BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Cry1Ab and Cry1F *Bacillus thuringiensis* (*Bt*) Corn Plant-Incorporated Protectants

U.S. Environmental Protection Agency
Office of Pesticide Programs
Biopesticides and Pollution Prevention Division

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I. Overview

A. EXECUTIVE SUMMARY

EPA made the decision to amend the registrations of eighteen (18) expiring *Bt* corn PIP registrations to extend the expiration dates. We conducted comprehensive assessments of each of these registrations, considering all toxicity and environmental effects data, data from insect resistance monitoring, and insect resistance refuge compliance reports, received and obtained since the last comprehensive evaluation of these products in 2001. Based upon our comprehensive assessment, we reached significant conclusions regarding the positive environmental impact of *Bt* corn PIPs, and we took several actions to strengthen the insect resistance management requirements to ensure continued success in the prevention of the evolution of resistance in target pests.

Since the commercialization of *Bt* crops, there have been a significant number of published field studies that, combined with the post-registration field studies required to be submitted to the Agency, have demonstrated that non-target invertebrates are generally more abundant in *Bt* cotton and *Bt* corn fields than in non-transgenic fields managed with chemical insecticides. Thus, these published and registrant-produced studies demonstrate that, not only are the *Bt* crops not causing any unreasonable adverse effects in the environment, but, arthropod prevalence and diversity is greater in *Bt* crop fields.

To strengthen insect resistance management of these corn PIPs and to address reports that compliance with the mandated refuge requirements has been decreasing, EPA is requiring enhanced compliance assurance programs (CAPs), and a phased requirement for seed bag labeling that clearly shows the refuge requirements. Also, given the increasing variety of PIP products and combinations, and the differing risk of resistance evolution that the various products represent, we are granting registrations for the corn PIP products for different time frames, based on assessments of their likelihood of forestalling the evolution of insect resistance. We are registering differing categories of products for differing time periods to reflect the assessed level of risk of resistance posed by the various corn PIP products. The scheme that we are following includes registration periods generally of five, eight, and twelve years; with the possibility of a fifteen-year registration period for products that are demonstrated to meet specified criteria. We retain, however, the discretion to register products for time periods differing from these defaults where circumstances warrant.

B. Cry1Ab AND Cry1F Bt CORN PLANT-INCORPORATED PROTECTANTS

1. Bt11, Cry1Ab *Bt* Corn

OPP Chemical Code: 006444

Pesticide Name: *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material (via elements of vector pZO1502) necessary for its production in corn (SYN-BTØ11-1)

Trade and Other Names: Bt11, YieldGard, Agrisure®, Attribute™

Uses: Full Commercial Use in Field Corn and Sweet Corn

Registrant:

Syngenta Seeds, Inc
P.O. Box 12257
Research Triangle Park, NC 27709-2257

Registrations: 67979-1 Bt Corn Event Bt11 with Cry 1Ab (Field Corn)
65268-1 Bt Corn Event Bt11 with Cry 1Ab (Sweet Corn)

2. MON810, Cry1Ab *Bt* Corn

OPP Chemical Code: 006430

Pesticide Name: *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material lnecessary for its production (Vestor PV-ZMCT01) in event MON 810 corn (OECD Unique Identifier: MON-ØØ81Ø-6)

Trade and Other Names: MON 810, Yieldgard®

Uses: Full Commercial Use in Field Corn

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

Pioneer Hi-Bred International, Inc.
P.O. Box 1000
Johnston, Iowa 50131-1000

Registrations: 524-489 Bt Corn Event MON 810 Cry1Ab
29964-7 1507 (POCry1F) x MON 810 (Cry1Ab)

3. TC1507, Cry1F *Bt* corn

OPP Chemical Code: 006481

Pesticide Name: *Bacillus thuringiensis* Cry1F protein and the genetic material (plasmid insert PHI8999A) necessary for its production in corn event DAS-Ø15Ø7-1

Trade and Other Names: Herculex™ I Insect Protection

Registrants: Mycogen Seeds
c/o Dow Agrosciences LLC
9330 Zionsville Road
Indianapolis, IN 46268-1054

Pioneer Hi-Bred International, Inc.
P.O. Box 1000
Johnston, Iowa 50131-1000

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

Registrations: 68467-2 Bt Corn Event TC1507 with PO Cry 1F
29964-3 Bt Corn Event TC1507 with PO Cry 1F
29964-7 1507 (POCry1F) x MON 810 (Cry1Ab)

Uses: Full Commercial Use in Field Corn

5. DAS-06275-8 moCry1F *Bt* corn

OPP Chemical Code: 006491

Pesticide Name: *Bacillus thuringiensis* var. *aizawai* strain PS811 Cry1F protein and the genetic material necessary for its production (plasmid insert PHP12537) in corn event DAS-Ø6275-8

Trade and Other Names: Mycogen Brand B.t. moCry1F

Registrants: Mycogen Seeds
c/o Dow Agrosciences LLC
9330 Zionsville Road
Indianapolis, IN 46268-1054

Registrations: 68467-4 Bt Corn Event DAS-06275-8 with MOCry1F

Uses: Full Commercial Use in Field Corn

C. FOOD CLEARANCE/TOLERANCE EXEMPTION LISTINGS

40 CFR Part 174.511 - Bacillus thuringiensis Cry1Ab protein in all plants; exemption from the requirement of a tolerance.

Residues of *Bacillus thuringiensis* Cry1Ab protein in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all food commodities.
[72 FR 20435, Apr. 25, 2007]

40 CFR Part 174.520 - Bacillus thuringiensis Cry1F protein in corn; exemption from the requirement of a tolerance.

Residues of *Bacillus thuringiensis* Cry1F protein in corn are exempt from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of corn; corn, field; corn, sweet; and corn, pop.
[72 FR 20435, Apr. 25, 2007]

II. Science Assessment

A. PRODUCT CHARACTERIZATION

Product characterization is critical to understanding the way in which the registered products were made and the unique characteristics that need to be assessed for each *Bt* plant-incorporated protectant. The product characterization data provide information on the specific transformation systems used for each product, on the actual DNA inserted into the plant, on the inheritance and stability of these traits in the plant, on biochemical characteristics of the *Bt* protein and on *Bt* protein expression levels for various plant tissues. Specific information and data for each of the registrations seeking renewal are included in tabular and descriptive formats.

The classifications that are found for each data submission are assigned by the EPA science reviewer and are an indication of the usefulness of the information contained in the documents and if the data meet the intent of the test guidelines. A rating of "ACCEPTABLE" indicates the study is scientifically valid and has been satisfactorily performed according to accepted EPA guidelines or other justified criteria. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. However, the studies may either have certain aspects not determined to be scientifically acceptable (SUPPLEMENTAL. UPGRADABLE) or that the studies have not been done to fulfill a specific EPA guideline requirement. If a study is rated as "SUPPLEMENTAL. UPGRADABLE," EPA always provides an indication of what is lacking or what can be provided to change the rating to "ACCEPTABLE." If there is simply a "SUPPLEMENTAL" rating, the reviewer will often state that the study is not required by current EPA guidelines or does not need to be reclassified as "ACCEPTABLE." Both ACCEPTABLE and SUPPLEMENTAL studies may be used in the risk

assessment process as appropriate.The following table summarizes the registered *Bt* protein-containing plant-incorporated protectant products being evaluated.

Table A1 - *Bt* Plant-incorporated protectant Products

Common Name and Cry Protein	OPP Chemical Code	Company	Plasmid ID	Plant/ Trade Name
*Bt*11 Cry1Ab *Bt* Corn	006444	Novartis	pZO1502	YieldGard, Attribute
MON810 Cry1Ab *Bt* Corn	006430	Monsanto	pvZMCT01* pZMBK07 pZMGT10**	YieldGard
Cry1F *Bt* Corn	006481	Mycogen-Dow & Pioneer/Dupont	PHI 8999	Herculex
moCry1F Bt Corn	006491	Mycogen-Dow	PHP12537	Mycogen Brand Bt Cry1F Event DAS-06275-8 Corn

* pvZMCT01 was a mixture of two plasmids
** Plasmid contains marker gene.

Transformation systems: Registered corn products were transformed using protoplast electroporation to introduce the desired DNA, methods employing bombardment of particles coated with DNA encoding the intended insert, or *Agrobacterium tumefaciens.*.

Each plasmid description includes a reference to the strains of *Bacillus thuringiensis* used as the source of the DNA sequence for the toxin protein. In addition, the sources for marker proteins, promoters, terminators and enhancers, as well as the fragment size, orientation and any modifications to the original DNA sequence to enhance expression in the plant are given. All the other DNA sequences introduced to improve or restrict expression of the introduced traits are also described. Finally, the plasmid discussion includes a description of any modifications made to the DNA (e.g., codon modifications to improve eukaryotic expression).

Characterization of the DNA Inserted in the Plant: Inserted DNA is characterized with Southern blot data of the DNA in the plant genome. The analysis usually consists of DNA isolation from the transformed plant, digestion of this DNA with several different endonucleases and hybridization of these restriction endonuclease fragments with labeled-DNA which is complementary to the introduced traits. This analysis includes not only probes specific for the entire insert, but also probes recognizing just the coding regions of the traits or DNA elements outside the coding region. Polymerase chain reaction (PCR) assays utilizing various specific and non-specific primers, genome walking, cosmid libraries and DNA sequencing have also been employed with sensitive Southern blotting techniques to more completely describe the inserted

DNA and surrounding regions. The information available from these blots can indicate the presence of all the elements of the expected insert as well as information about the possibility of deletions and other errors associated with DNA introduction by transformation. Comparison of Southern blots of genomic DNA, digested using a range of restriction endonucleases, can also reveal the copy number of the genes introduced and suspected linkage of the traits. Alternatively, the intensity of the radioactive label from binding the probe DNA can also estimate the number of insert copies incorporated in the plant genome. When the inserted DNA construct includes traits expressed only in bacteria and not expected to be expressed in the plant, data have been presented to indicate that there is no transcription or translation of the bacterial trait (e.g., *ori* and amp^r - discussed further in the horizontal gene transfer section).

Inheritance and Stability after Transformation: The data generated for this endpoint examine progeny from crosses between selected elite lines with the transformed *Bt* expressing line, looking for the independent segregation of the introduced traits in the progeny. Traditional breeding work done during the development of the plant line by backcrossing can reveal the linkage of the introduced traits as well as changes in trait expression. The inheritance data is the ratio of progeny expressing the hemizygous trait based on expected Mendelian inheritance. Stability data implies an examination of either the expression of the trait or tracking of the DNA itself over several plant generations. One of the main concerns with stability is spontaneous loss of the inserted DNA or loss of efficacy due to gene silencing. None of the *Bt* plant-incorporated protectant products showed independent assortment of the introduced traits (usually the marker protein and the *Bt* protein were examined). This indicates that the traits were on the same chromosome and closely linked (crossover events between the two traits were not detected).

The submissions that covered characterization of the actual DNA insert and stability/inheritance data are listed in the MRIDs for each product. These submissions are acceptable and fulfill this data requirement. It should be noted that stability and inheritance were not addressed with the registrations for MON810 (006430). However, considering the use of these crops for several growing seasons and the lack of reports relating to loss of efficacy due to *Bt* protein expression, this specific endpoint can be considered to have been addressed through commercial use.

Protein Characterization and Expression: For the *Bt* plant-incorporated protectants, data has been presented to demonstrate that the protein expressed from the inserted DNA is similar to what was produced in the source bacterium and is active as expected against the intended target insect. Some protein characterization data demonstrate that microbially produced *Bt* protein is the equivalent to that expressed in the plant. This apparent scientific tautology (where plant produced protein is the same as microbial protein is the same as the plant produced protein) has been used to justify the use of the microbially-produced protein as a test substance in toxicity tests. Because the expression level of these proteins is so low in plants, and the maximum hazard dose acute oral toxicity test is required as part of the human health risk assessment for these proteins, the ability to produce the protein in an industrial microbe is essential. The acute oral test requires between 2000 and 5000 mg of protein per kg bodyweight of test animal. Isolating the amount of purified protein required to dose several animals from *Bt*-expressing plants would be a tremendous burden involving harvesting and processing large volumes of plant material (ecological effects testing differs and is addressed in the ecological effects section of

this document). Proper characterization of the equivalency between these microbial proteins and plant expressed proteins provides an alternative to purifying the test material as the plant-produced protein from large volumes of tissue. These equivalency data were generated for all products registered to date.

Much of the characterization data describes the procedures used to isolate the protein or a highly *Bt* protein enriched fraction of plant extract. The tests done to support the equivalence of microbial and plant-produced *Bt* protein include: molecular sizing by SDS-PAGE and western blot analysis; immunorecognition using ELISA and western blot analysis; N-terminal amino acid sequencing; confirmation of the lack of glycosylation in the plant-produced protein; and bioactivity against a range of insects (often pest species including the target pest). Since the issues surrounding non-target effects are considered essential for the ecological effects assessment, these non-target pest tests are also covered in the ecological effects assessment.

The *Bt* protein expression level in various tissues throughout the growing season has been determined for each event. These data have been determined and presented (for the 2010 update), in terms of dry weight, as the amount of protein present in leaf, root, pollen, seed, and root tissue and whole plant. Although Bt11 Cry1Ab data have been provided for field corn, sweet corn data on the Bt11 expression remain as a data gap.

Table A2 - Comparative expression of Cry1F protein in moCry1F TC6275 and poCry1F TC1507 corn tissues

Tissue	**Growth Stage**	**Mean**	**Standard Deviation**	**Min/Max Range**	**Mean**	**Standard Deviation**	**Min/Max Range**
		TC6275			**TC1507**		
		(ng/mg Tissue Dry Weight)					
Leaf	**V9**[b] **R1** **R4** **Senescence**	17.3 28.5 44.8 0.71	3.41 5.38 16.8 1.14	10.7-23.8 16.5-36.7 35.8-109.2 0-3.0.9	12.1	6.2	0-24
Root	**V9**[b] **R1** **R4** **Senescence**	6.14 6.60 5.99 1.97	1.87 1.98 1.89 2.03	4.53-8.14 3.14-10.9 2.35-9.26 0.29-6.91			
Whole Plant	**V9** **R1** **Senescence**	6.22 7.16 2.47	1.16 1.45 0.41	4.98-7.87 5.32-9.57 1.95-3.07	5.2 3.6 1.6	1.9 1.1 0.6	2.6-6.8 2.5-4.7 0.9-2.4
Pollen	**R1**	3.67	0.34	3.09-4.60	21.9	2.9	16.4-27.2
Stalk	**R1**	11.0	2.67	6.77-16.4	5.8	1.7	3.3-10.3
Forage	**R4**	6.26	1.09	5.05-7.77	1.7	1.1	0-3.2
Grain	**Maturity**	1.14	0.27	0.62-1.68	2.2	0.8	0-4

[b] Recalculated Results

Table A4- Cry1Ab Concentrations on a Dry Weight Basis in Event Bt11 Hybrid Plants

Tissue	Location	Hybrid[1]	Developmental Stage		
			V9-V12	Anthesis	Seed Maturity
			mean□g Cry1Ab/gdw ± S.D.[2] (range)		
Leaves	Bloomington, Illinois	Event Bt11	25.88 ± 1.35 (23.89—27.63)	17.82 ± 1.54 (15.99—19.74)	16.84 ± 3.31 (12.17—19.59)
Roots	Bloomington, Illinois	Event Bt11	9.99 ± 0.59 (9.14—10.62)	6.41 ± 0.79 (5.67—7.72)	4.32 ± 1.52 (3.32—6.99)
Kernels	Bloomington, Illinois	Event Bt11	N/A[3]	N/A	1.45 ± 0.07 (1.39—1.56)
Pollen	Mackinaw, Illinois	Event Bt11	N/A	0.04 ± 0.00 (0.036—0.042)	N/A
Pollen	Monroeville, Indiana	Event Bt11	N/A	0.06 ± 0.02 (0.048—0.079)	N/A
Pollen	Seward, Nebraska	Event Bt11	N/A	<0.037	N/A

Table A3 - Average Cry1Ab concentration from Corn Event MON 810 corn tissues grown in four regions of the US.

Tissue (No. days post planting)	Cry1Ab conc. (ug/g dry weight)
Overseason Leaf (OSL)	
OSL-1 (21 days)	120 ± 15
OSL-3 (40 days)	46 ± 5.8
OSL-4 (50 days)	61 ± 17
OSL-5 (60 days)	51 ± 17
Overseason Whole Plant (OSWP)	
OSWP-1 (21 days)	120 ± 34
OSWP-5 (60 days)	25 ± 6.3
Forage (90 days)	7.6 ± 4.5
Pollen (60 days)	NA
Overseason Root (OSR)	
OSR-1 (21 days)	42 ± 9.5
OSR-3 (40 days)	20 ± 5.0
OSR-4 (50 days)	22 ± 3.7
OSR-5 (60 days)	19 ± 8.8
Forage root (90 days)	16 ± 6.0
Grain (125 days)	0.63 ± 0.06

NA = Not Applicable.

Residue Analytical Methods

Analytical methods and method validation (under OPPTS Guidelines OPPTS 860.1340) for the Cry1Ab corn were required in 2001 to complete the database. Although analytical methods had been submitted for Cry1F corn, additional confirmatory methods and standard EPA laboratory method validation were also necessary. The Agency has decided not to require validation of analytical methods by EPA's OPP Microbiology Laboratory at Fort Meade provided the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) has verified the performance of an appropriate qualitative rapid test kit. In the case of Cry1Ab and Cry1F corn, the Agency has confirmed that test kits have been verified by GIPSA and, therefore, the aforementioned requirements have been satisfied.

1. Product Characterization of Bt 11 Cry1Ab Corn (006444)

The corn line Bt 11 was produced by transforming another proprietary corn line with plasmid PZO1502 which contained *cry1Ab, pat* and *amp*^{r} genes. The registrant submission stated that prior to the transformation which resulted in Bt11, the plasmid pZO1502 was digested with the restriction endonuclease *Not* I with the intention to remove the *amp*^{r} gene from pZO1502. While no data was submitted to confirm removal of the *amp*^{r} gene from the transforming DNA, subsequent analysis showed that the *amp*^{r} gene was not present in Bt11 corn. The *cry1Ab* gene was also altered to improve its GC ratio for expression in corn and coded for a truncated form of the original protein. Both field corn and sweet corn containing the plant-incorporated protectant descend from the original Bt 11 transformant.

Data showed that the truncated Cry1Ab toxin could be extracted from corn leaf tissue and this purified material displays characteristics and activities similar to that produced in *E. coli* transformed to produce Cry1Ab. The purified tryptic core proteins from both plant and microbe were shown to be similar in molecular weight by SDS-PAGE, immunorecognition in western blots and ELISA, partial amino acid sequence analysis, lack of glycosylation and bioactivity against either European corn borer or corn earworm. This analysis justified the use of the microbially produced toxin as an analogue for the plant produced protein in mammalian toxicity testing.

The product characterization data supporting the registration of *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production (plasmid vector pZO1502) in corn is listed below.

Study Type	Result	MRID #
Transformation System	Corn line HE89 was transformed with plasmid *pZO1502* which contains genes for a truncated Cry1Ab, PAT and AMP^r. The *cry1Ab* gene was also altered to improve its GC ratio for expression in corn. (See MRID No. 437548-01 below which indicates the absence of the *amp*^{r} gene in the Bt11 and control plants.) CLASSIFICATION: ACCEPTABLE	431308-01
Inheritance and Stability after Transformation	The linkages of the *pat* and *cry1Ab* genes were shown by examining the progeny of two selfed generations derived from a population of corn plants segregating for the desired traits. None of the 2320 plants examined showed the two traits independently assorting which indicates that the loci are tightly linked. CLASSIFICATION: ACCEPTABLE	433526-02
Transformation System	The lack of any positive probe recognition for the plant genomic DNA samples indicate the absence of the *amp*^{r} gene in the Bt11 and control plants. The positive *amp*^{r} gene probe results for the plasmid DNA digest samples confirm that a fragment of a size consistent with the 7.2 Kb pZO1502 plasmid contained the *amp*^{r} gene. This would also be appropriate for any digest which had a single restriction cut site as these enzymes did according to the pZO1502 map. The probe results also indicate that a Not I digest would release the *amp*^{r} gene from the pZO1502 plasmid CLASSIFICATION: ACCEPTABLE	437548-01

Study Type	Result	MRID #
Protein Characterization and Expression	Data is presented showing that the truncated Cry1Ab toxin can be extracted from corn leaf tissue and this purified material displays characters and activities similar to that produced in *E. coli*. The similarities are shown in molecular weight after SDS-PAGE, immunorecogniton in western blots and ELISA of trypsin resistant core proteins, partial amino acid sequence analysis, lack of glycosylation and bioactivity against either European corn borer of corn earworm. CLASSIFICATION: ACCEPTABLE	433972-02
Protein Expression	Event Bt11 corn plant tissues from two field corn hybrids were harvested, analyzed by ELISA for expression of Cry1Ab. Cry1Ab was detected in all transgenic plant tissues sampled and at all developmental stages. Across all plant stages, mean Cry1Ab levels measured in leaves, roots and whole plants ranged from ca. 12-154 µg/g dry wt., 9-22 µg/g dry wt., and 6-70 µg/g dry wt., respectively. Mean Cry1Ab levels measured in kernels at seed maturity and senescence were ca. 2 µg/g dry wt. Cry1Ab levels in pollen were below the lower limit of quantification for pollen. Control sample levels were below the limit of quantification for all stages and tissues. Therefore, the level of Cry1Ab was generally similar between hybrids for each tissue type at each time point. The estimated total Cry1Ab levels per acre and per hectare over the growing season and across genotypes, ranged from mean levels of ca. 14/g acre (31 g/hectare) at whorl stage to ca. 125 g/acre (283 g/hectare) at kernel maturity, assuming a planting density of 26,500 plants per acre (65,500 plants/hectare). CLASSIFICATION: ACCEPTABLE	458798-03
Protein Expression	The values that are reported do not indicate vastly different expression levels for Cry1Ab in the sweet corn varieties compared to the field corn Bt11 x MIR 162 varieties tested. However, no Bt11 sweet corn tissue was analyzed. CLASSIFICATION: SUPPLEMENTAL for Bt11	478820-01
Analytical Method	The EnviroLogix Cry1Ab/Cry1Ac QuickStix™ Kit is designed to extract and detect the presence of Cry1Ab (*Bt*11 or Mon810 event) and Cry1Ac *Bt* proteins at the levels typically expressed in genetically modified corn grain. The sensitivity of the Cry1Ab/Cry1Ac QuickStix strips is 1% based on tests conducted with *Bt*11 corn. Blind studies with conventional corn spiked with *Bt*11 validated the 1% limit of detection. Likewise, corn grain from the following biotech events; Cry9C (StarLink), GA21 (Roundup Ready), NK603 (Roundup Ready), Cry3Bb1 and T25 (Liberty Link) were tested for cross reactivity with the QuickStix™ Kit. All results were negative indicating no cross-reactivity with the tested biotech events. Real-time and accelerated stability data indicate the QuickStix™ Kit (EnviroLogix Cat. #AS003BG) to be stable for 18 months at 4-8°C. CLASSIFICATION: ACCEPTABLE, upon clarification of product stability test data.	456867-01
Analytical Method	Further data on the analytical method as well as validation of an analytical method by EPA's OPP Microbiology Laboratory at Fort Meade is not required since the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) has verified the performance of a qualitative rapid test kit for detecting the presence of the MON 810 and Bt 11 Cry1Ab in grains and oilseeds.	

2. Product Characterization of MON810 Cry1Ab Corn (006430)

Monsanto's corn line MON 810 was produced by ballistically transforming another proprietary corn line with plasmid construct PV-ZMCT01. Plasmid construct PV-ZMCT01 consists of plasmids PV-ZMBK07 & PV-ZMGT10 ballistically introduced together. The MON 810 line of corn is similar to MON 801 corn in that they both were derived from transformation events utilizing PV-ZMCT01. The MON 810 only expresses a truncated version of Cry1Ab delta-endotoxin. MON801 expresses the full length version of Cry1Ab and the marker gene products. MON 810 and MON 801 were each transformed with the same plasmid construct (PV-ZMCT01). The MON 810 progeny express a slightly truncated version of Cry1Ab compared to MON 801, but the active site is still retained. The MON 810 progeny do not express detectable levels of the marker gene products found in MON 801 progeny. Some of the data used to evaluate MON810 corn was generated from MON801 corn. To justify this bridging of data from one corn transformation event to another, the company provided product characterization data to demonstrate the similarities and differences between the two transformation events.

