cry3Bb1 (MON 863 – which also applicable to MON 88017), EPA reviewed studies conducted on representative non-target species with several Cry3Bb1 protein variants and performed risk assessments on plants, wild mammals, birds, fish, aquatic invertebrates, estuarine and marine animals, earthworms, terrestrial non-target insects including honey bee adults and larvae, parasitic wasps, green lacewings, several lady beetle species, springtails (Collembola toxicity/reproduction), monarch butterflies, field evaluations of the effects of Cry3Bb1 exposure on non-target invertebrates, soil degradation/persistence studies and an endangered species impact assessment (EPA, 2003). In addition, gene flow and weediness assessments via pollen and Cry protein DNA uptake by plants and soil microorganisms were also performed. EPA concluded that the Agency has sufficient information to believe that there is no risk from the uses of Cry3Bb1 corn to non-target wildlife, aquatic, and soil organisms.

In 2007 an additional assessment of possible effects on Hungerford's crawling water beetle was performed for this registration. Hungerford's crawling water beetle species is currently known to occur in only six streams - five in mostly northern Michigan and one in Ontario, Canada. These are not major corn growing areas. The beetles are found in the cool riffles of clean, slightly alkaline streams. All streams where this beetle has been found have moderate to fast water flow, good stream aeration, inorganic substrate, with an open to partially open canopy just below beaver dams or similar human-made structures. Adults prefer gravel and cobble riffles while larvae occupy areas with slower current and dense growth of microalgae, especially Chara. Since the Hungerford's crawling water beetle larvae are reported to feed on filamentous algae (and possibly periphytic diatoms), no dietary exposure to anti-coleopteran Cry protein in corn tissue is expected. Therefore, the previous finding of No Effect (NE), direct or indirect, from cultivation of anti-coleopteran Cry protein containing corn to Hungerford's crawling water is confirmed.

At present, the Agency is aware of no identified significant adverse effects of Cry protein on the abundance of non-target organisms in any population in the aquatic or terrestrial field environment, whether they are animals, plants, pest parasites, pest predators, or pollinators. . Field testing and field census data submitted to the Agency show minimal to undetectable changes in the beneficial insect abundance or diversity. In corn fields densities of predatory and non-target insects are generally higher on Cry3Bb1 corn than non-Bt corn. Two year invertebrate abundance studies do not show a shift in biodiversity in Cry3Bb1 corn fields, except in cases where the predators are dependent on the pest insect as prey. In contrast, treatment with chemical pesticides, when studied, had significant effects on the total numbers of insects and on the numbers within the specific groups. To date the available field test data show that compared to crops treated with conventional chemical pesticides, the transgenic crops have no detrimental effect on the abundance of non-target invertebrate populations.

The movement of transgenes from Cry3Bb1 host plant into weeds and other crops has also been considered. The Agency has determined that there is no significant risk of gene capture and expression of Cry3Bb1 protein by wild or weedy relatives of corn in the U.S., its possessions or territories. The fate of Cry3Bb1 protein in soils and indirect effects on soil biota have also been evaluated (EPA, 2003; Icoz and Stotzky, 2007). The data show that most of the Cry protein

deposited into soil is quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time. It is also reported that the same degree of Bt Cry protein persistence takes place in soils that have been exposed to repeat Bt spray applications when compared to soil exposed to growing Bt crop. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil. Horizontal transfer from transgenic plants to soil bacteria has not been demonstrated (Sanvido, et al.2007). Published studies of Bt Cry protein in soil show no effect on bacteria, actinomyces, fungi, protozoa, algae, nematodes, springtails or earthworms (Saxena and Stotzky, 2001). In addition, new plants grown in Bt Cry protein-containing soil do not take up the Bt protein.

This assessment finds no hazard to the environment at the present time from cultivation of Cry3Bb1 protein expressing corn.