Study Type	Result	MRID #
Transformation System Characterization of the DNA Inserted in the Plant Protein Characterization and Expression	The digests of genomic DNA from corn line MON 80100 revealed that the two plasmids PV-ZMBK07 and PV-ZMGT10 had been inserted apparently at two locations. Full length copies of the cry1Ab, gox, nptII and cp4 epsps genes were found. Less than full length copies of all these genes were also found. Western blot analysis revealed that only Cry1Ab and CP4 EPSPS proteins were expressed at detectable levels in the corn plant. CLASSIFICATION: ACCEPTABLE	435332-01
Protein Characterization and Expression	The antiserum reactions revealed many western blot bands in both the Dipel® and the ECB resistant corn extracts not treated with trypsin. No bands clearly related to the Cry1Ab toxin were seen in the non-transformed plant extracts whereas a band comigrating with the full length Cry1Ac standard (similar in size to Cry1Ab) was seen in both Dipel® and ECB resistant corn. The tryptic digests of Dipel® and ECB resistant corn extracts revealed intensified bands that comigrated with the Cry1Ab tryptic core standard. Together these data infer that the same Cry1Ab protein is being produced in ECB resistant corn plants as is found in the microbial product. CLASSIFICATION: ACCEPTABLE	435332-03

Study Type	Result	MRID #
Characterization of the DNA Inserted in the Plant	The Southern blots with the two transforming plasmids PV-ZMBK07 and PV-ZMGT10 indicate that only a portion of the PV-ZMBK07 plasmid was successfully integrated. Western blots indicate that all the constructs tested (MON801, 802, 805, 809, 810, 813 and 814) produce delta endotoxin detectable as tryptic core with anti-Cry1Ac antiserum. The genes of the second plasmid used to transform the corn lines, PV-ZMGT10, which include *CP4 EPSPS* and *gox*, were not detected by Southern blot analysis using the PV-ZMGT10 plasmid as probe. These genes which confer glyphosate tolerance were apparently lost during development of the MON810 line since they had to be present for the original callus culture selection process but were not found in the final line described here. CLASSIFICATION: ACCEPTABLE	436655-01
Protein Characterization and Expression	The results of the western blot showed the trypsinized extracts of corn lines MON 802, 805, 809, 810, 813, and 814 expressed proteins that comigrated with the Cry1Ab protein as found in MON 801 and the same Cry1Ab protein purified from *E. coli.* These bands also reacted with antiserum #B6 specific for the tryptic core protein of Cry1Ab. These results indicate the trypsinized proteins found in all these plants were of same molecular size (63 kD) and immunoreactivity with the reference standards of Cry1Ab expressed in *E. coli* and corn line MON801. CLASSIFICATION: ACCEPTABLE	436655-03
Protein Characterization and Expression	The Cry1Ab protein produced in *E. coli* was shown by SDS-PAGE, western blot, N-terminal amino acid sequencing, glycosylation and bioactivity to be substantially equivalent to the plant produced Cry1Ab. The test results showed the tryptic core of the plant and microbial protein were of essentially identical SDS-PAGE mobility, immunoreactivity in western blot analysis and N-terminal amino acid sequence for the first 15 positions. A comparison of the dose response relationship of plant and microbial extracts against *Heliothis virescens* and *Helicoverpa zea* indicates that the tested proteins are of similar bioactivity. CLASSIFICATION: ACCEPTABLE. These results allow the substitution of the microbially produced Cry1Ab protein for the plant source in toxicology testing.	435332-04
Protein Expression	The concentration of MON 810 Cry1Ab in the tissues was determined by ELISA and calculated on a wet- and dry-weight basis. Cry1Ab was detected in all corn tissues at appreciable concentrations with the exception of pollen. CLASSIFICATION: ACCEPTABLE.	458789-01
Analytical Method	The independent laboratory validation is acceptable and the method conducted in the independent laboratory validation is suitable for an in-house performance verification at the OPP Microbiology Laboratory. CLASSIFICATION: ACCEPTABLE.	456964-01

Study Type	Result	MRID #
Analytical Method	The OPP Microbiology Laboratory determined that the initial corn samples (event free) provided by the registrant were not suitable for the in-lab validation as set forth in the independent laboratory validation conducted by Medallion Laboratories. A second set of corn samples provided by Monsanto was tested and was also found to be unsuitable for the in-lab validation. Both sets of corn samples provided by Monsanto had background levels of Cry1Ab sufficient to interfere with the method validation.	Protocol # PIP-2004-01, Laboratory Validation of MRID 45694-01, ILV of the SDI Check Bt1 Corn Lateral Flow Test Kit
Analytical Method	Further data on the analytical method as well as validation of an analytical method by EPA's OPP Microbiology Laboratory at Fort Meade is not required since the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) has verified the performance of a qualitative rapid test kit for detecting the presence of the MON 810 and Bt 11 Cry1Ab in grains and oilseeds.	

3. Product Characterization of Plant Optimized (PO) Cry1F Corn (006481)

A corn line of Pioneer Hi-Bred International and Dow Agrosciences / Mycogen was biolistically transformed with a linear *Pme*I fragment from plasmid pP8999 to produce line TC1507. This plasmid contains genes *cry1F*, *pat* and *kan*^{r} encoding the delta-endotoxin from *Bacillus thuringiensis* var. *aizawai* PS811, phosphinothricin acetyl transferase, and resistance to the antibiotic kanamycin, respectively. The *Pme*I fragment (6235 bp) derived from this plasmid was purified after plasmid digestion and used in the transformation process to eliminate the *kan*^{r} antibiotic resistance gene. The Cry1F protein expressed in transformed maize lines is a modified (synthetic, less than full length) form as compared to that from the bacterial isolate from which it is derived. This insecticidal protein confers resistance to the European corn borer (*Ostrinia nubilalis*) and feeding damage is significantly reduced or eliminated following expression of this gene in corn line TC1507. Expression of *cry1F* is under the control of the maize polyubiquitin promoter in line TC1507. The CaMV 35S promoter controls expression of the *pat* gene in this construct. The *pat* gene from *Streptomyces viridochromogenes* confers resistance to the herbicide glufosinate in corn lines accumulating this protein. Hybridization patterns indicate that one full length copy each of the *cry1F* and *pat* genes was integrated into the genome of line TC1507 and that no *kan*^{r} DNA was integrated. This suggests that one *Pme*I fragment from pP8999 integrated into the maize genome. In addition, there are one or two partial copies of the *cry1F* gene integrated into the genome which are most likely non-functional based upon the size of the fragments detected.

Study	***Result***	***MRID #***
Protein Expression	Maize plants (hybrids) from two locations grown under standard agronomic practices of the Midwestern Corn Belt were analyzed by ELISA for Cry1F protein content. The youngest leaf of expanding whorls at the V9 stage were collected from five plants per entry. Values of Cry1F protein for all four hybrids were similar, ranging from 1.52 to 2.63 pg/mg dry weight. Control hybrid A_M was negative for Cry1F as determined by ELISA. CLASSIFICATION: ACCEPTABLE	447148-04
Transformation System Inheritance and Stability after Transformation Protein Characterization	A modified (synthetic, less than full-length) form of the cry1Fa2 gene and the phosphinothricin acetyl transferase (pat) gene were inserted into maize plants by microprojectile bombardment. Three transformation events resulting from microprojectile bombardment will be evaluated under the proposed EUP: TC 1360, TC 1362 and TC 1507. Plants were analyzed for Cry1F by ELISA and PAT by application of glufosinate herbicide. Using a chi square analysis with a 95 % confidence interval, the expected Mendelian ratio of 1:1 was observed for both first and second generations for five inbreds with one exception; first generation TC 1632. Event TC 1507 has been analyzed for only the first generation and ratios (1:1) were as expected.CLASSIFICATION: SUPPLEMENTARY. The registrant should clarify the source of the ubiquitin exon and intron as being from the ubiquitin gene and not the promoter region. A determination of expression of the ubiquitin exon sequence is also needed and whether it alters the sequence of Cry1F.	447148-01
Transformation System	This submission represents a clarification of nomenclature as presented in a previous submission and review. Labeling (in a previous submission) of the Ubi DNA fragment on the plasmid map should have indicated that it includes the Ubi ZM promoter and the first exon and intron of the Ubi ZM gene. The Ubi exon and intron are included in this construct (PHI8999), however, they have no effect on the structure of the Cry1F product, only on the expression of the gene. Exon 1 contains no ATG start site for translation. A translation initiation sequence (Kozak consensus sequence) situated just upstream from the start site (first translated ATG) drives translation of the mature, spliced mRNA. CLASSIFICATION: ACCEPTABLE	450201-17
Characterization of the DNA Inserted in the Plant	The integration pattern of cry1F and pat genes introduced into event TC 1360 was analyzed by Southern blotting. Within the Southern analysis, two types of digests are employed to determine the complexity of DNA integration into the maize genome and to determine the copy number of integrated transgenes. Analysis of four of the progeny from event TC 1360 revealed the presence of two bands hybridizing to the cry1F probe; both bands appeared to hybridize with similar intensity. Hybridization to internal controls on the blot gave an indication of single copy integration and certainly no more than two copies of the insert integrated into the maize genome. When control plant DNA was probed, no hybridization was noted. TC 1360 and control DNA probed with the kan^r gene indicated no hybridization within these samples. CLASSIFICATION: ACCEPTABLE	447148-02

Study	***Result***	***MRID #***
Transformation System Characterization of the DNA Inserted in the Plant	A modified (synthetic, less than full-length) form of the cry1F gene and the phosphinothricin acetyl transferase (pat) gene were inserted into maize plants by microprojectile bombardment. Digestion of the genomic DNA of maize line 1507 with NheI or HindIII and Southern hybridization with probes specific for cry1F, kan^r and pat genes yielded indications of the complexity of the gene integration pattern and copy number. Hybridization patterns suggested that the copy number of introduced / integrated cry1F and pat genes is one. It is most likely that the TC 1507 line contains one functional cry1F gene and partial copies (1 or 2) of the gene which are non-functional. It is not possible with this technique, however, to discern the functionality of probed sequences. No kan^r DNA was introduced into line 1507 during transformation, as indicated by the lack of signal when 1507 genomic DNA was probed with the kan^r gene. There was no hybridization signal when the non-transformed maize line 13-1 was probed with pat or cry1F or kan^r. CLASSIFICATION: ACCEPTABLE	450201-02
Protein Characterization and Expression	Cry1F protein from maize 1507 pollen, grain, grain-derived feeds and a microbial source was evaluated biochemically using ELISA, SDS-PAGE and Western Blotting, and for bioactivity using insect bioassays. Control maize tissues were used to prepare comparable samples. Pollen from line 1507 contained Cry1F at 31 to 33 ng / mg pollen, while no Cry1F protein was detected in pollen from non-Cry1F plants. The purified maize-expressed Cry1F test substance was approximately 32 ng / mL extract. The comparable extract from non-Cry1F maize did not show any detectable Cry1F protein; the limit of detection (LOD) was 0.04 ng / mg sample. Coomassie stained gels indicated similar profiles for both control maize and Cry1F maize samples following SDS-PAGE. Antibodies directed against Cry1F detected this protein (64 kDa) in the Cry1F maize grain samples while there was no indication of any Cry1F protein in the control samples of grain. Pollen, maize-expressed Cry1F and microbially derived Cry1F were all active against the European Corn Borer larvae at the times tested. For the Tobacco Budworm larval bioassay, substances tested included maize grain, maize grain derived fish feed, and maize grain derived quail feed. Samples containing Cry1F maize grain and quail feed made from this grain had identical amounts of Cry1F protein based upon the GI_{50}s calculated. Comparison of control and Cry1F fish feed over four separate bioassays indicated that there was no statistical difference ($p = 0.05$) based upon ANOVA. Preparation of the fish feed sample reduced the biological activity of the Cry1F protein below sensitivity for the assay. CLASSIFICATION: ACCEPTABLE	450201-03

Study	***Result***	***MRID #***
Protein Expression	Protein expression values indicated substantial variability in protein levels for Cry1F in the tissues sampled. No definitive conclusions could be reached from the data presented when comparing levels of Cry1F in hybrid 1507 and inbred 1507 when examining pollen, silk, stalk, leaf, grain, whole plant and senescent whole plant samples. Since these hybrids and inbreds were grown in areas of Chile with similar climatic extremes to the maize growing areas of the U.S., it is anticipated that these values will represent those to be expected in the U.S. cornbelt. PAT expression was also not readily distinguishable when comparing inbred and hybrid expression values. The inability to detect PAT protein in the majority of samples, except leaf, is somewhat puzzling in that the plants demonstrated clear glufosinate tolerance at all field sites. Given the generally strong, non-tissue specific expression levels typically associated with the CaMV 35S promoter (driving pat expression), it is not readily apparent why more PAT protein was not detected in more samples. Its presence in leaf tissue was expected, however, the reason for the absence in many of these samples is less than clear. CLASSIFICATION: ACCEPTABLE	450201-04
Analytical Method	A double antibody sandwich test was developed to detect the Cry1F protein in homogenized maize grain samples using a rapid test method. A double antibody sandwich technique is used in the Lateral Flow Test Kit for Cry1F. Antibodies raised against the Cry1F protein are incorporated into the Lateral Flow test strip and coupled to a color reagent. When in contact with Cry1F protein, the antibodies bind Cry1F and a sandwich is formed, however, not all of the antibodies are coupled to the color reagent. The test strips contain two zones wherein capture of color reagent or antibodies can occur. One zone captures bound Cry1F and the other captures color reagent. Both zones display a reddish color when protein-antibody sandwich and / or unreacted color reagent are captured. When only one line (control) line is present, a negative sample is indicated, while the presence of two lines indicates the presence of Cry1F. The Cry1F Lateral Flow Test Kit accurately detected Cry1F protein in 30 of 30 corn kernels from Cry1F maize and indicated negative reactions for the 30 control maize kernels. This finding demonstrates the utility of using the Cry1F Lateral Flow Test Kit for detection of Cry1F protein in maize grain samples. This kit allows for a rapid qualitative determination of the presence of Cry1F protein. CLASSIFICATION: ACCEPTABLE	452793-01
Analytical Method	The results of this assay validation indicate that the ELISA based assay was suitable for the analysis of Cry1F as found in maize grain. Average recoveries from samples spiked with Cry1F protein (truncated microbial form) were between 67 and 107 %. Extractions from known Cry1F maize grain samples demonstrated that a sample as small as 50 mg could be properly extracted and quantified. CLASSIFICATION: ACCEPTABLE	452793-02

Study	***Result***	***MRID #***
Protein Characterization.	Standard techniques of protein chemistry were used to assess similarities between the bacterial and plant sources of the Cry1F protein. Additionally, insect mortality assays were performed to determine *in vitro* toxicity. An *in vitro* digestibility assay was done to determine that Cry1F was unstable under conditions simulating the gastric environment. This simulation of gastric conditions indicated that the toxin (from microbial source) was readily digested by pepsin. SDS-PAGE and Western blotting of plant and bacterial sources determined the presence of a 65 kDa protein corresponding to the trypsinized core of the d-endotoxin. Plant extracts contained 0.158 % Cry1F as determined by ELISA; control plants were negative. N-terminal sequencing of 5 aa determined that the microbial and plant expressed protein maintained this sequence intact. Glycosylation was not evident in Cry1F from either source. CLASSIFICATION: ACCEPTABLE	447149-03

4. Product Characterization of Maize Optimized (MO) Cry1F Corn (006491)

The mo*cry*1F (maize-optimized) gene codes for the identical truncated Cry1F protein as that expressed by maize plants containing the po*cry*1F (plant optimized) gene (MRID 447148-01). Codon changes were made to the gene to improve expression in moCry1F maize plants, but these codon changes do not alter the amino acid sequence of the protein as compared to that expressed in poCry1F maize plants. The first 605 amino acids of moCry1F and poCry1F are identical with the exception of an altered residue at position 604 (F604L). Because moCry1F and poCry1F proteins share an identical amino acid sequence, the protein equivalency and insect-pest spectrum data also support the moCry1F registration.

Specific moCry1F product characterization data (summarized below) indicate that plant-produced moCry1F protein is biologically, biochemically, and immunologically similar to that expressed in the source bacterium *B. thuringiensis* after trypsin digest. Southern blot data of restriction enzyme digests suggests that the insert in *B.t.* moCry1F corn line 6275 occurred as a simple integration of a partial copy of the T-DNA region (truncated at the 5' end) from plasmid PHP12537. One intact copy of *bar* (plant selectable marker gene, phosphinothricin acetyltransferase, *bar*) was confirmed. Southern blot analyses also revealed that tetracycline and spectinomycin resistance genes were not integrated into corn line 6275. Southern hybridization was used to assess the genetic stability of the insert in multiple generations of corn line TC 6275. Based on the segregation analyses, corn line 6275 exhibited stable Mendelian inheritance of the insert across the generations examined.

Study	***Result***	***MRID#***
Transformation System **Characterization of the DNA Inserted in the Plant**	**A synthetic, truncated *cry*1F transgene was optimized for maize expression and transformed into maize plants using *Agrobacterium tumefaciens* for plant transformation (called mo*cry1F*). The mo*cry*1F gene encodes a truncated, core**	**453186-01**

Study	*Result*	*MRID#*
	insecticidal toxin that is identical in amino acid sequence to the native Cry1F protein over the first 605 amino acids with the exception of an altered residue at position 604 (F604L). The codons for the remaining C-terminal (569) amino acids of the full-length protoxin, were removed from the transgene sequence. PCR verification of the *cry1F* gene indicated that the two genes segregate as a single gene. CLASSIFICATION: ACCEPTABLE	
Protein Expression	**A direct double antibody sandwich ELISA was developed to quantify Cry1F found in lyophilized tissues of mo*cry1F* maize. Samples of leaf, pollen and grain from the two locations in Chile all produced measurable levels of Cry1F for both transformed inbred and hybrid lines when expressed on a tissue dry weight or total extractable protein basis. Leaf tissue samples indicated a higher level of Cry1F expression or accumulation as compared to pollen and grain samples. The quality control samples included in the ELISA plates yielded 73.9 to 106.2% of predicted value, which is within the realm of variation typically seen in ELISA when protein are mixed and processed along with whole tissue samples Extractions from known moCry1F corn gain samples demonstrated that a sample as small as 50 mg could be properly extracted and quantified. CLASSIFICATION: ACCEPTABLE**	**453186-02**
Characterization of the DNA Inserted in the Plant	**The integration pattern of mo *cry1F* and *bar* genes introduced into event TC6228 plants was analyzed by Southern blotting. Control DNA spiked with PHP12537 DNA at concentrations equivalent to 1, 3 or 5 gene copies / genome was included as a positive control and a means to estimate copy number by comparing hybridization intensity. Control DNA without plasmid DNA was also included as a negative control. Data indicate that a single integration of a complete and functional transcriptional unit, representing the T-DNA of the binary plasmid PHP12537, is present in the modified corn line TC6228. Two antibiotic resistance genes, *spc* and *tet*, which are present in the region of the plasmid outside the T-DNA borders, were not transferred as determined by lack of hybridization with TC6228 DNA. CLASSIFICATION: ACCEPTABLE**	**452646-01**
Characterization of the DNA Inserted in the Plant	**Southern blot data from restriction enzyme digests suggest that there is a single insertion of a partial copy of the T-DNA region from plasmid PHP 12537 at one locus in corn line 6275. Restriction digests of corn line 6275 indicated that the *bar* gene was inserted intact and that the (mo)*cry1*F transcription unit was truncated on the 5' end up to and including the restriction enzyme site. The absence of the bacterial tetracycline and spectinomycin resistance genes and regions immediately outside of the left and right T-DNA borders was confirmed suggesting that only DNA contained within the T-DNA borders of plasmid PHP 12537 was integrated into maize line 6275. The inserted DNA was also characterized in two distinct generations of *B.t.* moCry1F maize line 6275, demonstrating the stability of the inserted**	**460193-01**

Study	*Result*	*MRID#*
	DNA. **CLASSIFICATION: ACCEPTABLE**	
Characterization of the DNA Inserted in the Plant	**This is a second study to characterize the inserted synthetic transgene *cry*1F in *B.t.* moCry1F maize line 6275 that contains the (mo)*cry*1F and *bar* genes (see first study, MRID#460193-01). DNA extracted from corn leaf tissue was examined by Southern blot analysis to characterize the T-DNA insert containing the (mo)*cry*1F and *bar* genes in transgenic corn event TC6275 . Southern blot data from this study confirm a single insertion of the T-DNA region from PHP 12537 in event TC6275 with a T-DNA truncation or alteration of the ubiquitin promoter and intron regions Additional border fragments at the 5' end resulting from *Eco*R I digestions suggested that the endonuclease restriction site at bp 1584 in the T-DNA insert was lost during integration into the corn genome.** **CLASSIFICATION: ACCEPTABLE**	**460193-02**
Protein Expression	**Cry1F and bar proteins were found in all transgenic lines, tissue types, and at almost all growth stages (none was detected in a few 6275H pollen samples and in most senescent leaf samples). On a dry weight basis, Cry1F levels ranged from a low of 0.71 ng/mg in senescent leaves (16.7 ng/mg in V9 leaves) in unsprayed transgenic lines to 44.8 ng/mg in R4 leaves; 3.7 ng/mg in pollen (unsprayed and sprayed transgenic lines); 10.4-11.0 ng/mg in stalks (unsprayed and sprayed transgenic lines); 5.82-6.26 ng/mg in forage tissue; 1.97 ng/mg in senescent roots (unsprayed transgenic line) to 6.60 ng/mg in R1 roots; and 1.08-1.14 ng/mg in grain (unsprayed and sprayed transgenic lines). On a dry weight basis, bar levels ranged from 0 ng/mg in senescent leaves to 682 ng/mg in R4 leaves (unsprayed transgenic line); 41 ng/mg in senescent roots to 373 ng/mg in R1 roots (sprayed transgenic line); 0-0.62 ng/mg in pollen (unsprayed and sprayed transgenic lines); 282-311 ng/mg in stalks (unsprayed and sprayed transgenic lines); 7-11 ng/mg in forage tissues (unsprayed and sprayed transgenic lines); and 23 ng/mg in grain (unsprayed and sprayed transgenic lines).** **CLASSIFICATION: ACCEPTABLE**	**460193-03**
Analytical Method	**This study demonstrates the high quantitative performance of the ELISA assay for the detection of the Cry1F truncated protein. The assay had a reproducible sensitivity of 0.5 ng/mL with an approximate 40-fold assay range of 0.4 to 17 ng/mL truncated Cry1F). The coefficient of variation (%CV) of the absorbance measurement within the curve is less than 10%. The cross-reactivity profile indicated little to no cross-reactivity or interference from a standard panel of agriculturally relevant recombinant proteins. The Cry1F assay kit was projected to be stable for approximately 1 year at 4°C.** **CLASSIFICATION: ACCEPTABLE**	**456856-01**
Analytical Method	**An independent laboratory, EPL Bio-Analytical Services,**	**456856-02**

Study	*Result*	*MRID#*
	validated Dow AgroSciences LLC analytical method GRME02.13 "Determination of Cry1F Insecticidal Crystal Protein in Corn Grain by Enzyme Linked Immunosorbant Assay" for accuracy, precision, and sensitivity. The LOQ of the method was confirmed as 0.75 µg/g (or 0.5 ng/mL) for corn grain extracts spiked with Cry1F. Average recoveries from samples spiked with Cry1F protein averaged 99 and 90 percent at the 0.075 and 0.15µg/g spike levels, respectively. The relative standard deviation (RSD) of replicate recovery measurement did not exceed 20 percent at or above the LOQ and interferences were negligible (<20% of the response of the Cry1F protein at the LOQ of 0.075 µg/g). The results of this assay validation indicate that the ELISA based assay is suitable for the analysis of Cry1F as found in corn grain. **CLASSIFICATION: SUPPLEMENTAL; Upgradable with appropriate documentation that this method works to quantify Cry1F expressed in transgenic Cry1F corn.**	
Analytical Method	**Dow AgroSciences analytical residue method GRM 02.30 "Determination of Cryl F ICP in Com Tissues by ELISA" and the associated ELISA kit from Strategic Diagnostics Inc.** **were demonstrated to be acceptable for the intended purpose. The concentration range validated for the method was 1.0 to 10.0 ng'mL (0. I to 1.0 ng'mg) and has a validated lower limit ofquantitation in all com tissues of 0.1 ng/mg. The Cryl F protein was recovered at acceptable levels from all** **tissues tested (Ieaf-V9, stalk-R I, whole plant-V9, -R I, -R4, -R6, root, pollen and grain). The assay proved to be specific for Cryl F protein and no matrix effects were detected in any ofthe com tissues tested. The Cryl F ELISA method was demonstrated to be acceptable for quantitative measurement** **of the CrylF protein in com tissues.**	**4665610-01**
Analytical Method	**Further data on the analytical method as well as validation of an analytical method by EPA's OPP Microbiology Laboratory at Fort Meade of an analytical method by the Fort Meade laboratory is not required since the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) has verified the performance of a qualitative rapid test kit for detecting the presence of the Cry1F in grains and oilseeds.**	

5. Product Characterization of MON 810 x TC1507 (Cry1Ab x Cry1F) Corn

Study Type	Result	MRID #
Characterization of the DNA Inserted in the Plant	Molecular analyses (restriction enzyme digests and Southern blots) were performed to compare the integrity of the transgenic inserts in events TC1507, DAS-59122-7, and MON810 corn with the transgenic DNA inserts in the combination PIP 1507×59122×MON810 corn product, produced by conventional plant breeding. The Southern blot data showed the predicted DNA hybridization patterns of the *cry*1F gene from TC1507 corn, the *cry*35Ab1 and *cry*35Ab1 genes from DAS-59122-7 corn, and the *cry*1Ab gene from MON810 corn. These data demonstrated equivalence among events because the transgenic inserts were stably integrated and retained when the parental lines are crossed to create 1507×59122×MON810 corn. In addition, event-specific PCR (polymerase chain reaction) methods detected and confirmed the presence of each expected transgenic DNA insert in the combination PIP corn event. This study was classified acceptable.	476778-01
Protein Expression	A field study was conducted using quantitative enzyme-linked immunosorbent assay (ELISA) methods to statistically compare the level of expression for several proteins in several combination PIP corn events. The conclusions for cry 1F and cry1Ab are summarized below: The levels of Cry1F, Cry1Ab and PAT proteins expressed in the 1507 × MON810 combination PIP corn event were compared to the individual parental events. No statistical differences were observed in any plant tissue for Cry1F, PAT, and Cry1Ab protein expression when 1507 × MON810 was compared to events 1507 and MON810. Based on these results, the protein concentrations of Cry1F and Cry1Ab expressed in the combination PIP event 1507 × MON810 are comparable to the respective protein concentrations of their respective single parental events. This study was classified acceptable.	478800-01

B. HUMAN HEALTH ASSESSMENT

1. Background

The basic premise relied on for the toxicology assessment is the fact that all the *Bt* plant-incorporated protectants are proteins. Proteins are commonly found in the diet and, except for a few well described phenomenona, present little risk as a mammalian hazard. In addition, for the majority of *Bt* proteins currently registered, the source bacterium has been a registered microbial pesticide which has been approved for use on food crops without specific restrictions. Because of their use as microbial pesticides, a long history of safe use is associated with many *Bt* products.

Several types of data are required for the *Bt* plant-incorporated protectants to provide a reasonable certainty that no harm will result from the aggregate exposure to these proteins. The information is intended to show that the *Bt* protein behaves as would be expected of a dietary protein, is not structurally related to any known food allergen or protein toxin, and does not

display any oral toxicity when administered at high doses. These data consist of an *in vitro* digestion assay, amino acid sequence homology comparisons and an acute oral toxicity test. The acute oral toxicity test is done at a maximum hazard dose using purified protein of the plant-incorporated protectant as a test substance. Due to limitations of obtaining sufficient quantities of pure protein test substance from the plant itself, an alternative production source of the protein is often used such as the *Bacillus thuringiensis* source organism or an industrial fermentation microbe. The justification for employing this alternative source of pure protein is the equivalence data discussed above under product characterization.

EPA believes that protein instability in digestive fluids and the lack of adverse effects using the maximum hazard dose approach in general eliminate the need for longer-term testing of *Bt* protein plant-incorporated protectants. Dosing of these animals with the maximum hazard dose, along with the product characterization data should identify potential toxins and allergens, and provide an effective means to determine the safety of these protein. The adequacy of the current testing requirements was discussed at the June 7, 2000 Scientific Advisory Panel (SAP) meeting. In their final report, the SAP agreed in principle with the methods used by EPA to assess the toxicity of proteins expressed in plants especially the maximum hazard dose approach.

2. In vitro Digestibility Assay

The intent of this assay is to demonstrate that the *Bt* protein is degraded into small peptides or amino acids in solutions that mimic digestive fluids. Usually only gastric fluid is tested since Cry protein is known to be stable in intestinal fluid, but in the initial *Bt* products registered, gastric and intestinal fluids were examined separately. In order to track the breakdown, the proteins were added to a solution of the digestive fluids and a sample was either removed or quenched at given time points (usually at time 0, one to several minutes later and one hour later). The time point samples were then electrophoresed on either an SDS-PAGE gel and further analyzed by western blot or tested in a bioassay against the target pest. All were degraded in gastric fluid in 0-7 minutes. All the *Bt* proteins tested in intestinal fluid were not affected by trypsin digestion as would be expected since this is similar to their behavior in the insect gut. In intestinal fluid, those *Bt* plant-incorporated protectants that are expressed as protoxin molecules broke down into the active toxin moiety and degraded no further.

As has been stated in several public fora, the *in vitro* digestibility test is basically a test to confirm the biochemical characteristic of instability of the protein in the presence of digestive fluids. The digestibility test is not intended to provide information on the toxicity of the protein or imply that similar breakdown will happen in all human digestive systems. The *in vitro* digestibility assay may also provide information about the potential of a protein to be a food allergen. The *in vitro* digestion assays confirm that the protein is being broken down in the presence of typical digestive fluids and is not unusually persistent in the digestive system. One of the limitations of the test is that it usually only tracks protein breakdown to fragments still recognized by the immunological reagents employed.

3. Amino Acid Homology and Heat Stability

Two additional characteristics that are considered as an indication of possible relation to a food allergen are a protein's ability to withstand heat or the conditions of food processing and its amino acid sequence when compared to known food allergens.

4. Acute Oral Toxicity

One of the bases for addressing the toxicity of proteins primarily through the use of acute oral toxicity is that, when demonstrated to be toxic, proteins are toxic at low doses (Sjoblad, *et al*., 1992). Therefore, when no effects are shown to be caused by the protein plant-incorporated protectants, even at relatively high dose levels in the acute oral exposure, the proteins are not considered toxic. The acute oral toxicity test is performed in mice with a pure preparation of the plant-incorporated protectant protein at doses from 3280 to over 5000 mg/kg bodyweight. None of the tests performed to date have shown any significant effects on the treated animals.

5. EPA Recommendation (2001)

The mammalian toxicity data continue to support the registrations of the *Bt* products described. EPA believes the data it currently has is sufficient to support the *Bt* plant-incorporated protectant registrations.

6. Human Health Assessment of Cry1Ab Crops, Including But Not Limited To:

Bt11 Cry1Ab Bt Corn (006444) and MON810 Cry1Ab Bt Corn (006430)

a. Toxicology Assessment

Mammalian toxicology data are available to examine the potential effects of Cry1Ab on human health and assess if the data support the registration of *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production (plasmid vector pZO1502) in corn (OPP PC Code 006444) and *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production in corn (OPP PC code 006430). *Bt* microbial pesticides, containing Cry proteins other than Cry1Ab, have been applied for more than 30 years to food and feed crops consumed by the U.S. population. These data would also support other Cry1Ab plant-incorporated protectants' human health assessments provided adequate information was submitted to show that the Cry1Ab proteins in question were biochemically and functionally similar to the proteins of the plant-incorporated protectants already examined.

1) Acute Toxicity

Study Type	Result	MRID #
Acute Oral Toxicity	Five male and five female mice received a single dose of 3,280 mg/kg of Cry1Ab protein by oral gavage. No animals died nor were there significant clinical signs as a result of the exposure. One female failed to gain weight between day 7 and day 14. All animals gained weight by the end of the study. Males gained more weight over the study than females. CLASSIFICATION: ACCEPTABLE Test substance is given a TOXICITY CATEGORY IV rating although highest dose administered is 3280 mg/kg due to lack of any evidence of a dose/effect relation.	433236-08
Acute Oral Toxicity	No test substance related deaths occurred. One female died within a day of BSA dose administration due to a perforated trachea. The majority of the animals failed to gain weight or showed a slight weight reduction. No treatment related trends in these losses was apparent. CLASSIFICATION: ACCEPTABLE. Test substance is given a TOXICITY CATEGORY IV rating although highest dose administered is 4000 mg/kg due to lack of any evidence of a dose/effect relation.	434680-01

2) Mutagenicity and Developmental Toxicity, Subchronic Toxicity, and Chronic Exposure and Oncogenicity Assessment

Data demonstrating no mammalian toxicity at high levels of exposure confirm the safety of the product at levels well above any possible maximum exposure levels anticipated for a plant-incorporated protectant. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which these plant-incorporated protectants were derived. [See 40 CFR Sec. 158.2130 and 158.2140.] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study.

The acute oral toxicity data submitted support the determination that the Cry1Ab protein is non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al*.1992). Since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry1Ab delta-endotoxin protein is not considered toxic. Because these proteins break down into their constituent amino acids, there would be no chronic exposure to the protein and therefore no need for chronic toxicity testing. Therefore, the mutagenicity, developmental toxicity, subchronic toxicity, chronic exposure and oncogenicity assessment studies were not required.

3) Effects on the Immune System

Since Cry1Ab is a protein, allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and

proteases, to be glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ab delta-endotoxin is degraded in two minutes by gastric fluid *in vitro* and is non-glycosylated. Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the delta-endotoxin in the crystal protein. Despite decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)2 have been made for various *Bacillus thuringiensis* microbial products claiming dermal allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. The reported reactions were determined to be due to non-Cry proteins produced during fermentation or to added formulation ingredients. Thus, the Cry1Ab protein is not expected to be a food allergen.

Allergenicity Endpoints of Cry1Ab Crops [*Bt*11 and MON810 *Bt* Corn (006444 & 006430)]

Study Type	Result	MRID #
In vitro Digestibility	The Cry1Ab protein from either maize or B.t.k. HD1-9 is rapidly degraded in the presence of pepsin. Using 1/1000 strength pepsin, a time course study shows that the introduced protein from either source degrades within 10 minutes to lack of any recognition in a western blot assay. CLASSIFICATION: ACCEPTABLE	433236-06
In vitro Digestibility	The tryptic core Cry1Ab protein is significantly degraded by 2 minutes incubation in gastric fluid but not significantly affected by 19.5 hours in intestinal fluid as monitored by western blot. The decrease in bioactivity of these digestions against tobacco budworm is similar to its loss of immunorecogniton in western blots CLASSIFICATION: ACCEPTABLE	434392-01
Amino Acid Sequence Homology	An amino acid database was constructed containing amino acid sequences of known protein allergens and gliadins. The B.t.k. HD-1 protein was compared to this database and no significant sequence similarity was identified. Based upon this data, there does not appear to be significant sequence similarity between HD-1 and known protein allergens and gliadins.	453849-01
Amino Acid Sequence Homology	The National Center for Biotechnology GenBank database, which contains all publicly available protein sequences, was queried to determine whether the Cry1Ab protein as expressed in transgenic maize Event Bt11 has any significant amino acid homology with protein sequences identified as toxins. No significant amino acid homology between Bt11 Cry1Ab to any non-*Bt* Cry proteins identified as, or known to be, toxins. There was a degree of homology observed between the *Bt* Cry31Aa1 parasporin protein and the *Bt*11 Cry1Ab protein. However, a global alignment of parasporin and *Bt*11 Cry1Ab demonstrated only 20.7% identity between the two proteins. Moreover, the Cry1Ab protein is a lepidopteran-specific toxin and is non-toxic to other non-lepidopteran species and mammals. Therefore, it is concluded that the degree of homology observed between the *Bt* Cry31Aa1 parasporin protein and the *Bt*11 Cry1Ab protein is not of toxicological relevance. CLASSIFICATION: ACCEPTABLE	458798-01

Study Type	Result	MRID #
Amino Acid Sequence Homology	Bioinformatics analyses indicate that there were no significant similarities (defined as ≥ 35% amino acid homology) between any of the sequential Bt11 Cry1Ab 80-amino acid peptides and any entries in the Syngenta Biotechnology, Inc. (SBI) Allergen Database, which contains all known and putative protein allergen sequences. Additionally, there were no alignments of eight or more contiguous identical amino acids between the Bt11 Cry1Ab protein and any of the proteins in the allergen database. Therefore, no biologically relevant structural similarities were observed between any known or putative allergen and the Cry1Ab protein produced in corn Event Bt11. CLASSIFICATION: ACCEPTABLE	458798-02
Amino Acid Sequence Homology	No significant sequence homology between any sequential MON 810 Cry1Ab amino acid peptides or alignments of eight or more contiguous identical amino acids between MON 810 Cry1Ab amino acid peptides and any entry in the AD3_1 database were identified. The closest similarity was to a wheat gamma-gliadin protein with 31.7% identity over 60 amino acid residues. No significant 8 amino acid identity stretches were discovered. // MON 810 Cry1Ab had no significant amino acid sequence homology as defined by the Codex suggested similarity criteria of 35% amino acid identity over 80 amino acids or any 8 amino acid short sequence identities to known allergenic or gliadin proteins. CLASSIFICATION: ACCEPTABLE	458789-02
Amino Acid Sequence Homology	The 818 amino acid sequence of event MON810 corn expressing Cry1Ab protein was sequentially searched and compared for protein sequence homology to known toxins contained in the NCBI Protein Sequence datasets. The BLASTP search showed no significant amino acid sequence homologies using the Cry1Ab protein sequence as a query with any proteins known to be toxins. Therefore, the Cry1Ab protein does not share any amino acid sequence homology with any proteins known to be toxins. **Classification: ACCEPTABLE**	477864-06
Amino Acid Sequence Homology	No identity matches of ~ 35% over ~ 80 amino acid residues were observed for the Cry1Ab protein sequence against the protein sequences of known allergens. In addition, there were no 8 or greater contiguous identical amino acid matches observed with the Cry1Ab protein sequence. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted allergen homology assessment for the Cry1Ab protein expressed in event MON810 corn (see MRID No. 458789-02). **Classification: ACCEPTABLE**	477864-04

Study Type	Result	MRID #
Heat Stability	This study shows that the commonly used methods of processing corn by milling reduce the level of Cry1Ab protein and lower the potential dietary exposure. Since Cry1Ab has no indications of causing adverse effects in either the acute oral study or by having similarities to known food allergens, there is no expectation that dietary exposure will be a hazard. However, the inactivation by processing further reduces the dietary risk by lowering the expected exposure.	Dien, B.S., R.J. Bothast, L.B. Iten, L. Barrios, and S.R. Eckhoff, (2002), Fate of Bt Protein and Influence of Corn Hybrid on Ethanol Production, Cereal Chemistry 79:582-585.

4) Effects on the Endocrine System (Updated July 2010)

As required under FFDCA section 408(p), EPA has developed the Endocrine Disruptor Screening Program (EDSP) to determine whether certain substances (including pesticide active and other ingredients) may have an effect in humans or wildlife similar to an effect produced by a "naturally occurring estrogen, or other such endocrine effects as the Administrator may designate." The EDSP employs a two-tiered approach to making the statutorily required determinations. Tier 1 consists of a battery of 11 screening assays to identify the potential of a chemical substance to interact with the estrogen, androgen, or thyroid (E, A, or T) hormonal systems. Chemicals that go through Tier 1 screening and are found to have the potential to interact with E, A, or T hormonal systems will proceed to the next stage of the EDSP where EPA will determine which, if any, of the Tier 2 tests are necessary based on the available data. Tier 2 testing is designed to identify any adverse endocrine related effects caused by the substance, and establish a quantitative relationship between the dose and the E, A, or T effect.

Between October 2009 and February 2010, EPA issued test orders/data call-ins for the first group of 67 chemicals, which contains 58 pesticide active ingredients and 9 inert ingredients. This list of chemicals was selected based on the potential for human exposure through pathways such as food and water, residential activity, and certain post-application agricultural scenarios. This list should not be construed as a list of known or likely endocrine disruptors.

The Cry1Ab protein is not among the group of 58 pesticide active ingredients on the initial list to be screened under the EDSP. Under FFDCA § 408(p) the Agency must screen all pesticide chemicals. Accordingly, EPA anticipates issuing future EDSP orders/data call-ins for all Registration Review cases, including those for which EPA has already opened a Registration Review docket for a pesticide active ingredient.

For further information on the status of the EDSP, the policies and procedures, the list of 67 chemicals, the test guidelines and the Tier 1 screening battery, please visit our website: http://www.epa.gov/endo/.

5) Dose Response Assessment

No toxicological endpoints were identified, therefore a dose response assessment was not required.

6) Dietary Risk Characterization

a) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry1Ab protein include information on the characterization of the expressed Cry1Ab delta-endotoxin in corn, the acute oral toxicity, and *in vitro* digestibility of the delta-endotoxin.

Adequate information was submitted to show that the Cry1Ab test material derived from microbial cultures were biochemically and functionally similar to the proteins produced by the plant-incorporated protectant ingredients in corn. Production of microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the conclusion that the Cry1Ab protein is non-toxic to humans. Therefore, because no effects were shown to be caused by these plant-incorporated protectants, even at relatively high dose levels (4000 mg/kg), the Cry1Ab delta-endotoxin protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectants was derived [See 40 CFR Sec. 158.2130 and 158.2140.] Further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study. Because the acute testing showed no toxicity, higher tier testing is not required.

Because Cry1Ab is a protein and the major exposure is dietary, food allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ab delta-endotoxin is degraded in two minutes by gastric fluid *in vitro* and is non-glycosylated. After decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)(2) have been made for various *Bacillus thuringiensis* microbial products claiming dermal allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. Thus, the Cry1Ab protein is not expected to be a food allergen.

Both (1) available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated because the lack of mammalian toxicity at high levels of exposure demonstrates the safety of the product at levels above possible maximum exposure levels.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA) which comprise (1) genetic material encoding these proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material (termed promoters, terminators and enhancers) that control the expression of the DNA encoding proteins. DNA is common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids as they appear in the subject active ingredient have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity in the acute exposures.

b) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of exposure (safety) will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that infants and children will consume minimal residues of this plant-pesticide and that there is a finding of no toxicity.

Thus, there are no threshold effects of concern and, as a result the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

c) Aggregate Exposure (Not Including Occupational Exposure) Risk Conclusions

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectants chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed corn products and drinking water. However, a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants has been demonstrated. The use sites for Cry1Ab delta endotoxin are all agricultural for control of lepidopteran insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity.

d) Cumulative Effects Risk Conclusions

The Agency has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, there are no cumulative effects.

e) Dietary Risk Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the United States population, including infants and children, to the Cry1Ab protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. We have arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the currently registered plant-incorporated protectants.

f) Occupational Exposure and Risk Characterization

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Worker exposure to the Cry protein via seed dust is also expected to be negligible because of the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity. If any unreasonable adverse effects caused by exposure to Cry1Ab are identified, these effects must be reported to the Agency as required by Sec. 6(a)(2) of FIFRA.

BPPD RECOMMENDATION: (2001 Conclusion)

There is a reasonable certainty that no harm will result from exposure to *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production in corn. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, EPA considers that the Cry1Ab tolerance exemption has been reassessed and meets the 408(c)(2) standard. The tolerance exemption citation for Cry1Ab in all plants follows.

§ 174.511 Bacillus thuringiensis Cry1Ab protein in all plants; exemption from the requirement of a tolerance.

Residues of *Bacillus thuringiensis* Cry1Ab protein in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all food commodities.
[72 FR 20435, Apr. 25, 2007]

2. Human Health Assessment of Cry1F Corn

Because both poCry1F and moCry1F share the same amino acid sequence, the toxicity of the Cry1F protein expressed in moCry1F and poCry1F plants is expected to be similar. The toxicity and allergenicity data submitted in support of the poCry1F registration are also adequate to support the registration of moCry1F corn

a. Mammalian Toxicity and Allergenicity Assessment

Data have been submitted demonstrating the lack of mammalian toxicity at high levels of exposure to the pure Cry1F protein. These data demonstrate the safety of the products at levels well above maximum possible exposure levels that are reasonably anticipated in the crops. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.2130 and 158.2140.] For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study, to verify the observed effects and clarify the source of these effects (Tiers II & III).

The acute oral toxicity data submitted support the prediction that the Cry1F protein would be non-toxic to humans. Male and female mice (5 of each) were dosed with 15 % (w/v) of the test substance, which consisted of *Bacillus thuringiensis* var. *aizawai* Cry1F protein at a net concentration of 11.4 %. Two doses were administered approximately an hour apart to achieve the dose totaling 33.7 mL / kg body weight. Outward clinical signs and body weights were observed and recorded throughout the 14 day study. Gross necropsies performed at the end of the study indicated no findings of toxicity. No mortality or clinical signs were noted during the study. An LD_{50} was estimated at >5050 mg / kg body weight of this microbially produced test material. The actual dose administered contained 576 mg Cry1F protein / kg body weight. At this

dose, no LD_{50} was demonstrated as no toxicity was observed. Cry1F maize seeds contain 0.0017 to 0.0034 mg of Cry1F / gram of corn kernel tissue.
When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al*.1992). Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry1F protein is not considered toxic. Further, amino acid sequence comparisons showed no similarity between Cry1F protein to known toxic proteins available in public protein databases.

Since Cry1F is a protein, allergenic sensitivities were considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, may be glycosylated and present at high concentrations in the food.

Data has been submitted which demonstrates that the Cry1F protein is rapidly degraded by gastric fluid *in vitro* and is non-glycosylated. In a solution of Cry1F:pepsin at a molar ratio of 1:100, complete degradation of Cry1F to amino acids and small peptides occurred in 5 minutes. A heat lability study demonstrated the loss of bioactivity of Cry1F protein to neonate tobacco budworm larvae after 30 minutes at 75 ºC. Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the d-endotoxin of the crystal protein. Additionally, a comparison of amino acid sequences of known allergens uncovered no evidence of any homology with Cry1F, even at the level of 8 contiguous amino acids residues.

The potential for the Cry1F protein to be a food allergen is minimal. Regarding toxicity to the immune system, the acute oral toxicity data submitted support the prediction that the Cry1F protein would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al*.1992). Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry1F protein is not considered toxic.

b. Aggregate Exposures

Pursuant to FFDCA section 408(b)(2)(D)(vi), EPA considers available information concerning aggregate exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectant chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed corn products and, potentially, drinking water. However a lack of mammalian toxicity and the digestibility of the plant-incorporated

protectants have been demonstrated. The use sites for the Cry1F protein are all agricultural for control of insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity demonstrated for the Cry1F protein.

c. Cumulative Effects

Pursuant to FFDCA Section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, we conclude that there are no cumulative effects for the Cry1F protein.

d. Determination of Safety for U.S. Population, Infants and Children

1) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry1F protein include the characterization of the expressed Cry1F protein in corn, as well as the acute oral toxicity, heat stability, and *in vitro* digestibility of the proteins. The results of these studies were determined applicable to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

Adequate information was submitted to show that the Cry1F test material derived from microbial cultures was biochemically and, functionally similar to the protein produced by the plant-incorporated protectant ingredients in corn. Production of microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the prediction that the Cry1F protein would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al*.1992). Since no effects were shown to be caused by Cry1F protein, even at relatively high dose levels (>5,050 mg test substance / kg body weight; 576 mg Cry1F / kg body weight), the Cry1F protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.2130 and 158.2140.] For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study to verify the observed effects and clarify the source of these effects (Tiers II & III).

Although Cry1F expression level data was required for an environmental fate and effects assessment, residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity.