D. Potential Interaction Between Cry1A.105, Cry2b2 and Cry3Bb1 Proteins (MRID 469513-05 & 469513-06)

The purpose of these studies was to characterize the potential for interaction between the lepidopteran-active proteins Cry1A.105 and Cry2Ab2 and the coleopteran-active protein Cry3Bb1. The Cry1A.105 and Cry1A.105 and Cry2Ab2 proteins were tested alone and in combination with either the Cry3Bb1 protein against European corn borer (ECB, *Ostrinia nubilalis*) and corn ear worm (CEW, *Helicoverpa zea*) in diet incorporation studies. Likewise, the Cry3Bb1 protein was tested alone and with the Cry1A.105 and/or the Cry2Ab2 proteins, against the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*). The activity of Cry1A.105 and Cry2Ab2 proteins was not significantly altered by the presence of Cry3Bb1, and the activity of Cry3Bb1 was not significantly altered by the presence of Cry1A.105 and/or Cry2Ab2. Collectively these data provide evidence that the proteins do not interact in an antagonistic, additive, or synergistic manner. This study, along with the interaction study between Cry1A.105 and Cry2Ab2 reviewed for the MON 89034 Experimental Use Permit indicate that MON 89034 x MON 88017 maize will not result in any unexpected interaction in an antagonistic, additive, or synergistic manner with regards to target insects. It is therefore extremely unlikely that the Cry1A.105, Cry2Ab2 and Cry3Bb1 proteins contained in a single plant will impart any hazard to non-target organisms exposed to these hybrids in the environment.

E. Environmental Risk Assessment for MON 89034 x MON 88017

I. Background

The first part of this document contains environmental risk assessments for Cry1A.105/Cry2b2 (MON 89034) and Cry3Bb1 (MON 88017) proteins. The MON 88017 assessment is based on the substantial similarity between the Cry3Bb1 protein in MON 88107 and the Cry3Bb1 protein produced in corn event MON 863 (EPA Reg. No. 524-528) which was granted registration in February 2003. The MON 863 environmental risk findings are summarized in Section C above. The Cry3Bb1 proteins produced in MON 88017 and MON 863 share an amino acid sequence

identity of >99.8%, differing only by one of 653 amino acids, and express similar protein levels in plant tissues (MRID No. 46185001). The Cry3Bb1 protein produced in MON 88017 is a variant of the wild-type Cry3Bb1 protein from *B.t.* subsp. *kumamotoensis* that protects the roots of corn plants from feeding damage caused by the coleopteran pest corn rootworm (*Diabrotica* sp.). MON 88017 also expresses the 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4 (CP4 EPSPS) which confers tolerance to glyphosate, the active ingredient in Roundup7 herbicides. The CP4 EPSPS protein is classified as a plant pesticide inert ingredient. MON 89034 is the second event in the triple stack providing protection against the European corn borer (ECB; *Ostrinia nubilalis*) and other lepidopteran pests.

II. MON 89034 x MON 88017 Environmental Risk Assessment

Monsanto is combining individual MON 89034 (Cry1A.105 and Cry2Ab anti-lepidopteran proteins) with MON 88017 (reviewed above). MON 88017 corn produces a Cry3Bb1 protein for protection against corn rootworm (Coleoptera). The MON 89034 x MON 88017 corn is obtained by conventional breeding of the stacked MON 89034 and single PIP trait MON 88017 corn lines. For environmental risk assessment purposes development of new non-target species data for the triple stack was not required because the non-target data and the environmental risk assessments reviewed above for the double stacked (MON 89034)and single (MON 88017) PIP lines are applicable to the MON 89034 x MON 88017 corn line. New data on the potential synergistic interaction between Cry1A.105, Cry2b2 and Cry3Bb1 proteins were developed to confirm this hypothesis. The results of the interaction study show that there is no change in the level of activity among susceptible insects when the three traits are combined. Therefore these data are also indicative of similarity in effects on non-target organisms of the stacked versus single trait hybrids. As a result the environmental risk assessment of the Cry1A.105/Cry2b2 x Cry3Bb1 proteins concludes that there will be no unreasonable adverse effects to the environment, including endangered species, by MON 89034 x MON 88017 corn.

The environmental assessment of the Cry1A.105, Cry2b2 and Cry3Bb1 stack, based on assessments on Cry1A.105, Cry2b2 and Cry3Bb1 proteins individually, indicates that no unreasonable harm will result to the environment from commercial cultivation of MON 89034 x MON 88017 corn. The Agency has also determined that MON 89034 x MON 88017 corn will have No Effect (NE), direct or indirect, on endangered and/or threatened species listed by the US Fish and Wildlife Service(USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds, terrestrial and aquatic plants, and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

Furthermore, the Agency believes that cultivation of MON 89034 x MON 88017 corn may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, Cry protein expressing corn requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of nonspecific conventional chemical pesticides is known to have an adverse effect on non-target beneficial organisms found living in the complex environment of an agricultural field. Many of these beneficial organisms are important integrated pest management controls (IPM) for secondary pests such as aphids and leafhoppers.