Both (1) available information concerning the dietary consumption patterns of consumers (and

major identifiable subgroups of consumers including infants and children); and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated. The lack of mammalian toxicity at high levels of exposure to the Cry1F protein demonstrates the safety of the product at levels well above possible maximum exposure levels anticipated in the crop.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA, RNA) which comprise (1) genetic material encoding these proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material, such as promoters, terminators, and enhancers, that control the expression of the genetic material encoding the proteins. DNA and RNA are common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids, as they appear in the subject active ingredient, have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

Study	Result	MRID #
Acute Oral Toxicity	Dosing of ten albino mice with bacterial cell protein containing the d-endotoxin of Bacillus thuringiensis var. aizawai at > 5050 mg/kg (0.576 g/kg of Cry1F) body weight resulted in no mortality and no observed gross abnormalities. All animals appeared normal during the study and all except one gained weight throughout the study. Classification: Acceptable. Toxicity category III based on dose given with no observable effect.	446911-01
Acute Oral Toxicity	This submission represents a clarification of test substance as presented in a previous submission and review. The acute oral toxicity study dosed mice at > 5050 mg microbial protein / kg body weight. The actual dose administered contained 576 mg Cry1F protein / kg body weight. At this dose, no LD_{50} was demonstrated as no toxicity was observed. The truncated form of the protein represents amino acids 28-612 of the Cry1F toxin sequence, whereas the plant-expressed form of Cry1F contains amino acids 1-605. The truncated form used in the oral toxicity study adequately represents that toxin to be found in the plant expression system. Classification: Acceptable.	450201-18

Amino Acid Sequence Homology	A modified (synthetic) form of the cry1Fa2 gene and the phosphinothricin acetyl transferase (pat) gene were inserted into maize plants by microprojectile bombardment. A database of available sequenced allergens and toxins was searched for similarity to both the less than full-length Cry1F and PAT proteins such that a level of eight, contiguous amino acid homology would be detected. This number of contiguous amino acids is considered to be the smallest antigenic portion of a protein (peptide) to induce an allergic reaction based upon T-cell recognition in a sensitized individual. The database search and comparison to known allergens from plant, bacterial, fungal and animal origins indicates that no significant amino acid homology exists for Cry1F or PAT with any of these proteins. For both proteins of interest, the lack of any significant amino acid homology indicates that the potential for an immunological response developing into a food allergy from consumption of these proteins is low. Classification: Acceptable.	449717-01
Amino Acid Sequence Homology	The 605 amino acid sequence of event TC1507 corn expressing Cry1F protein was sequentially searched for comparisons to known toxins contained in the NCBI Protein Sequence datasets. The BLASTP search showed no significant amino acid sequence homologies using the Cry1Ab protein sequence as a query with any proteins known to be toxins. Therefore, the Cry1F protein does not share any amino acid sequence homology with any proteins known to be toxins. **Classification: ACCEPTABLE**	477864-05
Amino Acid Sequence Homology	No identity matches of ~ 35% over ~ 80 amino acid residues were observed for the Cry1F protein sequence against the protein sequences of known allergens. In addition, there were no 8 or greater contiguous identical amino acid matches observed with the Cry1F protein sequence. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted allergen homology assessment for the Cry1F protein expressed in event TC1507 corn in (see MRID No. 449717-01). **Classification: ACCEPTABLE**	477864-01
Lack of cross-reactivity between Cry1F protein in Herculex I maize and dust mite Der p7 protein with human sera positive for Der p7-IgE	Using a six contiguous amino acid bioinformatics analytical search, a match was identified between the Cry1F protein of Herculex I (poCry1F) corn and the Der p7 protein, an allergenic protein of the dust mite, *D. pteronyssinus*. This study examined cross-reactivity between Cry1F protein in Herculex I maize and dust mite Der p7 protein with human sera positive for Der p7-IgE. There was no cross-reactivity; therefore, dust mite allergic individuals would not be expected to experience an allergic reaction from ingesting Cry1F. CLASSIFICATION: ACCEPTABLE	464440-01

Heat Stability	The Cry1F test substance was prepared in 10 mM potassium phosphate buffer (pH 7.5) and placed into a water bath at either 60, 75 or 90 ºC for 30 minutes, or into the refrigerator at 4℃. Application of treated Cry1F to the surface of an insect diet and measurement of growth inhibition of neonate tobacco budworm larvae, indicated that the Cry1F protein was labile to heat at and above 75 ºC. Classification: Acceptable.	452748-01

2) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408(B)(2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for the Cry1F protein and the genetic material necessary for its production. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

3) Overall Safety Conclusion (2001 Conclusion)

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1F protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the plant-incorporated protectants.

e. Other Considerations

1) Endocrine Disruptors (Updated July 2010)

As required under FFDCA section 408(p), EPA has developed the Endocrine Disruptor Screening Program (EDSP) to determine whether certain substances (including pesticide active and other ingredients) may have an effect in humans or wildlife similar to an effect produced by a "naturally occurring estrogen, or other such endocrine effects as the Administrator may designate." The EDSP employs a two-tiered approach to making the statutorily required

determinations. Tier 1 consists of a battery of 11 screening assays to identify the potential of a chemical substance to interact with the estrogen, androgen, or thyroid (E, A, or T) hormonal systems. Chemicals that go through Tier 1 screening and are found to have the potential to interact with E, A, or T hormonal systems will proceed to the next stage of the EDSP where EPA will determine which, if any, of the Tier 2 tests are necessary based on the available data. Tier 2 testing is designed to identify any adverse endocrine related effects caused by the substance, and establish a quantitative relationship between the dose and the E, A, or T effect.

Between October 2009 and February 2010, EPA issued test orders/data call-ins for the first group of 67 chemicals, which contains 58 pesticide active ingredients and 9 inert ingredients. This list of chemicals was selected based on the potential for human exposure through pathways such as food and water, residential activity, and certain post-application agricultural scenarios. This list should not be construed as a list of known or likely endocrine disruptors.

The Cry1F protein is not among the group of 58 pesticide active ingredients on the initial list to be screened under the EDSP. Under FFDCA § 408(p) the Agency must screen all pesticide chemicals. Accordingly, EPA anticipates issuing future EDSP orders/data call-ins for all Registration Review cases, including those for which EPA has already opened a Registration Review docket for a pesticide active ingredient.

For further information on the status of the EDSP, the policies and procedures, the list of 67 chemicals, the test guidelines and the Tier 1 screening battery, please visit our website: http://www.epa.gov/endo/.

3) Codex Maximum Residue Level

No Codex maximum residue levels exists for the plant-incorporated protectants *Bacillus thuringiensis* Cry1F protein and the genetic material necessary for its production in corn.

f. Tolerance Exemption

The tolerance exemption citation for Cry1F in corn follows.

§ 174.520 Bacillus thuringiensis Cry1F protein in corn; exemption from the requirement of a tolerance.

Residues of *Bacillus thuringiensis* Cry1F protein in corn are exempt from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of corn; corn, field; corn, sweet; and corn, pop.
[72 FR 20435, Apr. 25, 2007]

3. **Synergism Assessment for MON 810 x TC1507 (Cry1Ab x Cry1F) Corn**

Study	Result	MRID #
Synergism	A seven-day laboratory sensitive insect bioassay was conducted to determine if the combination PIP product 1507 x 59122 x MON810 has a synergistic effect in comparison to the individual parental events TC1507 (expressing Cry1F protein) and MON810 (expressing Cry1Ab protein) on target lepidopteran pests. The pests used in the bioassay were European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), fall armyworm (FAW, *Spodoptera furgiperda*), and corn earworm (CEW, *Helicoverpa zea*). The observed and expected larval mortality in the 1507 x 59122 x MON810 group were similar and mean larval weight of the survivors exposed to 1507 x 59122 x MON810 leaf tissue was not significantly different from the single parental events or the negative control of non-*Bt* maize. These results indicate that the Cry1F and Cry1Ab proteins act independently and do not have a synergistic or antagonistic effect on the target pests, other than an additive effect. Quantitative ELISA results also confirmed that the expression of each of the proteins in the combination PIP was not affected by the presence of the other protein. CLASSIFICATION: ACCEPTABLE	**476778-02**

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C. ENVIRONMENTAL ASSESSMENT

Background

In 2001, EPA conducted an environmental reassessment of the registered *Bt* plant-incorporated protectants. The general topics covered included gene flow and the potential for weeds to develop if pollen from *Bt* crops plants were to fertilize other plants, horizontal gene transfer, expression of *Bt* Cry proteins in plant tissues, ecological effects, especially considering the currently available data on monarch butterflies, and fate of *Bt* Cry proteins in the environment. Information used for the reassessment included the original data submissions for EPA registration, additional data submitted by the registrants (including that in the response to the December 1999 Data Call-in), public literature, results of workshops and scientific seminars, recommendations from the SAP, additional discussions with scientific experts, and other public comments.

In addition to those data, the 2010 environmental assessment includes all conditionally required data and new information the Agency has received regarding Cry1Ab and Cry1F proteins since 2001, including long-term field studies, avian dietary studies, and an updated assessment of the impacts of *Bt* proteins on monarch butterflies.

In 2005, the Agency conducted an environmental effects assessment of moCry1F, the insecticidal protein as expressed in maize line TC6275 (Hill 2005). The maize-optimized (mo) Cry1F gene encodes for the identical truncated Cry1F protein as expressed by maize plants containing the plant-optimized (po) Cry1F gene (MRID No. 447148-01). Codon changes were made to the gene to improve expression in moCry1F maize plants but these changes did not alter the amino acid sequence of the protein as compared to that expressed in poCry1F maize plants. Therefore, the specificity (i.e., toxicity toward target pests) of the Cry1F protein expressed in moCry1F and poCry1F plants should be similar. Testing with bacterially prepared Cry1F protein at levels greatly exceeding those found in maize optimized plants resulted in no adverse effect to several beneficial species including the monarch butterfly. Expression data reveal that moCry1F plants express somewhat lower concentrations of Cry1F protein in pollen and grain and higher concentrations in stalks, leaves, and roots than the poCry1F plants, though these expression differences are not expected to significantly change the exposure to Lepidopteran non-target organisms. Field monitoring for effects of poCry1F corn on non-target insects confirmed the absence of adverse effects to non-target organisms (MRID No. 450201-13). Data illustrating the soil degradation of poCry1F protein were submitted to the EPA in support of the registration of Herculex I (MRID No. 450201-05). These data support the registration of moCry1F corn because the two proteins share an identical amino acid sequence. EPA's assessment of the poCry1F studies submitted in support of the Herculex I registration (EPA Reg. No. 029964-3) are applicable to the moCry1F corn registration for reasons outlined above. These studies, along with those for Cry1Ab protein, will be summarized in this assessment in both a tabular format and a more descriptive format.

1. Tiered 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]

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 Environmental Protection Agency (EPA) uses a tiered (Tiers I–IV) testing system to assess the toxicity of a plant-incorporated protectant (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 endpoint. 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 EPA as Harmonized Office of Prevention, Pesticides, and Toxic Substances (OPPTS) Testing Guidelines (now Harmonized Office of Chemical Safety and Pollution Prevention (OCSPP) Testing Guidelines), Series 850 and 885 (EPA 712-C-96-280, February 1996).[2] These guidelines apply to microbes

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 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 General OCSPP Harmonized Testing Guidelines available from: http://www.epa.gov/ocspp/pubs/frs/home/guidelin.htm.

Series 850 Testing Guidelines available from:

http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/index.html

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, plant-incorporated protectants (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, or the Estimated Environmental Concentration (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 of test animals at the maximum hazard dose level. The Guidelines call for testing of one treatment group of at least thirty animals or three groups of ten 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%.

Failing the Tier I (10x EEC) screening at the MHD does not necessarily indicate the presence of an unacceptable risk in the field, but triggers the need for additional testing.[4] A less than 50% mortality effect at the MHD is taken to indicate minimal risk. Greater than 50% mortality, however, 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 OCSPP Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} (i.e., dose that will kill 50% of the test organisms within a designated period) 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 Adverse Effect Concentration (LOAEC), 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

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Series 885 Testing Guidelines available from: http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series885.htm.

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.

4 It is notable that the 10x 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 the highest dose in a worst-case scenario (sometimes referred to as the Highest Estimated Environmental Concentration or 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.

power, must be employed to evaluate the data from the definitive tests. Higher levels of replication, test species numbers, and/or repetition are needed to enhance statistical power in these circumstances.

Data that show less than 50% mortality at the maximum hazard dosage level (i.e., LC_{50}, ED_{50}, or LD_{50} >10x EEC) are sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. Also, 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 (U.S. EPA 1998).[6]

Validation: The tiered hazard assessment approach was developed for EPA by the American Institute of Biological Sciences (AIBS) and confirmed in 1996 as an acceptable method of environmental hazard assessment by a Federal Insecticide, Fungicide, and Rodenticide Act (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 PIPs; 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 genetically modified (GM) crop fields should be conducted. Testing of *Bacillus thuringiensis* (*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 (U.S. EPA 2000, 2001a, 2002, and 2004b).

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, however, does 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. EPA believes that maximum hazard dose Tier I screening testing presents a 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 I testing for short-term hazard assessment will be sufficient for most studies submitted in support of PIP registrations. If long-range adverse effects must be ascertained, however, then higher tier,

6 The established peer and EPA Science Board reviewed guidance on screening test levels of concern is 50% mortality at 5x environmental concentration for terrestrial and 10x for aquatic species. 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.

longer term field testing will be required. As noted above, the October 2000, SAP and the National Academy of Sciences (NAS 2000) recommended testing non-target organisms directly in the field. This approach, with an emphasis on testing invertebrates found in corn fields, was also recommended by the August 2002, SAP and was supported by several public comments. Based on these recommendations and due to the lack of baseline data on the potential for long-term environmental effects from the cultivation of PIP-producing plants, the Agency has required long-term field studies on invertebrate populations/communities and Cry protein accumulation in soils as conditions of past PIP registrations.

Since the commercialization of *Bt* crops, the number of field studies published in scientific literature, in combination with the post-registration field studies submitted to the Agency, has accumulated to a level where empirical conclusions can be made. As a result, the issue of long-range effects of cultivation of these Cry proteins on the invertebrate community structure in *Bt* crop fields has since been adequately addressed. Specifically, a meta-analysis[7] of the data collected from 42 field studies indicated that non-target invertebrates are generally more abundant in *Bt* cotton and *Bt* maize fields than in non-transgenic fields managed with insecticides (Marvier *et al.* 2007). In addition, a comprehensive review of short- and long-term field studies on the effects of invertebrate populations in *Bt* corn and cotton fields indicated that no unreasonable adverse effects are taking place as a result of wide-scale *Bt* crop cultivation (Sanvido *et al.* 2007). Another review of field tests published to date concluded that the large-scale studies in commercial *Bt* cotton have not revealed any unexpected non-target effects other than subtle shifts in the arthropod community caused by the effective control of the target pests (Romeis *et al.* 2006). 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.

Overall, the Agency is in agreement with the conclusions of these studies and, collectively, these results provide extensive data to support that *Bt* crops have not caused long-term environmental effects, on a population level, to organisms not targeted by *Bt* proteins. Based on these considerations, regulatory testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

2. Environmental Exposure Assessment

The EPA environmental exposure assessment is based on adverse effects at 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 when many species are tested or when 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

7 This research was funded by EPA grant CR-832147-01. The Bt crop non-target effects database can be found on the National Center for Ecological Analysis and Synthesis (NCEAS) Web Site: http://delphi.nceas.ucsb.edu/btcrops/.

effect is viewed as a concern only at 1X the field exposure.

The nominal protein expression levels for Cry1Ab and Cry1F, as determined by field and/or greenhouse conditions, are described below. Note that there may be variation between the *Bt* protein values reported by each company due to differences in the antibody-based reagents used for quantifying the *Bt* protein, or whether protein values in tissue are reported in fresh or dry weight. While these differences may make direct comparisons between the tissue expression levels reported by different companies difficult, the reported dry weight levels provide enough information to be used for risk assessment purposes especially when considered along with the reported tissue bioactivity values.

a. Cry1Ab Protein Expression in Bt11 and MON810 Corn Tissues

TABLE 1. Cry1Ab Concentrations on a Dry Weight Basis in Event Bt11 Hybrid Plants

Tissue	Location	Hybrid[1]	Developmental Stage		
			V9-V12	Anthesis	Seed Maturity
			mean µg Cry1Ab/gdw ± S.D. (range)		
Leaves	Bloomington, Illinois	Event Bt11	25.88 ± 1.35 (23.89—27.63)	17.82 ± 1.54 (15.99—19.74)	16.84 ± 3.31 (12.17—19.59)
Roots	Bloomington, Illinois	Event Bt11	9.99 ± 0.59 (9.14—10.62)	6.41 ± 0.79 (5.67—7.72)	4.32 ± 1.52 (3.32—6.99)
Kernels	Bloomington, Illinois	Event Bt11	N/A[3]	N/A	1.45 ± 0.07 (1.39—1.56)
Pollen	Mackinaw, Illinois	Event Bt11	N/A	0.04 ± 0.00 (0.036—0.042)	N/A
Pollen	Monroeville, Indiana	Event Bt11	N/A	0.06 ± 0.02 (0.048—0.079)	N/A
Pollen	Seward, Nebraska	Event Bt11	N/A	<0.037	N/A

TABLE 2. Average Cry1Ab concentration from Corn Event MON 810 corn tissues grown in four regions of the US

Tissue (No. days post planting)	Cry1Ab conc. (µg/g dry weight)
Overseason Leaf (OSL)	
OSL-1 (21 days)	120 ± 15
OSL-3 (40 days)	46 ± 5.8
OSL-4 (50 days)	61 ± 17
OSL-5 (60 days)	51 ± 17
Overseason Whole Plant (OSWP)	
OSWP-1 (21 days)	120 ± 34
OSWP-5 (60 days)	25 ± 6.3
Forage (90 days)	7.6 ± 4.5
Pollen (60 days)	NA
Overseason Root (OSR)	
OSR-1 (21 days)	42 ± 9.5
OSR-3 (40 days)	20 ± 5.0
OSR-4 (50 days)	22 ± 3.7
OSR-5 (60 days)	19 ± 8.8
Forage root (90 days)	16 ± 6.0
Grain (125 days)	0.63 ± 0.06

NA = Not Applicable.

b. Cry1F Protein Expression in MoCry1F TC6275 and PoCry1F TC1507 Corn Tissues

There is significantly increased Cry1F protein expression in moCry1F TC6275 stalks, whole plants, and forage sampled during the flowering (R1) stage in comparison to poCry1F TC1507. Conversely, there is significantly decreased Cry1F protein expression in moCry1F pollen and grain in comparison to poCry1F. These expression differences are not expected to significantly change the exposure to lepidopteran non-target organisms. The increased expression of Cry1F protein in stalks of moCry1F hybrids could potentially decrease the risk of resistance development by borers, while the decreased expression in pollen and grain should decrease the non-target exposure to Cry1F expressed in corn (Zabik *et. al* 2003).

TABLE 3. Comparative expression of Cry1F protein in moCry1F TC6275 and poCry1F TC1507 corn tissues (Table recreated from Zabik *et al.* 2003).

		TC6275 (moCry1F)			TC1507 (poCry1F)		
Tissue	**Growth Stage**	**Mean**	**Standard Deviation**	**Min/Max Range**	**Mean**	**Standard Deviation**	**Min/Max Range**
		(ng/mg Tissue Dry Weight)					
Leaf	**V9[b]**	**17.3**	**3.41**	**10.7-23.8**	**12.1**	**6.2**	**0-24**
	R1	**28.5**	**5.38**	**16.5-36.7**			
	R4	**44.8**	**16.8**	**35.8-109.2**			
	Senescence	**0.71**	**1.14**	**0-3.0.9**			
Root	**V9[b]**	**6.14**	**1.87**	**4.53-8.14**			
	R1	**6.60**	**1.98**	**3.14-10.9**			
	R4	**5.99**	**1.89**	**2.35-9.26**			
	Senescence	**1.97**	**2.03**	**0.29-6.91**			
Whole Plant	**V9**	**6.22**	**1.16**	**4.98-7.87**	**5.2**	**1.9**	**2.6-6.8**
	R1	**7.16**	**1.45**	**5.32-9.57**	**3.6**	**1.1**	**2.5-4.7**
	Senescence	**2.47**	**0.41**	**1.95-3.07**	**1.6**	**0.6**	**0.9-2.4**
Pollen	**R1**	**3.67**	**0.34**	**3.09-4.60**	**21.9**	**2.9**	**16.4-27.2**
Stalk	**R1**	**11.0**	**2.67**	**6.77-16.4**	**5.8**	**1.7**	**3.3-10.3**
Forage	**R4**	**6.26**	**1.09**	**5.05-7.77**	**1.7**	**1.1**	**0-3.2**
Grain	**Maturity**	**1.14**	**0.27**	**0.62-1.68**	**2.2**	**0.8**	**0-4**

[b] Recalculated Results

c. Fate in Soils and Indirect Effects on Soil Biota

Most of the Cry protein deposited into soil by *Bt* crops is quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time. It has also been reported that the same degree of *Bt* Cry protein persistence takes place in soils that have been exposed to repeat *Bt* spray applications as those exposed to *Bt*-expressing crops. Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are

needed to yield relevant data on persistence and natural variation. 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. Current studies of *Bt* in soil show no effect on bacteria, actinomyces, fungi, protozoa, algae, nematodes, springtails or earthworms. In addition, new plants planted in *Bt* Cry protein containing soil do not take up the *Bt* protein.

i. Fate of *Bt* Proteins in Soil

Soil organisms may be exposed to δ-endotoxins from current transgenic crops through roots, incorporation of above ground plant tissues into soil after harvest, or by pollen deposited on the soil. Root exposure may occur by feeding on living or dead roots or, theoretically, by ingestion or absorption after secretion of δ-endotoxin into the soil. The latter situation is the subject of a recent brief communication in the journal *Nature* by Saxena *et al.* (1999), and is discussed in more detail below. In addition, evidence suggests that some soil components (e.g. clays and humic acids) bind δ-endotoxins in a manner that makes them recalcitrant to degradation by soil microorganisms, without eliminating their insect toxicity. Therefore, exposure to δ-endotoxin bound to soil particles may also be a route of exposure for some soil organisms.

Experiments addressing amounts and persistence of δ-endotoxins in the soil have been submitted by registrants and reviewed for the current conditional registrations. In addition, a number of publications in the scientific literature have addressed the degradation of Cry proteins in the soil. These experiments consist of the incorporation of purified δ-endotoxin or transgenic plant material in soil in a laboratory setting. Cry protein DT_{50} (time to 50% degradation) studies were submitted for registration of corn expressing Cry1Ab and Cry1F proteins, and published studies are available for Cry1Ac cotton. Cry1Ab biodegraded in an estimated DT_{50} of 1.6 days as expressed in transgenic corn tissue and in 8.3 days for purified protein (Sims and Holden 1996). Based on a bioassay with the tobacco budworm (*Heliothis virescens*), a target species, purified Cry1F proteins incorporated into test soils biodegraded with a DT_{50} of approximately 3.13 days. Data submitted by Monsanto for Cry1Ac protein and transgenic Cry1Ac in cotton report DT_{50}'s of approximately 9-20 days for the purified protein and 41 days for the protein in cotton tissue (MRID No. 439995-09). Published data for Cry1Ab andy1Ac in cotton tissue or as purified protein report a DT_{50} range of 2.2 to 46 days, where measurable (in 4 of 11 experiments); DT_{50}'s for transgenic tissue were shorter than for purified protein in two of three experiments (Palm *et al.* 1994). DT_{50}'s for purified Cry1Ac in two different non-sterile soils were 22 days and 40 days (Palm *et al.* 1994). None of the studies discussed above have been performed under field conditions, although most have used field soil in laboratory microcosms.

Several studies indicate that Cry proteins bind to clays and humic acids (Crecchio and Stotzky 1998, Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998, Stotzky 2000b). The results of these studies suggest that such binding slows the rate of microbial degradation of these toxins (Stotzky 2000b). This protection is not absolute, however, since degradation has occurred under several experimental conditions. Several factors influence either the affinity of binding or the rate of degradation. In particular, pH near neutral generally increases degradation substantially. At pH above 5.8 to near neutral, degradation of Cry protein bound to clay minerals in soil was much faster than degradation at pH 4.9-5.0 (Tapp and Stotzky

1998). For example, it was found that Cry toxin added to nonsterile soil containing kaolinite or montmorillonite showed little degradation even after around six months at lower pH (pH~5), while substantial degradation occurred over this time period at higher pH (Tapp and Stotzky 1998).

Corn does not grow well below ~pH 5.6 (Aldrich *et al.* 1975), and therefore most corn growing soils are expected to be at a higher pH. Therefore, under most production conditions, corn would not be grown on soils that would inhibit the rate of degradation compared to what is seen at near neutral pH.

Tapp and Stotzky (1998) proposed that the more rapid degradation of Cry proteins at near neutral pH was due to a greater amount of microbial activity. The authors pointed out that even at pH near neutral, protein toxin activity (lethal concentrations against a sensitive bioassay) remained after six months and they interpreted these data as evidence of prolonged persistence of Cry protein in the soil. In these experiments, substantial degradation (loss of biological activity) typically occurred rapidly in the first several weeks, with much slower subsequent breakdown (Tapp and Stotzky 1998). A similar pattern was observed in some experiments performed by others over a range of Cry1Ab and Cry1Ac protein concentrations from ~2-700 ng toxin/g soil (Palm *et al.* 1994, Palm *et al.* 1996). These experiments suggest that testing for persistence in the field should be determined over sufficiently long periods to assure an accurate assessment of degradation.

These results must be interpreted cautiously with regard to implications for persistence in the field. Field deposition of Cry protein is associated with plant material (pollen or crop residue) or plant root exudates (e.g. carbohydrates and amino acids) which typically stimulate microbial activity and reproduction (Cheng and Coleman 1990, Griffiths *et al.* 1999, Jensen and Soerensen 1994, Meharg 1994). Many of the experiments examining persistence of Cry proteins reported in the published literature have apparently been conducted in bulk soils or soil components. Bulk soil generally does not support populations of microorganisms as high as in the rhizosphere or in soils where plant residues are incorporated. Research suggests typical ratios of 5-20 for rhizosphere to bulk soil microbes, with rhizosphere populations commonly 100-fold higher than in bulk soil (Atlas and Bartha 1993). Therefore, degradation rates under field conditions may be higher than those shown in bulk soil experiments.

This conclusion is supported by data submitted by Sims (Sims and Holden 1996) where the DT_{50} of free Cry protein alone (in bulk soil) is about 5-fold higher than in ground corn tissue added to soil (although proteases from the corn tissue might also contribute to degradation). In addition, Donegan *et al.* (1995) found that microbial populations increased 100-1000 fold with the addition of plant material (cotton) with or without Cry1Ab or Cry1Ac proteins. Palm *et al.* (1996) found more rapid degradation of truncated Cry1Ab or Cry1Ac proteins when incorporated with cotton crop residues more often than when purified protein was used. In these microcosm experiments, toxin DT_{50}s, where possible to determine, varied greatly from 2.2 to 46 days (microbial populations in these previously dried, re-hydrated, soils were not determined). These experiments relied on ELISA of extracted Cry proteins to quantify residual Cry protein in the soil, so it is not clear what fraction of the extracted protein may have retained biological

activity. The extraction efficiency was reported to be about 27-60%, with lower efficiency corresponding to higher clay and organic content, and it is unclear whether un-extracted protein degraded at a similar rate as the extracted protein and retained biological activity. Other research (Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998), however, suggests that at least some of the clay-bound fraction of Cry protein is more resistant to extraction and degradation while retaining biological activity.

Some experiments that show relatively long persistence of Cry proteins in the soil do not consider rates of degradation, reporting instead only the duration of protein activity or presence of protein. These experiments sometimes begin with very high concentrations of the protein compared to the amounts found in the plant. For example, Tapp and Stotzky (1995) and Koskella and Stotzky (1997) used approximately 100 µg Cry protein/g soil and approximately 100-300 µg toxin/g soil, respectively, in their experiments, while *Bt* plants typically produce less than 10 µg/g plant tissue and the concentration in soil from incorporation is typically estimated to be at the PPB to low PPM levels. In addition, the bioassays or immunoassays used to detect the protein in the soil are very sensitive, able to detect the protein at concentrations of around 5-10 PPB (ng/g). Depending on the experiment, reductions of 10^3 to 10^5 may be required to reduce the amounts of Cry protein added to soil below detectable levels. Therefore, in order to predict persistence under field conditions it is important to know starting concentrations as well as degradation rates. Other experiments discussed above used amounts of Cry protein more representative of many plant tissues in *Bt* plants.

To summarize, available data suggests that the DT_{50} of Cry1Ab or Cry1Ac proteins, incorporated in corn plant residues or as free toxin in non-sterile soil, typically range from approximately 1.6-22 days but have been measured to be as long as 46 days and data showed DT_{50} for Cry1F to be 3.13 days. As suggested by Palm *et al.* (1996), DT_{50} may be expected to vary significantly depending on soil conditions. Conditions that favor microbial growth, however, including presence of metabolizable organic matter such as crop residues or rhizosphere secretions, and near-neutral pH, will favor shorter DT_{50}'s. Binding of Cry protein to clays and humic acids reduces microbial degradation rates compared to other soil components or media, but, based on current data, it cannot be concluded from these results that persistence is greater than demonstrated in available experiments. Furthermore, microcosm and laboratory data in non-sterile soils and near neutral pH suggest that most of the Cry protein deposited in soil may be quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time.

It is important to consider a number of factors expected to influence persistence under actual field conditions: humic acid and clay content of the soil; clay type; pH; moisture; soluble ion content and type; and temperature. Varying conditions can affect microbial activity, composition, and population levels, along with binding affinity of Cry proteins for soil components. Persistence of Cry proteins could therefore vary considerably. The conditions examined by the registrants, however, generally replicate common field soil conditions, although performed in a laboratory setting. Field tests of Cry protein degradation under a range of conditions typical of *Bt* crop cultivation yield relevant data on persistence and natural variation.

ii. Secretion of Cry Proteins by Plant Roots

Saxena *et al*. (1999) reported that Cry protein was exuded by the roots of transgenic corn plants in laboratory studies. In this study, Bt11 and nontransgenic corn plants were grown in Hoagland's solution or in sterile or nonsterile soil (sandy loam with 6% montmorillonite clay added, pH 6.0-6.5) (Saxena *et al.* 1999, Stotzky 2000d). Results of this study showed that the amount of total protein found in the medium averaged 105 µg per plant, as determined by the Lowry method. Although it is possible that the observed Cry1Ab protein was the result of small pieces of plant tissue (e.g. root cells or root hairs), it is unlikely since the Hoagland's solution was centrifuged, which removed cellular debris (Stotzky 2000a). In addition, the simple protein band pattern reported (66kDa) by Saxena *et al.* (1999) was the same as the Cry1Ab protein and found in *Bt* corn exudates only after 7 and 15 days. Nonetheless, the procedure used to isolate the Cry protein from soil, including vortexing in extraction buffer prior to centrifugation, could have ruptured or lysed cellular material.[8] In addition, small root material is difficult to separate from the rhizosphere soil used in the subsequent assays. The SDS-PAGE protein profile from non-sterile soil was reported to contain many more bands than from the SDS-PAGE profile from the Hoagland's solution (Stotzky 2000d). This indicates either extraction of plant cellular proteins and/or microbial proteins, possibly in addition to exuded or secreted proteins. SDS-PAGE gels of extracted proteins from the sterile soil treatment were not performed (Stotzky 2000d), so such data may have been expected to result in a protein profile similar to that from the Hoagland's solution if plant cellular material was not included.

While the data generally support the deposition of Cry protein in the Hoagland's solution external to corn root cells, the evidence concerning the soil experiments is not conclusive. Based on the reported methods, it is possible that both exuded and cellular protein could have contributed to the soil results. Alternatively, Cry1Ab protein may not have been exuded in the soil experiments, and may be an artifact of growth in Hoagland's solution experiment. This distinction is important, because it cannot currently be ruled out that a substantial portion of the insecticidal or immunological activity observed after 25 days in the soil experiment was due to plant associated Cry protein, which could have been protected from microbial degradation. Rhizosphere soil, which is the region very close (e.g. soil adhering to plant roots) to the surface of the roots, was used in this study. The concentration of Cry protein in this soil sample was probably higher than for soil further from the roots.

Relatively long persistence of Cry proteins is not surprising when the starting amounts are high and the assays for the protein (bioassay or immunoassay) are very sensitive. Saxena *et al.* (1999) reported that the 66KDa band disappeared after 25 days when the soil was no longer sterile. With a single incorporation at the concentrations estimated by Saxena *et al.* (1999), and DT_{50} estimates submitted by the registrant, it is possible that Cry protein would not be detectable after 25 days of exposure in the soil, even with sensitive bioassays or immunoassays (Sims and

[8] The composition of the EnviroLogix extraction buffer is proprietary, but Karen Larkin (personal telephone communication, March 22, 2000) confirmed that it is intended, in combination with vortexing, to lyse plant cells.

Holden 1996). The presence of Cry protein activity after 25 days in nonsterile soil appears to support either the persistence or continuous exudation of Cry protein, or both.

Results of the Saxena *et al.* study suggest that exposure of soil organisms to Cry protein may be continuous during the growing season, as well as after incorporation of plant residues. Some proteins may be secreted from the roots of plants in significant amounts by an active export process. These amounts may be much higher than the incidental amounts that might be released by other processes (e.g. sloughing off of root cells) and could lead to continuous exposure of soil organisms to Cry proteins. Experiments indicate that leakage of cytoplasmic proteins into the soil is at most incidental (Borisjuk *et al.* 1999, Denecke *et al.* 1990). Therefore, Cry protein exuded into the soil may have different risk implications than a single incorporation of Cry protein containing plant material.

Proteins secreted into the soil by plant roots are limited in number and specialized for that purpose, containing specific endoplasmic reticulum (ER) export signals in the form of short amino terminal amino acid sequences that target the protein to the lumen of the ER, and other short sequences targeting the protein into the apoplast (Borisjuk *et al.* 1999, Denecke *et al.* 1990, Rusch and Kendall 1995, Vitale and Chrispeels 1992). Cry proteins are not expected to be secreted into the soil because they are cytoplasmic proteins in *Bacillus thuringiensis* and, in particular, because no ER secretion peptide sequence has been identified in these proteins (Kostichka *et al.* 1996). A bacterial secretion peptide has been found only for a CryV protein that has been shown to be exported, or secreted, from *B. thuringiensis* (Kostichka *et al.* 1996). All other known Cry proteins, including those registered, form intracellular inclusions of Cry protein, and are not secreted. Other transgenic cytoplasmic proteins have been shown to be efficiently secreted only when a known ER signal peptide sequence is specifically added, otherwise these proteins remain cytoplasmic (Borisjuk *et al.* 1999, Denecke *et al.* 1990).

Available data for other secreted proteins suggest that the amount of a secreted protein found in culture medium may be as high or higher than the amount associated with plant tissue after several days growth (Borisjuk *et al.* 1999). In Bt11 corn roots, Cry1Ab is expressed at an average of 20.2 μg/g total root protein. It is difficult to predict whether this level of root expression of Cry1Ab is consistent with active secretion, based on the roughly estimated amounts of Cry1Ab found in the media (soil or Hoagland's solution) reported by Saxena *et al.* (1999).

The corn plants used in the exudation experiments were identified as NK4640*Bt*, which correspond to Syngenta Bt11 corn lines. This variety of corn contains a modified *cry1Ab* gene. Previous soil fate data supplied by the registrant was from experiments performed with nonsterile soil in the laboratory and consisted of replicated single incorporations of transgenic plant material or purified Cry protein. All other studies of Cry protein stability in the soil performed for registration or other purposes also used a single incorporation of purified protein or protein in the transgenic plant, with subsequent monitoring of residual activity using sensitive bioassays or other means, such as immunoassays. Single incorporation studies were performed because it was believed that most of the exposure of soil organisms to the Cry proteins would be through the incorporation of plant residues after harvest and to a lesser extent due to pollen shed.

Degradation studies were performed by Monsanto, registrant of MON 810 corn, which also contains the *cry1Ab* gene. In these studies, Cry1Ab protein degraded in non-sterile soil with a 1.6 d DT_{50} when ground plant material is used, and 8.3 d for the purified Cry1Ab protein in soil. The ground tissue used in this study would be expected to have a significantly increased surface area compared to crop residue incorporated at the end of a growing season. This increase in surface area might reduce degradation times. Use of ground tissue has been criticized (NAS/NRC 2000). Cry1Ab protein bioactivity of corn tissue incubated without soil decreased with an estimated DT_{50} of 25.6 days, and a DT_{90} of 40.7 days. After one week in soil, approximately 1% and 10% of the original levels of B.t.k. protein remained in leaf and stalk tissue, respectively. After three weeks, B.t.k. protein was still detected in the stalk tissue, but the level in transgenic leaves was similar to the background levels seen in control leaf tissue. B.t.k. protein apparently binds to soil particles, making quantitative extraction difficult. Biological activity, assessed by European corn borer bioassay, is reduced to control levels after three weeks of incubation in soil (MRID No. 436960-01).

Degradation of Cry1F in the soil was demonstrated in the study submitted in support of the poCry1F registration (MRID No. 450201-05). Based on a bioassay with the tobacco budworm (*Heliothis virescens*), a target species, purified Cry1F proteins incorporated into test soils biodegraded with a half-life of approximately 3.13 days. The amount of Cry1F protein in an acre of corn (if 25,000 corn plants/acre at harvest were left in the field) is approximately 20.5 g/acre. As a result, the expected maximum environmental concentration (EEC) for poCry1F protein was calculated to be 23 micrograms /kg dry soil (15 cm deep). Whole moCry1F plants at the R1 stage contain 7.16 ng of Cry1F/mg of tissue (dry weight) whereas poCry1F plants at the R1 stage contain 3.6 ng of Cry1F/mg of tissue. Therefore, we can roughly approximate that the EEC for moCry1F at the R1 stage would be 34.5 micrograms/kg dry soil (15 cm deep). Although EPA is aware of no evidence to indicate that prolonged exposure to trace amounts of Cry protein in the soil affects non-target organisms, the Agency felt that the submitted data did not sufficiently address the issue of residual Cry protein accumulation in the soil.

The October 2000 Scientific Advisory Panel (SAP) concluded, in SAP Report No. 2000-07 (March 12, 2001), that published data did not, at that time, adequately address the persistence of Cry proteins from *Bt* crops in the soil. Since it is difficult to correlate the relevance of the published laboratory data to field situations, the SAP recommended field studies be conducted in established *Bt* fields in a variety of soil types and climatic conditions. The SAP suggested an investigation of amount, accumulation and persistence of biological activity of Cry proteins in the soil. The SAP also concluded, however, that this data was not necessary for an EPA preliminary risk assessment but may be needed for a final assessment. In general, the Panel believed that studies on the mechanism of how Cry proteins enter the soil (e.g., secretion, shedding of root hairs, and degradation of biomass pollen) were primarily of academic interest, whereas knowledge of the potential environmental impacts was important for risk assessment. EPA agreed that some of these data would be useful in completing the database for a future assessment, and required supplementary studies regarding Cry protein degradation in soil.

The Agricultural Biotechnology Stewardship Technical Committee (ABSTC) submitted a research protocol for a *Bt* Cry protein soil accumulation study on March 15, 2002. We found the proposed protocol acceptable with the additional requirement of an ELISA (letter from USEPA to ABSTC 05/08/02). The ABSTC submitted a response to the Agency's letter, including a revised protocol, on June 5, 2002, which was accepted by EPA with the provision that the detailed protocol of the insect bioassay also be submitted and accepted. The studies have since been conducted and the results are summarized below.

1) Three-Year Soil Persistence of Cry1Ab (MRID No. 460224-01)
This study, which fulfills the soil fate data requirement for MON 810 field corn, Bt11 field corn, and Bt11 sweet corn, was submitted to EPA jointly by Syngenta Seeds, Pioneer Hi-Bred and Monsanto Company, the member companies of ABSTC. Bioassay results indicated that ECB larvae fed a diet containing 15% *Bt* corn soil exhibited no toxic response to the diet mixture, as measured through growth inhibition of test larvae (LOD 0.03 µg/g soil). Based on these findings, the study author concluded that Cry1Ab protein does not accumulate and persist in soil. However, Cry1Ab protein may have been present in test soil at concentrations that were below the LOD. Consequently, it may be more accurate to state that after three years of continuous Cry1Ab field corn production, Cry1Ab 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.

2) Three-Year Soil Persistence of Cry1F (MRID No. 471207-01)
This study, which fulfills the soil fate data requirement for TC1507 and TC6275, was submitted to EPA by Mycogen Seeds c/o Dow AgroSciences LLC. The results showed no detectable Cry1F protein residues and no biological activity observed in the soil of Herculex I corn, demonstrating Cry1F protein rapidly degrades in various soil matrices after 3 years of continuous cropping with Herculex I corn and is not expected to exude from the roots or be present in the sloughed-off root cells. This field study confirms previously conducted laboratory experiments [Herman *et. al* (5)] and the Agency's risk and benefits assessment for Cry1F corn and the 2001 Reassessment of *Bt* PIPs, which predicted that Cry1F protein rapidly degrades in the soil.

iii. 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 (Sanvido *et al.* 2007; Oliveira *et al.* 2008). In addition, *Bt* toxin released from root exudates and biomass of *Bt* corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil (Saxena and Stotzky 2001). Other research findings conclude no *Bt*-related risks have evolved from the decomposition of *Bt*-corn leaves for the meso- and macrofauna soil community (Hönemann *et al*. 2008). 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 *Bt* 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 as variations in seasons and weather, plant growth stage, and plant varieties, independent of being genetically engineered, are also responsible for significant shifts in soil microbial communities. To date, most studies with genetically engineered crops have shown minor or no effects on soil microbes beyond the variation caused by the factors listed above.

3. Non Target Wildlife Hazard Assessment

Prior to registration of the first *Bt* plant-incorporated protectants in 1995, EPA conducted ecological risk assessments for all *Bt* Cry proteins expressed in potato, corn, and cotton. EPA evaluated studies of potential effects on a wide variety of non-target organisms that might be exposed to the *Bt* protein, e.g., birds, fish, honeybees, ladybugs, parasitic wasps, lacewings, springtails, aquatic invertebrates and earthworms. Such non-target organisms are important to a healthy ecosystem, especially the predatory, parasitic, and pollinating insects. These risk assessments demonstrated that *Bt* Cry proteins expressed in transgenic plants do not exhibit detrimental effects to non-target organisms in populations exposed to the levels of Cry protein found in plant tissue. While EPA was aware of potential adverse effects on many species of Lepidoptera from Cry1 proteins, the Agency did not believe that non-target Lepidoptera would be exposed to sufficient amounts of *Bt* protein to cause an unreasonable deleterious effect, nor that *Bt* crops would threaten the long-term survival of a substantial number of individuals in the populations of these species. At that time, even though EPA knew that *Bacillus thuringiensis var. kurstaki* was toxic to Lepidoptera, EPA also concluded that threatened or endangered species of butterflies and moths would not be at risk because they would not be exposed to *Bt* Cry1protein in *Bt* crops.

Published field testing results and field test data voluntarily submitted to EPA by the registrants also shows minimal to undetectable changes in the abundance of beneficial and other non-target insect populations. In some cases the densities of predatory and non-target insects are reported to be higher on *Bt* crops than on non- *Bt* crops. These results are discussed below and are described in supporting assessment documents.

In light of recent environmental effects concerns from commercialization of *Bt* crops the Agency has reviewed new and existing data regarding non-target wildlife effects for *Bt* crops with a special emphasis on Lepidoptera and monarch butterflies and reevaluated the sufficiency of data to support continued registration of *Bt* crops.

Although *Bt* Cry proteins are very specific in their activity to certain insect species, EPA has examined the toxicity of the Cry proteins to birds, fish, honeybees and certain other beneficial insects. Because of the extensive scientific literature on the susceptibility of lepidopteran species to *Bt* Cry1 proteins, EPA assumes that Cry1 proteins would be toxic to butterflies if they were exposed to high levels of the protein and, therefore, did not require lepidopteran toxicity data. EPA nevertheless required data on Collembola (springtail) and earthworm species to ascertain

effects on beneficial soil invertebrates because soil exposure to *Bt* Cry proteins was a possibility. In the honeybee study, effects studies on brood as well as adults were required when exposure to the *Bt* Cry protein in pollen was expected. Evaluations of risk to other non-targets that may be affected by the *Bt* pollen, specifically the monarch butterfly, were conducted in 1999, 2000 and 2001-2002 by the USDA, ABSTC and university scientists in the USA and Canada (ABSTC, 2001, Dively *et. al.* 2004). The reports from these studies are summarized below and in the supporting DCI review document (Rose 2001).

Bt delta endotoxins are proteins and, unlike inorganic chemicals, do not have the potential to bioaccumulate causing delayed effects. An accumulation through the food chain is therefore not expected to take place, and there are no data to support this possibility for protein substances. The basic biological properties of proteins also make *Bt* Cry proteins readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports of soil binding under certain circumstances, the bound Cry proteins are also reported to be rapidly degraded by microbes upon elution from soil. The same sources report that *Bt* proteins in the soil of *Bt* corn fields have no detectable effect on soil invertebrates or culturable microbial flora. The *Bt* Cry proteins do not have any characteristics in common with persistent, bioaccumulative chemicals that are transferred through the food chain. Therefore, chronic effects testing of protein substances is not routinely performed.

a. Summary of Non-Target Organism Toxicity Testing on Corn Bt 11 (006444) and MON810 (006430)

TABLE 4. Tabular results of non-target wildlife testing for Cry1Ab proteins

OCSPP Guideline No.	Study	Results	MRID No.
885.4380	Larval Honey Bee Testing	*Btk* HD-1 protein at 20 ppm showed no toxicity to larval honey bees. An LC_{50} was not possible to calculate since this was a single dose test. Therefore, the NOEL is greater than 20 ppm.	434392-02
885.4380	Non-Target Adult Honey Bee Testing	There were no statistically significant differences among the various groups. However, sizable mortality occurred in all treatments. *Btk* HD-1 protein at 20 ppm resulted in a mean mortality of 16.2%. Because mortality was observed at the single dose tested, a NOEL could not be determined from this study, but it was less than 20 ppm. 20 ppm was determined to be significantly higher than exposure conditions in the environment.	434392-03
885.4380	Verification of Test Substance from Nontarget Insect and Honey Bee Testing	Test substance was stable for up to 7 days in 1:1 honey:sucrose solution. Test material was bioactive.	434680-02
885.4340	Non-Target Insect Testing - Green Lacewing Larvae	*Btk* HD-1 protein at 16.7 ppm showed no toxicity to green lacewing larvae after 7 days. The NOEL is greater than 16.7 ppm.	434680-03
885.4340	Non-Target Insect Testing - Ladybird Beetles (*Hippodamia convergens*)	*Btk* HD-1 protein at 20 ppm showed no toxicity to ladybird beetles (*Hippodamia convergens*). The NOEL is greater than 20 ppm.	434680-05
885.4340	Non-Target Insect - Parasitic Hymenoptera	*Btk* HD-1 protein at 20 ppm showed no toxicity to *Brachymeria intermedia*. Since this is a single dose study, an LC_{50} cannot be calculated. The NOEL is greater than 20ppm.	434680-04
N/A	Evaluation of Transgenic Corn Event Bt11 in Broiler Chickens	In a six-week feeding study with 1600 young broiler chicks, diets prepared with transgenic N7070Bt corn or N7070Bt corn that had been sprayed with Liberty herbicide produced growth, feed conversion rates, carcass yields, and survival rates similar to diets prepared with non-	456521-01

OCSPP Guideline No.	Study	Results	MRID No.
		transgenic corn. Birds fed N7070, N7070Bt, or N7070Bt-Liberty corn had similar body weights and feed conversion ratios throughout the test. There were no significant differences in the carcass yield of birds (48-days old) receiving either transgenic or non-transgenic corn. The study fulfills the conditional requirement for a six-week avian study.	
N/A	Comparison of broiler performance when fed diets containing YieldGard® Corn, YieldGard® x Roundup Ready®, parental line, or commercial corn hybrids	To compare the nutritional value of YieldGard and YieldGard x Roundup Ready to their respective parental lines, as well as to other non-transgenic corn varieties, groups of broiler chickens were fed prepared diets containing one of the corn lines for 42 days. Eight corn lines (treatments) were evaluated in a randomized complete block design. Each treatment group consisted of 10 pens (5 pens of 10 males each and 5 pens of 10 females each), for a total of 100 birds/treatment (800 birds overall). Mortality was observed throughout the study, and at test end survivors were examined for performance, carcass yield, and meat composition. Most of these mortalities were apparently due to sudden death syndrome and ascites. No mortalities were determined to be related to the treatment diet, and no other adverse effects were observed. There were no biologically significant differences in performance, carcass yields or meat composition between any of the groups tested.	456118-01
885.4050	Avian Oral Toxicity in Northern Bobwhite Quail	No treatment related mortality or differences in food consumption, body weight or behavior occurred in birds fed 50,000 or 100,000 ppm transgenic corn meal derived from Monsanto's MON 80187 corn line (which contains Cry1Ab protein) relative to birds fed corn meal made from parental corn lines which did not express *Bt* protein. Although this study utilized Monsanto's *Bt* corn for testing, the test material was considered sufficiently similar to the Bt11 corn grain to bridge the data.	435332-05
885.4240	Corn Pollen Containing the Cry1Ab Protein: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*).	Monsanto submitted this study to support their MON 810 corn. The study is scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. These results indicate that *Daphnia magna*, a sensitive aquatic invertebrate species, is not affected by a 48 hour exposure to 100 mg of Cry1Ab protein containing MON810 corn pollen/L. This study adequately address potential aquatic toxicity concerns for MON 810 corn pollen expressing Cry1Ab protein. Mon 810 pollen is preferred over Bt11 pollen as a test material in studies supporting Bt11 corn. However, given the equivalent Cry1Ab expression in Bt11 and MON 810 corn (< 90 ng Cry1Ab/g dry wt.pollen) and the lack of treatment related effects seen in any *Bt* corn pollen *Daphnia magna* studies, the data requirement is satisfied for Bt11. The data suggest that at the expected environmental concentration the proposed use of Cry1Ab protein in corn is not likely to have any measurable effects on aquatic invertebrates.	442715-02
885.4240	*Daphnia magna* Study and Bridging Rationale	Novartis cited the Ciba Seeds (now Novartis Seeds) Event 176 acute 48 hr. study (MRID No. 433236-10) to support Bt11. This 48-hour static renewal toxicity study of Event 176 maize pollen containing *Bt* Cry1Ab Cry protein was conducted using *Daphnia magna*. Test daphnids were dosed at five concentration levels, including a maximum hazard dose of 150 mg/L (nominal) of water. No mortalities were observed at any of the treatment levels tested. The 48-hour EC_{50} was determined to be greater than 150 mg/L. The NOEC was found to be >150 mg/L. These results indicate that *Bt* Cry1Ab protein expressed in corn showed not toxicity at 150 mg/L to Daphnia magna. In view of the above results, no freshwater aquatic invertebrate hazard is expected from the use of this product. Bt11 pollen is preferred over Event 176 pollen as a test material in studies supporting Bt11 corn. However, given the low level of expression of Cry1Ab in Bt11 pollen [(< 0.55 micro g Cry1Ab/ g protein) or (< 90 ng Cry1Ab/g dry wt.pollen)] compared to Event 176 pollen [80.63 micro g Cry1Ab/g protein) or (12.36 micro g Cry1Ab/g dry wt. pollen)] and the lack of effects seen in the cited *Daphnia magna* study using Event 176 pollen, the data requirement is satisfied for Bt11.	433236-10 442742-01
885.4200	Evaluation of the European Corn Borer Resistant corn Line MON	Feed per fish, feed conversion ratios, final weight, percentage weight gain and survival were not significantly different between fish fed the control MON 800 diet when compared to those fed the diet containing	438879-01