The overall result of cultivation of corn expressing Cry proteins is that the number of chemical insecticide applications for nontarget pest control is reduced for management of multiple pest problems.

F. References

DeWald, Chester. (1999). Personal communication. plant breeder and geneticist, USDA-ARS, Woodward, OK, December, (580_256_7449).

DeWald, C.L., P. Sims, Y. Li, and V.A. Sokolov. (1999). A novel cytoplasm for maize. Maize Genetics Conference Abstracts. 41:P114.

Edelstein, R. (2007). Review of Human Health and Product Characterization Data for Registration for *B. thuringiensis* Cry1A.105 and Cry2Ab2 Proteins and the Genetic Material Necessary for their Production in MON 89034 Corn. U.S. Environmental Protection Agency. Washington, D.C.

Holm, L, J.V. Pancho, J.P. Herberger, and D.L. Plucknett. (1979). In: *A geographical atlas of world weeds*. (pp. 391). John Wiley and Sons, New York.

Hunter, M. (2006). Review of "Evaluation of the Potential for Interactions Between *Bacillus thuringiensis* Proteins Cry1A.105 and Cry2Ab2" for Monsanto's MON 89034 X MON 88017 Maize Experimental Use Permit 524-EUP-OT. July 6, 2006.

Icoz, I, and G. Stotzky (2007). Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil. Transgenic Research, September 13, 2007.

Marvier, M., McCreedy, C., Regetz, J. & Kareiva, P. A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316, 1475–1477 (2007).

Milofsky, T. and Z. Vaituzis (2006). Review the soil fate study submitted in support of ABSTC's Cry1Ab corn registrations. Biopesticides and Pollution Prevention Division. U.S. Environmental Protection Agency. Washington, D.C.

Rosi-Marshall E. J., J. L. Tank, T. V. Royer, M. R. Whiles, M. Evans-White, C. Chambers, N. A. Griffiths, J. Pokelsek, and M. L. Stephen. (2007). Toxins in transgenic crop byproducts may affect headwater stream ecosystems. PNAS , vol. 104, no. 41, 16204–16208.

Sanvido,O., Romeis, J., Bigler, F. (2007). Ecological Impacts of Genetically Modified Crops: Ten Years of Field Research and Commercial Cultivation. Adv Biochem Engin/Biotechnol 107: 235–278

Saxena, D. and Stotzky, G. (2001) *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. Soil Biol. Biochem., 33, 1225–1230

Schoper, J. (1999). Personal communication. Geneticist, Pioneer Hi-Bred International, Johnston, IA, (515-270-3544).

USEPA (2000). SAP report No 99-06. Sets of scientific issues being considered by the Environmental Protection Agency regarding: Section I - Characterization and Nontarget Organism Data Requirements for Protein Plant Pesticides. Dated February 4, 2000. Available at the EPA website: http://www.epa.gov/scipoly/sap/1999/index.htm#december

USEPA (2001). SAP Report No. 2000-07. Sets of scientific issues being considered by the Environmental Protection Agency regarding: Bt plant-pesticides risk and benefit assessments. Dated March 12, 2001. Web site: http://www.epa.gov/scipoly/sap/2000/october/octoberfinal.pdf

USEPA (2002). SAP Report No. 2002-05. A set of scientific issues being considered by the Environmental Protection Agency regarding: Corn rootworm plant-incorporated protectant nontarget insect and insect resistance management issues. Dated November 6, 2002. Web site: http://www.epa.gov/scipoly/sap/2002/august/august2002final.pdf

EPA. (2003). Ecological hazard assessment for *Bacillus thuringiensis* Cry3Bb1 protein, EPA Reg. No. **524-LEI**

Vaituzis, Z. and R.I. Rose. (2000). Reassessment of *Bt* crop effects on nontarget wildlife. Biopesticides and Pollution Prevention Division. U.S. Environmental Protection Agency. Washington, D.C.

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Chemical Name: Bacillus thuringiensis Cry3Bb1 protein and the genetic material necessary (vector ZMIR39) for its production in corn
Bacillus thuringiensis Cry2Ab2 protein and the genetic material necessary (vector PV-ZMIR245) for its production in corn
Bacillus thuringiensis Cry1A.105 protein and genetic material necessary (vector PV-ZMIR245) for its production in corn

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