OCSPP Guideline No.	Study	Results	MRID No.
	801 as a Feed Ingredient for Catfish.	transgenic corn from the test line MON 801. Body composition data exhibited no significant differences in percentage moisture, fat, or ash, with higher protein content in the test fish on a dry weight basis. This difference in protein content disappears when one expresses the results on a wet weight basis. Data in this study are consistent with historical controls for catfish grown at the Delta Research and Extension Center. Although this study utilized Monsanto's *Bt* Cry1Ab corn for testing, the test material was considered sufficiently similar to the Bt11 Cry1Ab corn grain to bridge the data.	
885.4340	Effect of Cry1Ab, on *Folsomia candida* and *Xenylla grisea* (Insecta: Collembola).	In the cited study, purified *Btk* insecticidal proteins derived from *E. coli* (200 ppm), including Cry1Ab protein, had no observable toxicological effect on two species of Collembola: *Folsomia candida* and *Xenylla grisea*. The Agency has required Novartis to submit a Collembola study using leaf material rather than bacterially-derived Cry1Ab.	439416-01
885.4340	Chronic Exposure of *Folsomia candida* to Corn Tissue Expressing Cry1Ab Protein.	This study determined that the LD_{50} of lyophilized MON 810 corn leaf tissue containing the Cry1Ab protein to Collembola (*Folsomia candida*) over a 28-day exposure period is greater than 50% (by weight) of the diet. The no-effect-level for mortality was 50% of the diet. This same concentration in the diet had no effect on the reproduction of Collembola. According to the sponsor, the estimated concentration of Cry1Ab protein was 50.6 µg/g in lyophilized tissue and 6.27 µg/g in fresh tissue. The control substance was lyophilized leaf tissue from the non-transgenic corn line MON 823 which has a genetic background similar to the MON 810 line but does not carry the gene responsible for the Cry1Ab protein. Thiodicarb was used as a positive control or reference substance. While this study is useful in characterizing effects of Cry1Ab corn tissue on Collembola and satisfies the requirement for MON 810 corn, it does not adequately characterize the effect of *Bt*-11 corn tissue on Collembola since possible treatment related effects were observed in a *Bt* corn Collembola study.	MRID No. 442715-01
N/A	Field Surveys of non-Target Invertebrate Populations in Corn Field Studies of Non-Target Invertebrate Populations in *Bt* Corn	On March 15, 2002, a report titled "Field Surveys of Non-Target Invertebrate Populations in *Bt* Corn" was submitted on behalf of the Non-Target Organism Subcommittee (NTO Subcommittee) of the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) members (MRID No.45652001). EPA reviewed this submission and indicated that final reports of the in-progress studies and the results of any additional research should be submitted at a later date. On March 15, 2006, the NTO Subcommittee submitted the document "Field Studies of Non-Target Invertebrate Populations in *Bt* Corn" (MRID No. 467846-01). This submission included complete references and additional information that had become available. Most papers focused on large-scale field studies using transgenic corn, including discussions of study design and non-target invertebrate populations. Overall, the published literature did not report any consistent adverse impacts on non-target invertebrates as a result of multi-year commercial *Bt* corn cultivation. Slight reductions in some invertebrate predator populations were seen; however, these are an inevitable result of all pest management practices which tend to result in reductions in the abundance of the pests as prey.	456520-01 467846-01
N/A	Effects on Monarch Butterfly Larvae after Continuous Exposure to Cry1Ab-Expression Corn Pollen During Anthesis	Effects on the monarch butterfly (*Danaus plexippus*) from continuous exposure of larvae to natural deposits of *Bt* and non-*Bt* corn pollen on milkweed (*Asclepias syriaca*) were measured in five studies using Cry1A(b)-expressing hybrids (events BT11 and MON810) and non-transgenic corn in Maryland, Iowa, and Ontario, Canada. First instars were exposed beginning at 3 to 4 and 6 to 7 days after initial anthesis. Overall mean pollen densities during larval development in the first and second bioassays were 163 and 170 grains/cm^2, respectively. Pollen density on milkweed plants in the non-*Bt*, BT11, and MON810 hybrids averaged 163, 155, and 174 grains/cm^2, respectively, during the first bioassay and 172, 173, and 158 grains/cm^2, respectively, in the second bioassay. There were no statistically significant differences in pollen density between assays or among hybrid types. The number of anthers on the leaves was positively correlated with the amount of pollen deposited	461620-01

OCSPP Guideline No.	Study	Results	MRID No.
		on the leaves. The study included the results of a risk assessment simulation model that projected, over the range of the Corn Belt, an increased risk of 0.6% in mortality to monarch larvae from prolonged exposure to *Bt* corn pollen. The increased risk to monarch larvae when considering all of North America was 0.3%.	
885.6200	Cry1Ab Insecticidal Protein: An Acute Toxicity Study with the Earthworm in Artificial Soil Substrate	The 14-Day LC_{50} value for earthworms exposed to Cry1Ab insecticidal protein derived from *E. coli* in an artificial soil substrate was determined to be greater than 200 mg/kg (ppm), which was the single concentration tested. There were no statistically significant effects at the single dose tested, therefore the NOEL is greater than 200 ppm. Although this study was graded supplemental, *Bt* Cry1Ab proteins expressed in the corn plant are not expected to generate a toxic effect in the earthworm, therefore, no additional follow-up of this study is required.	438879-02

b. Summary of Non-Target Organism Toxicity Testing on Corn TC1507 (006481) and TC6275 (006491)

TABLE 5. Tabular results of non-target wildlife testing for Cry1F proteins

OCSPP Guideline No.	Study	Results	MRID No.
885.4150	Wild Mammal Testing, Tier I	Mammalian wildlife exposure to moCry1F protein is considered likely; however, the mammalian toxicology data submitted for the Human Health Assessment for poCry1F indicates that there was no significant toxicity to rodents from acute oral testing at the maximum hazard dose. Based on the bridging data in combination with the poCry1F rodent study, no hazard to mammalian wildlife is anticipated from moCry1F.	446911-01
885.4050	Dietary Toxicity Study with the Northern Bobwhite (*Colinus virginianus*)	The dietary LC_{50} for corn grain (meal) expressing *Bt* var. aizawai protein in corn grain when fed to juvenile northern bobwhite quail (*Colinus virginianus*) for five days was determined to be greater than 100,000 ppm (10% of corn meal). The NOEC was 100,000 ppm and there were no treatment-related mortality or behavioral changes observed in comparison to the control replicates. These data were determined to be insufficient to make a hazard assessment from repeated exposure(s) to higher doses of *Bt* corn and a six week study with 60 to 70% corn in the diet was deemed necessary to assess hazards from chronic exposure of wild and domesticated fowl. Therefore, the study was determined to be supplemental. However, the additional study was submitted (see below) and the study is now upgraded to acceptable.	450201-12
Non-guideline	Nutritional Equivalency of *Bt* Cry1F Maize-Poultry (Cobb x Cobb) Feeding Study	In a six week study where commercial broiler chickens were fed a diet containing 54.21%-57.03% *Bt* Cry1F line 1507 and control diets there was no statistically significant difference found in mortality, mean body weight, mean daily weight gain, or mean food conversion.	456220-01
885.4380	Honey Bee Larva Testing, Tier I	The data show that at the expected environmental exposure the proposed use of Cry1F protein in corn is not likely to have any measurable deleterious effects on the honey bee (*Apis mellifera*). There was no treatment mortality or behavior change observed between the dosed and control replicates. LC_{50} > 64 ng Cry1F in 2 mg pollen /larva and 640 ng Cry1F protein /larva. Based on the bridging data in combination with this study, Cry1F protein as expressed in corn pollen should have no detectable adverse effects on honey bee larvae or their development into healthy adults.	450415-03, 453078-05 (supplement)
885.4340	Non-target Insect Testing, Tier I with Green Lacewing Larvae (*Chrysoperla carnea*)	Green lacewing larvae fed a concentration of *Bt* poCry1F protein at15x the expected rate found in corn pollen (pollen expressing 32 ng Cry1F/mg pollen) resulted in no mortality or signs of toxicity or abnormal behavior over a 13 day period (>20% control mortality period). The LC_{50} and NOEC was determined to be >15x the concentration of poCry1F found in pollen and the was determined to be > 480 ppm a.i	450201-09, 453078-01 (supplement)

OCSPP Guideline No.	Study	Results	MRID No.
		(the test concentration). Mortality and pupation rate were comparable between the treatment and control group. Based on the bridging data in combination with the poCry1F green lacewing study moCry1F should have no detectable adverse effects on *Chrysoperla carnea* in the field.	
885.4340	Non-target Insect Testing, Tier I with the Ladybird Beetle (*Hippodamia convergens*)	Adult lady beetles fed a concentration of *Bt* Cry1F protein at at 15x the expected rate found in corn pollen (pollen expressing 32 ng Cry1F/mg pollen) resulted in no mortality or signs of toxicity over a 29 day period. Therefore, the NOEC and the LC_{50} were determined to be >15x the concentration of Cry1F found in pollen determined to be > 480 ppm a.i (the test concentration). The test insects were exposed to a dose of active ingredient approximating the amount that would be ingested by the beetles feeding on aphids under field conditions. As a result, no discernible beneficial beetle population effects are expected from the proposed uses of the Cry1F producing corn.	450201-10, 453078-02 (supplement)
885.4340	Non-target Insect Testing, Tier I with the Parasitic Hymenoptera (*Nasonia vitripennis*)	Parasitic Hymenoptera fed a concentration of *Bt* Cry1F protein 10x the expected rate found in corn pollen (expressing 32 ng Cry1F/mg pollen) showed no mortality or signs of toxicity or abnormal appearance or behavior of surviving wasps in the treatment or control group over a 12 day period. The test was terminated after 12 days because 20% mortality was reached in the negative control. The NOEC and the LC_{50} were determined to be > 320 ppm a.i (10x field rate when calculated for pollen expressing 32 ng Cry1F/mg pollen). No hazard to parasitic Hymenoptera at field use rates is expected from the cultivation of Cry1F containing corn.	450201-11, 453078-03 (supplement)
885.4340	Non-target Insect Testing, Tier I with Neonate Larvae of the Monarch Butterfly (*Danaus plexippus*)	First instar Monarch larvae fed a 10,000 ng/mL diet (the highest rate tested) showed no mortality after seven days of feeding. There was some growth inhibition at 10,000 ng/mL diet. LC_{50} > 10,000 ng/mL and NOEC <10,000 ng/mL. Since doses equivalent to 10,000 ng/mL diet are not likely to occur in nature, it was concluded that Cry1F protein will not pose a risk to monarchs.	451311-02
885.4340	Collembola Testing, Tier I (*Folsomia candida*)	Collembola (*Folsomia candida*) were fed three treatment levels (12.5, 3.1, 0.63 mg/kg) of Cry1F protein every two to three days for 28 days representing 79x, 388x, and 1560x that which would be encountered in the field with no observable treatment mortality or behavior change. Results of the study indicate that levels of Cry1F that might occur in the field are not expected to adversely effect the soil invertebrate Collembola species. LC50 and NOEL >12.5 mg Cry1F/kg soil.	450201-07
850.6200	Acute Toxicity Study with the Earthworm (*Eisenia fetida*) in an Artificial Soil Substrate	Earthworms, *Eisenia fetida*, fed 2.26 mg poCry1F/kg dry soil, representing up to 100X the estimated concentration present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants did not have adverse effects. LC50 and NOEL >2.26 mg Cry1F/kg dry soil. Based on the results of this study, it is not likely that Cry1F transgenic corn plantings will have adverse effects on earthworms.	450201-06, 453078-04 (supplement)
N/A	Non-target Insect Field Survey	Sticky traps were set out weekly for six weeks. In addition, ten plants in the center row were visually evaluated for beneficial arthropods weekly for six weeks. Beneficial insects counted were: lady beetles (*Cycloneda munda* & *Coleomegilla maculata*), predacious carabids, brown lacewings (Hemerobiidae), green lacewings (*Chrysoperla plorabunda*), minute pirate bugs (*Orius insidiosus*), assassin bugs (Reduviidae), damsel bugs (Nabidae), ichneumonids and braconids (parasitic wasps), damselflies and dragonflies (Odonata), and spiders (Arachnida). Data included counts of adult and larval lady beetles and lacewings when appreciable numbers were collected. Results from the study indicated that the transgenic corn lines TC1507 and 1360 did not adversely affect the number of beneficial arthropods in the field. In general, line TC1507 showed larger numbers of beneficial insects. The field census study adequately addressed potential concerns for Cry1F protein expressed in corn to non-target insect populations. However, the Agency	450201-13

OCSPP Guideline No.	Study	Results	MRID No.
		recommended that the monitoring continue into the first few years of commercial use of Cry1F corn crops in order to confirm the single season Ano effects@ findings and to gather long-range non-target insect effects and abundance data.	
N/A	Non-target Exposure and Risk Assessment for Environmental Dispersal of Cry1F Maize Pollen. (A probabilistic risk assessment)	This study was conducted with poCry1F to consider the exposure of non-target species including endangered Lepidoptera species to field corn pollen expressing the Cry1F delta endotoxin by evaluating pollen dissemination. The Cry1F concentration found in pollen occurring on milkweeds near the edge of *Bt* corn fields was predicted. Distance of pollen dispersal, levels of Cry1F expression in pollen, milkweed distribution and biomass from the edge of the field, pollen grain physical properties, and spatial-temporal availability of Cry1F to monarch larvae was determined. According to a probability-log plot demonstrating lepidopteran species susceptibility to Cry1F, 99% of lepidopteran species exhibit an LC_{50} of $\exists$0.06 $\Phi g\ g^{-1}$ which is 290-fold lower than the geometric mean LC_{50} (12.4 $\Phi g\ g^{-1}$) and lower than the most sensitive lepidopteran species. The toxicity threshold, or no effect level for monarch neonates, for the Tier 1 risk assessment was determined to be 10 $\Phi g\ g^{-1}$ diet. When fed up to 10 $\Phi g\ g^{-1}$ Cry1F microbial toxin in diet, neonate monarch larvae were not affected. The toxicity threshold, or no effect level for monarch neonates, for the Tier 1 risk assessment was determined to be 10 $\Phi g\ g^{-1}$. The log-probability plot of the *Bt* LC_{50} for lepidopteran species shows that the EEC does not exceed the LC_{50} for 98% of the intergenera population beyond 1 m from the field edge. The LC_{50} is not exceeded for 90% of the population 0.2 m from the edge. For monarch larvae, the no effect level (10 $\Phi g\ g^{-1}$) occurs near the 50^{th} percentile intergenera LC_{50}. Since there is a rapid fall-off in exposure with distance, there is limited potential for non-target effects beyond the immediate field border. In addition, the estimated risk quotients (ratio of exposure to effect) demonstrate a lack of concern for monarchs (or other lepidopteran species) beyond 1 m from the field edge. The RQ in the corn field was 0.096. Finally, pollen from moCry1F plants express less Cry1F protein than poCry1F plants, further reducing non-target exposure.	450415-02
885.4240	A 48-Hr Static Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*)	There were no overt signs of toxicity to daphnids (*Daphnia magna*) exposed to 100 mg *Bt*-pollen/L - (maize pollen containing the *Bt* Cry1F delta-endotoxin). The 48-hr EC50 was > 100 mg a.i./L. The NOEC was >100 mg a.i./L. These data show that there will be no adverse effects on daphnia from incidental field exposure to transgenic corn pollen containing Cry1F.	450201-08
885.4200	Freshwater Fish Testing, Tier I	The Agency previously waived static renewal toxicity tests for freshwater fish due to the lack of substantial exposure to poCry1F protein in runoff and corn pollen. However, the registrant submitted a study in support of the potential moCry1F registration. Juvenile rainbow trout (*Onchorhynchus mykiss*) were fed a standard fish diet containing 100 mg Cry1F ICP a.i./kg of diet for eight days with no mortality or sublethal effects. The LD_{50} was determined to be greater than 100 mg a.i./kg of diet. The actual concentration of the test material in the diet was not determined and therefore this study is supplemental.	450442-01 460193-06
850.1075	Fish Acute Toxicity Test, Freshwater and Marine	Study was not required for this product because of very low or no potential for exposure.	None assigned

c. Non Target Wildlife Risk Characterization

i. Terrestrial Wildlife

1) Mammalian Wildlife

The human health data submitted to EPA indicate that there is no significant toxicity to rodents from acute oral testing at the maximum hazard dose for Cry1Ab or poCry1F. The data submitted in support of poCry1F, combined with the bridging data summarized in Section 2.b. of this assessment (Table 3), also indicate that no hazard to mammalian wildlife is anticipated from moCry1F. In addition, there are reports of no adverse effects on livestock after several years of feeding with *Bt* corn. Mammalian wildlife exposure to the *Bt* Cry proteins is considered likely; however, the mammalian toxicology information gathered to date does not show a hazard to wild or domesticated mammals.

2) Avian Species

When administered by oral gavage at a dosage up to 2,000 mg protein/kg body weight, *Bt* corn has no apparent effect upon bobwhite quail after 14 days. A study with a non-commercial line of MON 80187 showed no mortality or differences in food consumption, body weight, or behavior when bobwhite quail were fed 50,000 or 100,000 ppm Cry1Ab in corn meal. (Although this study utilized Monsanto's *Bt* corn for testing, the test material was considered sufficiently similar to the Bt11 corn grain to bridge the data.) In addition, there are reports of no adverse effects from the commercial poultry industry after several years of using *Bt* corn in poultry feeds.

The dietary LC_{50} value for corn grain (meal) expressing *Bacillus thuringiensis* var. *aizawai* Cry1F protein in corn grain when fed to juvenile northern bobwhite for 5 days was determined to be greater than 100,000 ppm (10 % corn meal). The No Observed Effect Concentration (NOEC) was also 100,000 ppm. The study is scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates.

Though the above data indicate that the *Bt* Cry1 protein produced in corn does not show a hazard to birds, EPA determined that they were not sufficient to make a final hazard assessment from repeated exposure(s) to higher doses of *Bt* corn. In 2001, a six-week study with 60 to 70% corn in the diet was conditionally required to assess hazards from chronic exposure of wild and domesticated fowl. These studies have since been conducted, and the results are summarized below.

a) Cry1Ab
i) Bt11 (MRID No. 456521-01)
In a six-week feeding study with 1600 young broiler chicks, diets prepared with transgenic N7070Bt corn or N7070Bt corn that had been sprayed with Liberty herbicide produced growth, feed conversion rates, carcass yields, and survival rates similar to diets prepared with non-transgenic corn. Significant differences were observed, however, in survival by sex during portions of the study; males had increased mortality during the finishing (days 35-42) and overall (days 0-42) periods. The study author attributed this to the males being more susceptible to heat stress. There were also some erratic differences in mortality by sex during the grower phase (days 21-35), but these were not seen on a cumulative basis (days 0-42), and were attributed to chance. Birds fed N7070, N7070Bt, or N7070Bt-Liberty corn had similar body weights and feed conversion ratios throughout the test. Birds fed NC2000 corn had significantly decreased

weights for each portion of the study, and a significantly decreased adjusted feed conversion ratio for the starter (days 0-21), finishing (days 35-42), and cumulative (days 0-42) periods. The study author attributed these decreases to an interaction between the climatic conditions and the NC2000 diet formulation, which was more hygroscopic than the others, thus affecting the flow of the feed into the feeder pan. Evidence of restricted feed flow was seen in two pens during the finishing period, and these were excluded from the statistical analysis for feed conversion ratio. There were no significant differences in the carcass yield of birds (48-days old) receiving either transgenic or non-transgenic corn. The Agency concurs that the diets with transgenic corn produced growth, carcass yields, and meat quality similar to those obtained with the non-transgenic isoline. No valid comparison to the NC2000 corn can be made, however, as it is unclear whether the superior performance of the N7070-series corns compared to that of the NC2000 was due to inferiority of the NC2000 corn or to formulation differences in the NC2000 diet. The study fulfills the conditional requirement for a six-week avian study.

ii) MON 810 (MRID No. 456118-01)

YieldGard and YieldGard x Roundup Ready corn produce the Cry1A(b) protein, which confers insect protection. In addition, YieldGard x Roundup Ready expresses the maize EPSP synthase gene, which confers tolerance to glyphosate. To compare the nutritional value of YieldGard and YieldGard x Roundup Ready to their respective parental lines, as well as to other non-transgenic corn varieties, groups of broiler chickens were fed prepared diets containing one of the corn lines for 42 days. Eight corn lines (treatments) were evaluated in a randomized complete block design. Each treatment group consisted of 10 pens (5 pens of 10 males each and 5 pens of 10 females each), for a total of 100 birds/treatment (800 birds overall). At study start, two additional birds were included in each pen to compensate for potential losses due to mortality and dehydration. On study day 7, the pens were culled to 10 birds per pen. Mortality was observed throughout the study, and at test end survivors were examined for performance, carcass yield, and meat composition. Chick mortality was as expected during the first 7 days of the study. Mortality during days 7-42 (6%) was slightly higher than normal, but was random across treatments, ranging from 2% to 11% for the different corn lines. Most of these mortalities were apparently due to sudden death syndrome and ascites. No mortalities were determined to be related to the treatment diet, and no other adverse effects were observed. There were no biologically significant differences in performance, carcass yields or meat composition between any of the groups tested. Based on these results, the two Cry1Ab corn lines tested do not produce adverse effects in broiler chickens through dietary exposure.

b) Cry1F (MRID No. 456220-01)

The registrant submitted a six-week study as required by the EPA. Two hundred forty-five male broiler chickens (Cobb x Cobb) were fed diets containing commercial corn-soy type ration with either reference yellow dent corn, *Bt* Cry1F maize 1507 hybrid, or a non-transgenic control hybrid corn (five replications each) for six weeks. The broilers were fed a starter ration (54.21% corn) for the first 20 days and then a grower ration (57.03% corn) for days 21 through 42. There were no statistically significant differences in mean percent mortality, mean body weight, mean daily weight gain, or mean feed conversion among any of the treatments.

Furthermore, moCry1F grain contains less Cry1F protein (1.14 ng/mg) as compared to poCry1F grain which contains 2.2 ng/mg of Cry1F protein (dry weight tissue; Zabik et. al 2003). The six week broiler study, in conjunction with the initial bobwhite quail study submitted in 2001, is sufficient to demonstrate that there should be no discernible detrimental effects to wild or domesticated fowl from the proposed uses of Cry1F producing corn including moCry1F.

ii. Aquatic Species

There is no evidence for sensitivity of aquatic (including endangered) species to Cry proteins. Toxicity studies with aquatic organisms do not show a hazard for fish or invertebrates exposed to either *Bt* corn pollen or to bacterially expressed Cry protein. It has also been demonstrated that farm fish food mix made from corn seed containing the *Bt* protein does not contain detectable active *Bt* Cry protein; therefore, farmed fish would not be exposed to *Bt* Cry proteins. In addition, aquatic exposure from *Bt* crops is extremely small. A simple standard pond scenario (1 hectare pond, 2 meters deep draining a 10 hectare watershed planted with corn) was used to develop a worst case EEC for Cry1Ab and Cry1F proteins (high protein expression level) on the basis of corn pollen loadings from airborne pollen deposition and agricultural runoff. Airborne pollen deposition will result in water concentrations less than 78 ng Cry1Ab protein/L when based on conservative estimates for pollen dispersal. The contribution of Cry1Ab to the pond through agricultural runoff is comparable (66 ng L-1 based on GENECC). Thus, total water concentration of less than 144 ng Cry1Ab protein/L is projected under worst case conditions (Wolt 2000). Airborne pollen deposition results in water concentrations of approximately 1.25 ng Cry1F/mL and the contribution of Cry1F to the pond through agricultural runoff is <0.15 ng/mL. Thus, total water concentration of 1.4 ng Cry1F protein/L is projected under worst case conditions.

1) Aquatic Invertebrates

The major source of *Bt* Cry proteins in fresh water is corn pollen. Toxicity studies with corn pollen containing Cry1Ab proteins conducted using *Daphnia magna* show an acute EC_{50} was >100 mg/L in one study and 150 mg/L in another. The LOEC (lowest observed effect concentration) was found to be 150 mg/L. The amount of pollen was considered to exceed the 144 ng Cry1Ab protein /L projected aquatic exposure in the fields under worst case conditions. Toxicity studies with corn pollen containing Cry1F proteins conducted using the sensitive aquatic indicator species *Daphnia magna* show the no-mortality concentration and NOEC to be >100 mg a.i./L. There were no overt signs of toxicity to daphnids exposed to 100 mg *Bt* Cry1F pollen/L. The amount of pollen tested was considered to well exceed field exposure. The data for poCry1F were bridged to support the moCry1F registration; moCry1F pollen contains less Cry1F protein (3.67 ng/mg) as compared to poCry1F pollen, which contains 21.9 ng/mg of Cry1F protein (dry weight tissue; Zabik *et. al* 2003).

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* studies are unacceptable because they are 850 Series OCSPP Guideline studies. 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 magna* study as per the 885 series OCSPP 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.

UPDATE (September 2010): Since the 2007 Rosi-Marshall et al. publication, numerous researchers have published peer-reviewed studies that identify issues with the scientific merit and relevance of the original caddis fly study (Swan et al. 2009, Jensen et al. 2010, summarized by Beachy et al. 2008, Parrott 2008, and Wolt and Peterson 2010). In response to comments received on the proposed terms and conditions for the extension of the 2010 expiring *Bt* corn registrations, EPA conducted a literature review of these recently published studies. Criticisms of the Rosi-Marshall et al. study included several findings: (1) adverse effects were not caused by toxicity of Cry1A but, rather, by other differences between plant test substances (Jensen et al. 2010); (2) the abundance of Trichoptera in streams containing residues of Cry1A was not reduced (Chambers et al. 2007); and (3) while post-harvest crop residue was identified as the most likely route of exposure (Carstens et al. 2010), aquatic exposure to biotech crops has been shown to be limited temporally and spatially with low to negligible exposure concentrations of Cry proteins in post-harvest crop tissues (Swan et al. 2009, Griffiths et al. 2009, Jensen et al. 2010, Wolt and Peterson 2010, Carstens et al. 2010). In light of these results, EPA is waiving the requirement for additional aquatic invertebrate studies to assess hazard to aquatic shredder species for existing Cry protein PIP registrations.

Questions have been raised for using corn pollen in aquatic invertebrate testing with *Daphnia magna* because corn pollen is thought to be too large for ingestion by these filter feeders (EcoStrat, 2000). However, there is some observational evidence that daphnids do ingest pollen. As indicated in some study reports reviewed by the Agency, daphnids were actually yellow in color, which can be indicative of ingestion of the test material, with no treatment mortality or behavior change compared to untreated controls. Also, if the pollen is not ingested, or excreted without digestion when presented to *Daphnia*, then there will not be any exposure, and therefore no risk to *Daphnia* in the aquatic environment.

2) Freshwater Fish

The requirement for a freshwater fish static renewal toxicity study has been waived based on a lack of any substantial exposure of fish to the *Bt* Cry proteins produced in *Bt* crops (Wolt 2000). Farm fish diets made with corn containing the Cry proteins do not adversely affect susceptible target insect larvae, as bioassay testing and analyses using ELISA indicate that Cry protein is not detectable in the fish feed samples. Therefore, farm fish eating a food mix made from corn containing the *Bt* delta endotoxin would not be exposed to detectable active *Bt* Cry protein.

Despite acceptable data waiver justification, Dow AgroSciences submitted a freshwater fish study in support of the moCry1F registration (MRID No. 460193-06). Juvenile rainbow trout

(*Onchorhynchus mykiss*) were fed a standard fish diet containing 100 mg Cry1F ICP a.i./kg (100 ppm) of diet for eight days with no mortality or sublethal effects. The LD_{50} was determined to be greater than 100 mg a.i./kg of diet. The study is considered supplemental, however, because the actual concentration of the test material in the diet was not determined.

In view of the lack of demonstrated toxicity and exposure, no aquatic hazard is expected from the continued uses of *Bt* Cry protein in *Bt* 11, MON810, TC1507 and TC6275 corn crops.

iii. Plants

Since the active ingredient in this product is an insect toxin (*Bt* endotoxin) and EPA is unaware of demonstrated toxicity to plants, the plant toxicity studies have been waived.

iv. Non-target Invertebrates

All of the insect studies submitted in support of the registration of Cry1F used maximum hazard dose concentrations of poCry1F (TC1507) corn pollen. As shown in Section 2.b. of this assessment (Table 3), there is higher Cry1F protein expression in poCry1F corn pollen (21.9 ng/mg tissue) as compared to 3.67 ng/mg tissue in moCry1F (TC6275) pollen (dry weight tissue; Zabik *et. al* 2003). Therefore, the lack of discernible detrimental effects to non-target insects from poCry1F demonstrated in the studies below strongly suggests that there should also be no effects to non-target insects from moCry1F. Although moCry1F corn does have higher Cry1F protein expression than poCry1F corn in plant tissues most likely to be exposed to the soil dwelling Collembola and earthworms, the Collembola and earthworm studies conducted for poCry1F used concentrations of Cry1F protein that exceed those that would be seen from the proposed uses of either poCry1F or moCry1F corn. This suggests that the proposed uses of Cry1F protein in corn are not likely to have any measurable population effects on soil invertebrates including Collembola and earthworms.

1) Honey Bees

a) Cry1Ab

Feeding tests were conducted on both honey bee larvae and adults for Cry1Ab proteins. At a single dose of Cry1Ab, 20 ppm showed no adverse effects to larval honey bees under the test conditions. The NOEL for Cry1Ab was determined to be greater than 20 ppm. In adult honey bees no statistically significant differences were seen among the various treatment and control groups.

Concerns have been raised as to whether the honey bee larvae that were dosed with pollen containing Cry proteins were actually exposed to the proteins. The pollen has to be pre-digested by nurse bees (which, conversely, may also inactivate the Cry protein) in order to be palatable to larval honeybees (EcoStrat, 2000). However, small amounts of pollen are known to be fed directly by nurse bees in the hive (Winston, 1987). In addition, the Agency has other laboratory studies on file in which aqueous mixtures of purified Cry protein had been added to the diet of honeybee larvae maturing within honeycomb brood cells, or to a 1:1 (by volume) honey-water

mixture for adult honeybees. No adverse effect was observed in larvae or adults. This conclusion has been confirmed by hive studies in the field.

An adult honeybee study (Schur *et al.* 2000) was conducted as a semi-field study in Germany using field-grown *Bt* Cry1Ab corn plants, and honeybee colonies placed inside tents of plastic gauze placed over areas of the cornfields. Three replicate tents (1 colony/tent) containing *Bt* corn and three replicate tents containing non-transgenic corn were evaluated during the period of pollen shed, and the bee colonies were observed for an additional 30 days following pollen shed. The study showed no adverse effects of *Bt* corn pollen containing high levels of Cry1Ab protein on adult honeybee survival, foraging frequency, behavior or brood development during the 7-day period of pollen shed. Following the pollen exposure period, the hives were removed from the tents and observed for an additional 30 days for effects on brood development. No effects on brood development were associated with field exposure to *Bt* Cry1Ab corn pollen.

b) Cry1F

The reviewed capped honey bee brood cell study where larvae were fed Cry 1F corn pollen and pure Cry1F protein showed normal larval development and emergence of healthy adult honey bees. This study shows that at levels higher than the expected environmental exposure, the proposed use of Cry1F protein in corn is not likely to have any measurable deleterious effects on the honey bee (*Apis mellifera)*. The data showed no significant difference between treatment mortality or behavior change between the dosed and control replicates. As a result, no discernible detrimental effects to honey bees are expected from the proposed uses of the Cry1F-producing corn. The data adequately address potential toxicity concerns for foraging honey bees exposed to Cry1F protein expressed in corn pollen in the field. In addition, since corn is wind pollinated, few honey bees are expected to be exposed.

2) Lady Beetles

a) Cry1Ab

Lady beetle (*Hippodamia convergens*) predator toxicity studies submitted at the time of registration demonstrate that corn pollen containing the anti-lepidopteran Cry proteins do not cause detectable adverse effects to lady beetles. Purified Cry1Ab protein at 20 ppm also showed no adverse effects or behavior changes. The test insects were exposed to the active ingredient at approximately the dose that would be ingested by the beetles feeding on aphids under field conditions.

b) Cry 1F

Adult lady beetles (*Hippodamia convergens)* fed a concentration of *Bt* Cry1F protein at 15x the expected rate found in corn pollen resulted in no mortality or signs of toxicity over a 29 day period. Therefore, the NOEC was determined to be >15x the concentration of Cry1F found in pollen and the LC_{50} was determined to be > 480 ppm a.i (the test concentration). The submitted study shows that corn containing the Cry1F protein should not cause significant adverse effects

to lady bird beetle predators. The test insects were exposed to a dose of active ingredient approximating the amount that would be ingested by the beetles feeding on aphids under field conditions. As a result, no discernible beneficial beetle population effects are expected from the proposed uses of the Cry1F producing corn. This conclusion is confirmed by adult and larval lady beetle abundance found in the field census study. These studies adequately address potential concerns for Cry1F protein expressed in corn to beneficial beetles.

3) Parasitic Hymenoptera

a) Cry1Ab

No adverse effects were observed when a maximum hazard dose of 20 ppm Cry 1Ab was tested on *Brachymeria intermedia.* The NOEL therefore is greater than 20 ppm and no adverse effects to Hymenoptera are expected from exposure to Cry1Ab protein in the field.

b) Cry1F

Parasitic Hymenoptera *(Brachymeria intermedia*) fed a concentration of *Bt* Cry1F protein at 10x the expected rate found in corn pollen showed no mortality or signs of toxicity over a 12 day period. Therefore, the NOEC was determined to be >10x the concentration of Cry1F found in pollen. The LC_{50} was determined to be > 320 ppm a.i (the test concentration). As a result, no adverse effects to parasitic wasps are expected from field exposure to Cry1F protein producing corn. The conclusions are also confirmed by the parasitic wasp abundance found in a field census study submitted with the application.

4) Green Lacewing

a) Cry1Ab

The studies submitted to support the initial registration showed no significant adverse effects to green lacewing larvae at a maximum hazard dose of 16.7 ppm Cry1Ab protein in a 7 day feeding study. The NOEL, therefore, was greater than 16.7 ppm and no adverse effect to green lacewing was expected as a result of exposure to Cry1Ab protein at field concentrations.

Since that time, there have been several publications proposing that transgenic *Bt* plants may create serious impacts on non-target organisms that feed on pests exposed to the transgenic proteins. The reported harmful effects of *Bt* corn on larvae of the beneficial predatory insect green lacewing stem largely from the work of Hilbeck *et al*. (1998a 1998b, 1999). EPA performed a formal review of the first two studies on the effects of *Bt* corn intoxicated prey and pure *Bt* corn protein on lacewing (DP Barcode D236803 and D250457). If these laboratory results are taken at face value, the adverse effects are so slight as to suggest no significant impact on beneficial insects in the field.

Hilbeck *et al.* (1998a) reported slightly elevated mortality and prolonged development time in lacewing larvae reared on *Bt* intoxicated prey (the European corn borer - ECB). The authors

subsequently reassessed these results (Hilbeck *et. al.* 1998b) and reported that there are no significant reproductive effects from *Bt* corn protein. The authors concluded that "...surviving, unaffected *C. carnea* developed at rates similar to those in the untreated control" and "from this, we conclude that total developmental time until adult eclosion is not an appropriate parameter for detecting Cry1Ab protein effects." (Hilbeck, *et al.* 1998b). The second study (Hilbeck *et al.* 1998b) used defined quantities of pure *Bt* protein and there was significant mortality only in an *artificial* diet test group; no significant mortality was observed when the artificial diet was supplemented with *E. kuehniella* eggs (a natural diet). Therefore, this study does not demonstrate any adverse effects to lacewing larvae under simulated field feeding habits where the lacewing larvae have a choice of natural diet in the field. Moreover, in this study, the concentration of pure Cry protein to which the larvae were exposed was 100 micro grams /ml of diet and continuous, and therefore not reflective of Cry1Ab exposures that may occur under field conditions – either by exposure to plant tissues, pollen or by consumption of exposed prey species, such as ECB larvae. The dosage used in these studies is at least 30 times that found in most corn tissues in the field.

In a tritrophic study published in 1999 (Hilbeck *et al.*, 1999) an intermediate prey not susceptible to *Bt* was fed purified *Bt* protein in an artificial diet and then was presented to lacewing larvae. The study noted effects at no lower than 50 microgram levels, in contrast to the nanogram-level exposure which would be encountered in corn tissues in the field.

Generally, these findings do not show any detrimental effects at Cry protein exposure levels in the field. The laboratory results were seen only at exposure to microgram quantities, whereas in the field the exposure is only to nanogram amounts. In addition, any surviving ECB larvae would normally be within the corn plant most of their larval life and not available for consumption by chrysopids. (ECB larvae live within the corn stalk, not on stalk surface). The authors concluded that "...trials investigating predation efficiency and predator performance under field conditions are necessary before conclusions regarding the potential ecological relevance of the results presented in our paper can be drawn" (Hilbeck, *et al,* 1998b). Field studies have already been published on the effects of *Bt* crops on insect predators showing no significant differences in the density of beneficial insects, including green lacewings. In addition, Pilcher *et al.* (1997a) showed no significant differences in growth or mortality of *Coleomegilla maculata* (lady beetle), *O. insidiosus* (minute pirate bug), and *Chrysoperla carnea* (green lacewing) feeding on non-transgenic and *Bt*-expressing pollen in the laboratory.

b) Cry1F

Green lacewing larvae fed a concentration of *Bt* Cry1F protein at 15x the expected rate found in corn pollen resulted in no mortality or signs of toxicity due to feeding on Cry1F over a 13 day period. Therefore, the NOEC was determined to be >15x the concentration of Cry1F found in pollen and the LC_{50} was determined to be > 480 ppm a.i (the test concentration). These laboratory findings do not show significant detrimental effects and provide data that show a lack of risk to beneficial insects at Cry1F levels that will be encountered in the field use situation. These findings confirm published field studies on the effects of *B.t.* crops on insect predators showing no significant differences in the density of beneficial insects, including green lacewings.

The conclusions are also confirmed by the adult and larval green lacewing abundance found in a field census study submitted with the application.

5) Soil Invertebrates

The FIFRA Scientific Advisory Panel (USEPA 2001) does not believe that Collembola and earthworms are appropriate indicator species for Cry1Ab testing because of the Lepidoptera-specific nature of the Cry1Ab protein. When it initially reviewed the applications for the products that were registered in 1995, EPA considered requiring studies evaluating effects upon the representative beneficial soil invertebrates Collembola and earthworms. EPA was concerned (1) that such soil organisms may be subject to long-term exposure as a result of soil incorporation of crop residues (or when crop residues are left on the soil surface) and (2) that adverse effects on such soil organisms could result in an accumulation of plant detritus in fields. Recent reports of exudation of Cry proteins by corn roots throughout the growth season add to this concern. However, EPA understands that routine agronomic practices have included the long term use of chemical insecticides, which have adverse effects on soil organisms, but there has not been an accumulation of significant amounts of plant detritus in soils (Pimentel & Raven, 2000). Thus, *Bt* crops, which are expected to have less impact on these species than chemical pesticides, should not result in any increased build up of plant detritus or Cry proteins at toxic levels. Supporting this conclusion are data required by the EPA that indicate that such proteins are known to degrade rapidly in field soils. Therefore, significant soil buildup and effects to non-target soil organisms are not anticipated. This has been confirmed by Saxena and Stotzky (2001), who report that *Bt* Cry proteins released from root exudates and biomass of *Bt* corn has no apparent effect on earthworms, nematodes, protozoa, algae, bacteria, actinomyces and fungi in soil, in spite of the fact that enough detectable Cry protein is bound to soil particles to show toxicity to the target pest. These results suggest that despite its presence in soil, the Cry protein released in root exudates of *Bt* corn, or from the degradation of the biomass of *Bt* corn, is not toxic to a variety of organisms in the soil environment. Stotzky (2000) also reported that the same degree of *Bt* Cry protein persistence takes place in soils that have been exposed to repeat *Bt* microbial spray applications. In addition, new plants grown in *Bt* containing soil do not take up the *Bt* protein.

a) Earthworms

Earthworm feeding studies submitted to the Agency for all of the registered Cry proteins demonstrate that the Cry proteins are not toxic at the expected environmental concentration. Concerns have been raised as to whether the earthworms actually ingested the *Bt* Cry proteins when these are incorporated into the soil in the test systems used (EcoStrat, 2000). This question is mainly of academic importance. For hazard assessment purposes it is sufficient to know that the earthworms were not harmed when presented with the *Bt* Cry proteins in their soil environment. If they do not ingest it in the test soil, likewise they will not ingest it in the field. The earthworms do, however, ingest the *Bt* Cry proteins with the soil without harmful effects. Saxena and Stotzky (2001) reported that there were no significant differences in the percent mortality and weight of earthworms after 40 days in soil planted with *Bt* or non-*Bt* corn or not

planted, or after 45 days in soil amended with biomass of *Bt* or non-*Bt* corn or not amended. However, the toxin was present in both the casts and guts of the worms in these tests.

i) Cry 1Ab

The 14-Day LC_{50} value for earthworms exposed to Cry1Ab insecticidal protein derived from *E. coli* in an artificial soil substrate was determined to be greater than 200 mg/kg (ppm), which was the single concentration tested. There were no statistically significant effects at the single dose tested. Although this study was graded supplemental, *Bt* Cry1Ab proteins expressed in the corn plant are not expected to generate a toxic effect in the earthworm; therefore, in light of recent recommendations by the FIFRA Scientific Advisory Panel (USEPA, 2001) that invertebrates known not to be affected by the Cry proteins specific for insects not be tested, no additional follow-up of this study is required.

ii) Cry1F

The submitted data show that Cry1F protein has no measurable deleterious effects on earthworms, a representative beneficial soil invertebrate species. This suggests that the proposed uses of the Cry1F protein in corn are not likely to have any measurable population effects on beneficial soil invertebrates. The one limit test concentration of 2.26 mg Cry1F/kg dry soil represented more than 100X the estimated concentration present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants. This concentration is higher than any amount of Cry protein that may be present in the soil during any stage of the growing season (such as from root exudation). Based on the results of this study, Cry1F transgenic corn plantings will have no adverse effects on earthworms.

b) Collembola

i) Cry1Ab

Monsanto's original application for registration included a study on Collembola exposed to 200 ppm of Cry1Ab proteins derived from *E. coli*. The study showed no adverse effects, but EPA classified the study as supplemental because the test substance was not leaf tissue containing Cry1Ab. Subsequently, Monsanto submitted a new study using lyophilized corn leaf tissue containing the Cry1Ab protein in the MON810 corn line. The estimated concentration of Cry1Ab protein was 50.6 µg/g in lyophilized tissue and 6.27 µg/g in fresh tissue. The control substance was lyophilized leaf tissue from the non-transgenic corn line MON 823 which has a genetic background similar to the MON 810 line but does not carry the gene responsible for the Cry1Ab protein. Test substances included corn powder at 0.5, 5.0, and 50% of the diet. Mortality was assessed every 7 days for the duration of the 28-day test. Additional observations were also made with respect to growth, egg production, and egg hatch. For the corn powder treatments and controls, no mortalities occurred in the treatment or control groups. Likewise, there was no significant difference in reproduction between the treated group and either control group. The study was scientifically sound and no treatment mortality or behavior change was

observed between the dosed and control replicates. The study also showed that at field use rates reproduction of the test insects will not be impaired.

The Collembola studies submitted to the Agency for most of the registered Cry proteins showed no adverse effects at maximum hazard doses. Novartis (now Sygenta) had cited the MON 810 leaf tissue study to support their Bt11 corn plant-incorporated protectant. While this study is useful in characterizing effects of Cry1Ab corn tissue on Collembola and satisfies the requirement for MON 810 Cry1Ab corn, it does not adequately characterize the effect of Bt11 corn tissue on Collembola. The requirement for a Collembola study which includes control plant lyophilized leaf tissue from non-transgenic parental corn lines and lyophilized leaf tissue containing the Bt11 plant-incorporated protectant is not fulfilled. However, in light of recent recommendations by the FIFRA Scientific Advisory Panel (USEPA, 2001) that invertebrates known not to be affected by the Cry proteins specific for insects of different orders not be tested, this requirement can be waived.

ii) Cry1F

Since Collembola feed on decaying plant material in the soil, they may be exposed to Cry1F protein in corn found in the field. A study was conducted to determine if there may be adverse effects of Cry1F on Collembola. The study is scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates after 28 days. The results of this study indicate that at levels that would reasonably be expected to be found in the field, collembola were not affected by chronic exposure to Cry1F protein. The exposure rates in this study are 1560-, 388-, and 79-fold higher than the expected field concentration. The reviewed data show that *Bacillus thuringiensis* Cry1F corn protein has no measurable deleterious effects on collembola (*Folsomia candida*), a representative beneficial soil insect species. This indicates that the proposed uses of the Cry1F protein in corn are not likely to have any measurable population effects on beneficial soil insects.

As discussed above, EPA does not believe that to date there are any valid data demonstrating specific adverse impacts of plants expressing *Bt* Cry1 proteins on beneficial soil invertebrates. To the contrary, EPA believes that available scientific data and information indicate that cultivation of *Bt* crops has a positive effect on soil flora, when compared to the most likely alternative, use of non-selective synthetic chemical pesticides.

6) Non-Target Insect Abundance Studies

Data available to date indicate no difference in the number of total insects or the numbers of specific orders between the transgenic crop plots and either the isogenic or wild type control crops. No shift in the taxonomic distribution of insects was seen, except in cases where the predators are dependent on the pest insect as prey as their major food source.

Pilcher *et al.* (1997b) conducted limited size field studies in two consecutive years with *Bt* corn. No differences were observed in the number of predators colonizing either isogenic control corn or *Bt* corn in 1994. In 1995 more predators were seen on *Bt* corn than on control corn. The

authors concluded that *Bt* corn pollen did not affect predator abundance. They also concluded, however, that the absence of significant differences may have resulted from plot size. Due to the small plot sizes separated by only one buffer row, pollen from *Bt* corn and isogenic corn may have been mixed by wind. They concluded that the inconsistent results between the two years indicate that larger scale studies are necessary for significant data.

Orr & Landis (1997), studied the oviposition of European Corn Borer (Lepidoptera: Pyralidae) and impact of natural enemy populations in transgenic high pollen level Cry1Ab versus isogenic corn. No significant differences in *O. nubilalis* egg populations, or its predators or parasitoids were observed. Mortality factors exerted by predators were consistent in all plots. The corn type did not appear to have an impact on these factors. Larval parasitism was not significantly different and therefore probably density-independent.

Obrycki (1997) performed a study to determine the effects of transgenic corn expressing *Bt* Cry protein on the abundance of predatory insects in corn fields. He found that the average number of predatory insects was not significantly different between the sprayed and unsprayed plots on four of the five observation days over a seven week period. Conventional pesticide spray drift was suspected as the reason. No significant difference in abundance was found between the *Bt* and the non *Bt* plots on any of the five observation days. Similar numbers of Coccinellid eggs, larvae, and pupae were observed on the transgenic and the non-transformed corn plants. Higher numbers of Chrysopid eggs, *Orius insidious* (Anthocoridae), Nabidae, and Arachnida were observed on the *Bt* corn, but not at statistically significant levels.

Lozzia (1999) reported a biodiversity and structure of ground beetle assemblages (Coleoptera Carabidae) trial in Cry1Ab *Bt* corn and the effects on non-target insects conducted in four trials in North Italy over a 2-year period. No statistical difference was evident in the total number of carabids. There was no decreasing trend in the biodiversity indices from the first to the second year and considering the data as a whole, the two years appear comparable. The difference in biodiversity recorded for some indices was not due to the presence of transgenic corn. The aerial fauna as a whole for both years and both localities was not different. Similarly, abundance of aphids, leaf hoppers, other Homoptera, thrips, leaf beetles, spiders, lady beetles, parasitic Hymenoptera, other Hymenoptera, and Diptera was not different. The number of arthropods was higher, but not significantly, in the transgenic corn. Several sampling methods and visual checking showed that there was no significant difference in abundance, composition or biodiversity of non target arthropods in isogenic and transgenic corn crops. The data show that the transgenic plants do not lead to an increase or decrease of any insect populations. It appears that Cry1Ab proteins do not directly affect the phytophagous species nor do they have "any indirect influence on other trophic levels or activities such as behavior, oviposition or predators-prey."

Nuessly & Hentz (1999) conducted 4 studies using Novartis (now Sygenta) Seeds' Attribute Bt11-derived Cry1Ab sweet corn hybrids and conventional sweet corn hybrids grown under local practices in four Florida locations. Noted in the reports were increases in species diversity in the corn plots, i.e. there were generally higher populations of beneficial and non-target insects as

compared to the conventional control plots, associated with the significantly decreased use of broad-spectrum insecticides (organophosphates, carbamates and synthetic pyrethroids).

Three single-year field surveys of non-target invertebrates in *Bt* Cry1F corn have been submitted to EPA, the results of which are summarized in the table below:

TABLE 6. Field surveys of non-target invertebrates in *Bt* Cry1F corn.

Study	# Years	Study Design	Taxa Collected	Top Line Results
Field survey of beneficial arthropods associated with *Bt* Cry1F maize (Higgins 1999) **MRID No. 450201-13**	1 year 1999	**TC1507 & 1360:** 2 treatments: Cry1F and non-*Bt* near isoline; plot size: 28 x 4 m; 1 site (Iowa) 4 reps; sampling: visual and sticky trap observations before, during, and after anthesis	lady beetles, predacious beetles, lacewings, insidious flower bugs, assassin bugs, damsel bugs, parasitic wasps, damsel or dragonflies, and spiders.	Cry1F maize lines had no effect on the presence of beneficial arthropods compared with non-*Bt* near isolines; generally, TC1507 showed larger numbers of beneficial insects
Field study of non-target arthropods associated with *Bt* var. aizawai Cry1F maize **MRID No. 456520-01**	1 year (2000)	**TC1507:** 3 treatments: Cry1F, non-*Bt* near isoline, and non-*Bt* near isoline treated with foliar insecticide (lamba-cyhalothrin); plot size: 24 x 6 m; 1 site (France) 4 reps; sampling: visual observations 7X during growing season	thrips, insidious flower bugs, leafhoppers	Cry1F showed no significant impact on the non-target arthropods. Insecticide treatment significantly reduced non-target arthropod populations
Field survey of non-target arthropods associated with *Bt* Cry1F maize **MRID No. 456480-01**	1 year (2001)	**TC1507:** 2 treatments: Cry1F and non-*Bt* near isoline; plot size: 3.5 A; 1 site (Iowa); sampling: visual and sticky trap observations before, during, and after anthesis	community census	Abundance of key taxa showed no consistent reduction in the Cry1F field, although fewer parasitic hymenoptera were observed.

In October 2001, EPA approved amendments to the *Bt* corn registrations of Agricultural Biotechnology Stewardship Technical Committee (ABSTC) member companies (EPA Reg. Nos. 524-489, 67979-1, 65268-1, 29964-3 and 68467-2), which extended the registrations of these products until October 2008. As a condition of these registrations, EPA required the registrants to either submit existing studies submit a protocol for new field survey studies. On March 15, 2002, a report titled "Field Surveys of Non-Target Invertebrate Populations in *Bt* Corn" was submitted on behalf of the Non-Target Organism Subcommittee (NTO Subcommittee) of the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) members (MRID No. 45652001). EPA reviewed this submission and indicated that final reports of the in-progress studies and the results of any additional research should be submitted at a later date.

On March 15, 2006, the NTO Subcommittee submitted the document "Field Studies of Non-Target Invertebrate Populations in *Bt* Corn" (MRID No. 467846-01). This submission included complete references and additional information that have since become available. Most papers focused on large-scale field studies using transgenic corn, including discussions of study design and non-target invertebrate populations. EPA reviewed the submitted information and, overall, the published literature did not report any consistent adverse impacts on non-target invertebrates as a result of multi-year commercial *Bt* corn cultivation. Slight reductions in some invertebrate predator populations were seen; however, these are an inevitable result of all pest management

practices which tend to result in reductions in the abundance of the pests as prey. The continually expanding body of literature provides EPA, academia, and the public with a better understanding of the impact of transgenic crops on non-target organisms and provides useful information and considerations for those conducting large-scale field studies.

As anticipated, there are reports of *Bt kurstaki* Cry protein toxicity to some lepidopteran species in isolated, high dose laboratory studies. At present, however, EPA is aware of no identified significant adverse effects of *Bt* Cry proteins on the abundance of non-target beneficial organisms in a population in the field, whether they are pest parasites, pest predators, or pollinators. Published field testing results and field scouting data submitted to EPA show minimal to undetectable changes in the beneficial insect abundance or diversity. Results indicate no difference in the number of total insects or the numbers of specific orders between the transgenic crop plots and either the isogenic or wild type control crops when these are grown without chemical pesticide treatment. In commercial fields densities of predatory and non-target insects are generally higher on *Bt* crops than non-*Bt* crops primarily because the *Bt* crops are not subjected to the same number of applications of nonspecific pesticides. Generally no shift in the taxonomic distribution of insects was seen in *Bt* crops, 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 insect populations. However, yearly insect census estimates from representative fields will continue to be required.

7) Non-Target Lepidoptera

The toxicity of *Btk* to butterflies is a well known and a widely published phenomenon. For the purpose of its risk assessment of *Bt* plant products, EPA accepted that *Bt* proteins could be toxic to Lepidoptera and relied exclusively on lepidopteran exposure to *Bt* Cry protein. Since the exposure to butterflies and moths from the agricultural uses of *Bt* was not expected to be as high as in forest spraying (where no widespread/recurring or irreversible harm to lepidopteran insects was observed), *Bt* crops likewise were not expected to cause widespread or irreversible harm to non-target lepidopteran insects. Published preliminary data of toxicity of high doses of *Bt* to monarchs in the laboratory do not translate into exposure to toxic levels in the field. In light of the recent reports expressing concern for monarch conservation efforts, however, this conclusion has been reevaluated and much research effort has been devoted to this issue.

The weight of evidence of the published and recent research data reviewed indicate that milkweeds in the corn fields and to within 1 meter of cornfields are unlikely to be dusted with toxic levels of *Bt* pollen from the currently registered *Bt* corn varieties, MON810, Bt11 and TC1507. In addition, the distribution of corn pollen within and outside of corn fields, the distribution of milkweeds within corn habitat and other types of habitat, monarch oviposition and feeding behavior, limited temporal overlap between monarch larvae and pollen shed (and similar issues) in much of the corn growing regions of the United States indicate a low probability of demonstrable adverse effects of *Bt* corn pollen on monarch larvae.

Based on the review of the submitted DCI data, the Agency concludes that the published monarch toxicity information is not sufficient to cause undue concern of harmful widespread effects to monarch butterflies at this time. In the event that continuing studies demonstrate a substantial reduction in monarch butterflies attributable to *Bt* corn pollen, especially as the percentage of *Bt* corn planting increases, and should new data indicate unanticipated risks to other non-target Lepidoptera, particularly risks to threatened or endangered species, the Agency will institute appropriate risk management practices. The following is a discussion of the studies available to date on the field toxicity of Cry1 proteins to non-target Lepidoptera species.

a) Black Swallowtail Butterfly

Wraight *et al*. (2000) performed a field study to assess whether mortality of early instar black swallowtails was associated either with proximity to a field of Cry1Ab *Bt* corn or with levels of *Bt* pollen deposition on host plants. Potted host plants were infested with first instar black swallowtails and placed at intervals from the edge of a field of *Bt* corn (MON 810). There was no relationship between mortality and proximity to the field or pollen deposition on host plants. Moreover, pollen from these same plants failed to cause mortality in the laboratory at the highest pollen dose tested (10,000 grains/cm^2), a level that far exceeded the highest pollen density observed in the field (200 grains/cm^2). The authors conclude that *Bt* pollen of the variety tested is unlikely to affect wild populations of black swallowtails.

b) Karner Blue Butterfly and Other Threatened or Endangered Species

In the preliminary BRAD EPA concluded that there was a possibility that off-site pollen flow from *Bt* corn fields might potentially have adverse effects on federally listed threatened or endangered Lepidoptera because of the selectivity of Cry1 proteins for certain lepidopteran species. EPA noted, however, that the majority of listed lepidopteran species have very restricted habitat ranges. Examination of an overlay map showing the county level distribution of lepidopteran species relative to corn production counties in the US as listed by the U.S. Fish and Wildlife Service (USFWS, 1997) shows that as a rule, listed lepidopteran species do not occur in agricultural areas where corn is grown nor is corn considered a host plant for these species. The map clearly indicates that any potential concern regarding range overlap with corn production is restricted to the Karner blue butterfly *(Lyceides melissa samuelis)*. The butterfly is found along the northern extent of the range of wild lupine (its host plant), where there are prolonged periods of winter snow pack, primarily in parts of Wisconsin, Michigan, Minnesota, Indiana, New Hampshire and New York. Wild lupine grows on dry, sandy soils in pine barrens, oak savannah, forest trails and previously disturbed habitats such as utility rights-of-way, military installations, airports, highway corridors, sand roads and abandoned sand pits (US Fish and Wildlife Service, 2000a, 2000b). No corn is grown in the area in New Hampshire where Karner blue butterflies are found.

EPA concluded that because Cry1 proteins are broadly active against Lepidoptera, some activity against the Karner blue would not be surprising. Toxicity testing of Karner blue larvae directly, however, is not possible due to its endangered status. Previous studies that tested the

susceptibility of lepidopterans to Cry1 proteins resulted in different LC_{50} values for different species in the same genus. For example the Cry1Ab LC_{50} for *Spodoptera exigua* is estimated at 3,180 ng/mL diet and 95,890 ng/mL diet for *Spodoptera frugiperda* (Luttrell *et al*. 1999). Herms *et al*.(1997) performed a study demonstrating that the Karner blue larvae were susceptible to a formulated microbial *Bt* product based on the *Bt kurstaki* HD-1 strain that contains Cry1 proteins. Therefore, it can be assumed that the Karner blue may be susceptible to Cry1Ab, and perhaps to Cry1F, if levels of toxin in ingested pollen are high enough to adversely affect Karner blue butterflies. But, Herms *et al*. (1997) also showed that the Karner blue has susceptibility similar to the gypsy moth to a microbial *Bt* formulation containing Cry1 proteins. Since the gypsy moth is known to be less susceptible to Cry1Ab protein than European corn borers, and levels of *Bt* pollen found in the field are not toxic to European corn borers (the target pest), levels toxic to the Karner blue are also not expected. Nonetheless, to be as protective as possible with respect to any potential effects on this endangered species, EPA in its preliminary BRAD and at the October 2000 SAP considered whether registrations of *Bt* corn could potentially affect the Karner blue. EPA also initiated contacts with the Fish and Wildlife Service to obtain information helpful to the Agency in assessing whether the *Bt* corn registrations could actually have an impact on the Karner blue. In addition to interacting with the FWS, EPA continued to receive data and information, and to refine its analyses of whether the *Bt* corn registrations could affect the Karner blue.

The Karner blue requires wild lupine *(Lupinus perennis)* as an oviposition substrate and larval food source. In considering the potential risk of Cry1Ab and Cry1F proteins to Karner blue larvae, key issues to be addressed are: (1) whether the amount of corn pollen shed from *Bt* corn fields onto wild lupine would constitute a hazard to the Karner blue; (2) whether there are wild lupine growing in the areas immediately adjacent to corn fields that are reestablished from fallow fields; (3) the extent of transport of corn pollen shed from corn fields; and (4) whether there is overlap between the period of pollen shed from corn fields with the period of Karner blue larval emergence.

Hazard to Karner Blue. The Agency considers the most sensitive species tested to be a useful indicator of potential effects on endangered or threatened species. During the time period since EPA determined preliminarily that *Bt* corn could potentially affect Karner blue, the Agency has received and obtained additional data. These data have enabled the Agency to conduct an ecological risk assessment for potential impacts to the Karner blue. Following EPA's standard procedures for ecological risk assessment for endangered species, no effect is expected if there is a safety factor of 10X between the estimated environment concentration (EEC) of the pesticide and the LC_{50} or LD_{50} to the most sensitive species tested (USEPA, 1986). As described below, EPA has determined the ratio between the EEC and LD_{50} on the most sensitive species tested for Cry1Ab and Cry1F pollen protein.

Toxicity of pollen from the currently registered Cry1Ab *Bt* corn products to Karner blue larvae is estimated to be very low. At least 12 lepidopteran species have been tested to determine LC_{50} levels for Cry1Ab (MRID 455122-00). The most sensitive species tested is the monarch butterfly. Researchers have determined that the concentration producing no mortality whatsoever is greater than (>) 4000 pollen grains/cm^2 of leaf surface (Hellmich, *et al*, 2001).

Thus the actual LD_{50} for monarchs is likely to be substantially higher. Since the EEC is 300 pollen grains /cm^2 or less at the field edge, and 200 at one meter and 75 at three meters,[9] the ratios of the EEC/LC_{50} (with >4000 pollen/cm^2 as that LC_{50}) have been conservatively calculated to be 1:>13.3 for Cry1Ab at the field edge, 1:>20 at one meter and 1:>53 at two meters from the field edge (Vaituzis, *et al*, 2001).

Similarly, toxicity of pollen from the currently registered Cry1F *Bt* corn products to Karner blue larvae is estimated to be very low. For Cry1F, at least 16 lepidopteran species have been tested to determine LC_{50} levels (Wolt, 2000). The most sensitive species tested is the diamondback moth. Because no data were submitted on toxicity of Cry1F in corn pollen, the LC_{50} obtained on the diamondback moth with pure cry1F protein was converted to an LC_{50} in terms of pollen grains and compared to the EEC in terms of the amount of Cry protein per gram of leaf tissue at the 300 pollen grains/cm^2 (of leaf tissue) level in the field. The ratios of the EEC/LC_{50} have been calculated to be 1:172 for Cry1F at the field edge, 1:263 at one meter and 1:690 at two meters from the field edge. (Vaituzis, *et al*, 2001).

Overlap of wild lupine and corn. Based on its assessment of all relevant data and information, EPA has determined that the potential exposure of Karner blue to *Bt* corn pollen is limited because corn and lupine do not generally overlap. Wild lupine does not occur at all in corn fields. Moreover, wild lupine is not expected to grow adjacent to corn fields But, in one case brought to the attention of EPA, farm land can be taken out of production for conservation purposes in Wisconsin. Where farmland is taken out of production, and fields allowed to lie fallow, wild lupines might invade such fields. In these instances, it is possible that the Karner blue could be present on such lupines. When EPA initially began the *Bt* reassessment, the Agency was concerned that lupines occurring adjacent to such reestablished corn fields could potentially contain Karner blue larvae that could possibly be adversely affected by pollen shed from *Bt* corn. EPA has received information that indicates that the Karner blue is not expected to occur in proximity to such reestablished fields. The Wisconsin Department of Natural Resources states that most agricultural operations do not support habitat for the Karner blue, nor present a threat to the continued existence or recovery of the Karner blue in Wisconsin. Wisconsin Statewide Karner Blue Butterfly Habitat Conservation Plan and Environmental Impact Statement, Wisconsin Department of Natural Resources (2000) (http://www.dnr.state.wi.us/org/land/er/publications/karner/karner.htm). Moreover, while EPA received a comment to the effect that "[t]he Karner Blue is documented as occurring adjacent to corn fields", examination of the cited reference proves the opposite.[10]

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[9] The data collected for the DCI provide a deposition curve of pollen distribution outside a corn field. A conservative estimate of about 300 pollen grains (frequency of occurrence 0.017) per square centimeter is found at the edge of a corn field and the levels drop off rapidly within a few meters of the corn field edge (Pleasants, *et al*, 2001).

[10] A commenter cited Andow, *et al*. (Andow., D.A., Lane, C.P., D.M. Olson, *Use of Trichogramma in Maize - Estimating Envt'l Risk*, in H.M.T. Hokkanen and J.M. Lynch, *Biological Control Benefits and Risks* (Cambridge U. Press 1995)) in support of the proposition that the Karner blue is "documented as occurring adjacent to corn fields." Examination of this paper on *Trichogramma* demonstrates that the brief discussion of the Karner blue does not support the stated proposition. What the paper does state is that: "the Karner blue [is] known to occur in counties of Minnesota where maize is widely grown." Hokkanen, p. 102. The Karner blue "occur[s] in sites near agricultural fields." *Id.* (citing personal communication). "The Karner blue is a specialist feeder on wild lupine (Lupinus perennis Fabaceae) in sandy soils intercalated in the oak savannah habitat near the Mississippi River in Minnesota. This area is surrounded by agricultural lands." *Id.* Given the limited extent of pollen transport when shed from corn fields, EPA considers the term "adjacent," when applied to corn fields in this context, to be most appropriately considered as 0-3 meters from the field edge. EPA does not agree that any of the quotations identified in the Andow paper is supportive of the statement that "[t]he Karner Blue is documented as occurring adjacent to corn fields." (Emphasis supplied). Of greater interest is a very brief section of the Andow paper entitled *Actual distances to Karner blue habitats*.

Overlap of corn pollen shed and larval emergence. Karner blue larvae are relatively less likely to be feeding during, or following the whole period of corn pollen shed. An analysis of pollen shed overlap with Karner blue larvae has been submitted to EPA and reviewed (MRID 455129-01). This report indicates that there are 35 counties where Karner blue butterflies are found and corn is grown. EPA has received additional data from U.S. FWS indicating that there are 38 counties where the Karner blue is found and corn is grown. For 11 of these counties, no overlap of pollen shed for certain hybrids and Karner blue larvae is expected. For other counties, the possible overlap does not happen every year nor for more than a day or two in the life of the feeding larvae. For example, these data show that in some instances there might be one day of overlap every seven years. In addition, if pollen does fall on wild lupine plants, the studies done on corn pollen shed for the monarch butterfly Data Call-In (DCI), have shown that rain and wind remove large amounts of pollen. *Bt* protein in corn pollen also degrades relatively rapidly in sunlight. (Pleasants, *et al*, 2001). The rapid removal of corn pollen from plant leaves, and the rapid degradation of *Bt* endotoxin in corn pollen reduces the likelihood that Karner blue larvae will encounter *Bt* endotoxin.

Thus, on the basis of new data and information received and obtained on the potential impact of *Bt* corn on Karner blue, EPA has conducted an ecological risk assessment using the best data available, and determined that there will be no effect on the Karner blue from the *Bt* corn registrations. This determination is based on a number of factors including (1) if wild lupine were to grow adjacent to *Bt* corn fields, the amount of corn pollen shed from such fields onto the wild lupine would be insufficient to constitute a hazard to the Karner blue; (2) relevant data and information indicate that there will be relatively little, if any, wild lupine growing in the areas immediately adjacent to corn fields that are reestablished from fallow fields; (3) the amount of corn pollen shed from corn fields to adjacent areas is low; (4) available data suggest that there may be limited overlap between the period of pollen shed from corn fields with the period of Karner blue larval emergence.

As with all aspects of these registrations, however, EPA will continue to evaluate *Bt* corn agricultural practices, ongoing research, and endangered and threatened species implications, and will continue working with other Federal and State agencies as new information becomes available.

In its entirety, this section states:

> We have conducted intensive surveys of the distribution of Karner blue in Winona county. The five Karner blue habitats in the area are only 0.5-0.9 km (mean 0.66 km) from the nearest agricultural field. A more vivid picture of the proximity of Karner blue habitat to agricultural land is illustrated in fig. 10.8, which shows one of the larger populations of Karner blue in Minnesota (each square indicates the location of at least one Karner blue adult in its typical habitat of oak savanna woodlands. This population is only 0.6 km from the nearest agricultural field, which has been planted with maize. These data provide further evidence that the potential risk from releases of *T. nubilale* is not negligible.

Id. at 111-12. Thus, "intensive surveys" by Andow of the Karner blue in Minnesota demonstrates that the five Karner blue populations identified exist from 500 to 900 meters from the nearest agricultural lands (with a mean distance of 660 meters). Given that EPA considers that the relevant data on corn pollen transport supports a finding that "adjacent to corn fields" should be considered as the area from 0-3 meters from the field, EPA does not consider these data to support the proposition that the Andow paper "documents" that the Karner blue "occur[s] adjacent to corn fields."

EPA has also determined that there are no indirect effects on endangered and threatened plant species, such as impacts on lepidopteran pollinators that are important and/or essential to an endangered or threatened plant. Working with U.S. FWS, EPA has identified an endangered bog orchid that is pollinated by a non-endangered hawk moth. While some hawk moths might be found in and around corn fields, they feed and oviposit on numerous plant species. Therefore, exposure of the hawk moth to *Bt* endotoxin is expected to be low. Moreover, even if the hawk moth is susceptible to Cry1 proteins, the number of hawk moths exposed to a lethal concentration should be insignificant to negligible based on the toxicity analysis for the most susceptible species as discussed above. Therefore, EPA determines that exposure to *Bt* corn will not sufficiently suppress the pollinator to affect the endangered plant species.

c) Monarch Butterfly

In 1999, *Bt* corn registrants submitted two research reports (DP Barcode D255949) to EPA on potential effects of *Bt* corn pollen on monarch butterflies (*Danaus plexippus* Linneaus): Losey, *et al* (Cornell) and Jesse and Obrycki (Iowa State). In the Losey *et al*. study, pollen collected from *Bt* (Bt11 N4640 *Bt* corn) corn was applied by gently tapping a spatula of pollen over milkweed leaves *(Asclepias syriaca* Linnaeus) which had been lightly misted with water. Pollen density was set to visually match densities on milkweed leaves collected from corn fields. Five three-day-old monarch larvae from a captive colony were placed on each leaf. The larvae reared on leaves dusted with pollen from *Bt* corn ate less, grew more slowly, and suffered higher mortality than larvae reared on leaves dusted with untransformed corn pollen or leaves without pollen. Larval mortality after 4 days of feeding on leaves with *Bt* pollen was significantly higher (44%) than the mortality either on leaves dusted with untransformed pollen or on control leaves with no pollen (both 0%).

Jesse & Obrycki used *Bt* field corn pollen (Event 176) covered leaf samples taken from within and at the edge of corn fields (80-217 pollen grains/cm^2) to assess mortality. The samples were fed under laboratory conditions to monarch butterfly first instar larvae. The authors found a 19% mortality in larvae feeding on the *Bt* corn pollen treatment from leaves within and at the edge of the corn field within 48 hours, compared to 0% on non-*Bt* corn pollen exposed plants and 3% in the no pollen controls.

These reports were reviewed by the Agency. The reviews concluded that the preliminary controlled studies without exposure data are not conducive to conventional risk assessment procedures for *Bt* corn pollen effects on monarch butterflies without additional field study information. The reports of *Bt* corn pollen toxicity to monarch caterpillars did, however, result in a number of steps taken by the Agency to more fully assess and understand the possible effects of transgenic corn expressing an insecticidal protein from *Bacillus thuringiensis* (*Bt* corn) on non-target lepidopteran species, particularly monarch butterflies (*Danaus plexippus*). To help identify the level of exposure and other risks to monarch butterflies, on December 15, 1999 EPA issued a monarch butterfly adverse effects data call-in (DCI) notice to the registrants of *Bt* corn products under its FIFRA Section 3(c)(2)(B) authority. On December 9, 1999 (USEPA, 2000), and again on October 18-20, 2000 (USEPA, 2001), the Agency presented current and possible new data requirements to evaluate ecological effects, including the monarch question, to a

FIFRA Scientific Advisory Panel for their recommendations. In addition, EPA consulted with monarch butterfly experts and USDA to better understand the effect of *Bt* corn pollen on monarch butterflies. Until more definitive data and information were available about the potential risks of *Bt* corn pollen to monarch butterflies and other lepidopterans, EPA requested that registrants instruct their customers who are planting non-*Bt* corn refuges (for resistance management) to place the non-*Bt* corn refuge between *Bt* corn and habitats such as prairies, forests, conservation areas, and roadsides as a precautionary measure. However, in light of the recently reviewed DCI research data showing that monarchs appear to breed on milkweed inside corn fields and that toxic levels of *Bt* pollen do not accumulate outside corn fields, this recommendation no longer appears necessary.

The DCI called for information in five basic areas relating to the potential exposure of non-target lepidopterans, particularly monarchs to *Bt* corn pollen. These include: the distribution of monarch butterflies, milkweed plants and corn; corn pollen release and distribution in the environment; toxicity of *Bt* corn Cry proteins and *Bt* corn pollen to lepidopterans; monarch egg laying and feeding behavior; and monarch population monitoring. The Agency has reviewed the submitted DCI data and incorporated the findings into this reassessment. The DCI data is a result of an ABSTC and USDA coordinated research effort, with additional research by independent university and government workers.

Subchronic toxicity studies conducted since the DCI was initiated have shown that monarch larvae feeding on corn pollen expressing MON 810, Bt11, TC1507 or TC6275 at pollen levels found in corn fields do not demonstrate observable adverse effects on survival, weight, or other fitness parameters (e.g., developmental change, weight gain, percent survival to pupation, pupal weight (mg), percent emergence from pupae, adult weight (mg), or adult wing length) (Stanley-Horn *et al*. 2001). Risk from other factors such as destruction of over wintering habitat, weather, predators, physiological stress, human activity (Taylor 1999) and conventional chemical insecticide use (Stanley-Horn *et al*. 2001) are a much greater and more widespread threat to monarch populations than the use of *Bt* corn. The potential reduction of insecticide use that may result from planting *Bt* corn will most likely benefit monarch populations as well as other beneficial insects, especially in popcorn and sweet corn production.

The submitted data demonstrated that levels of MON 810 and Bt11 corn pollen toxic to monarchs would probably not occur under natural field conditions. The mean pollen density of all the studies was found to be 170 inside the corn fields and 63 grains/cm2 at the edge. The highest average corn pollen densities monitored in the field were 586 in Maryland (Stanley-Horn *et al*. 2001) and 900 grains/cm2 found in one Iowa corn field (Pleasants *et al*. 2001). In a worst case scenario, pollen deposition when no rainfall occurred was approximately 1400 grain/cm2 (Pleasants *et al*. 2001). Research conducted in response to the DCI showed that Cry1Ab corn pollen densities of >4000 grains/cm2 do not show mortality to monarchs (Hellmich *et al*. 2001). These studies have also shown that the order of monarch sensitivity to Cry proteins is Cry1Ab > Cry1Ac > Cry9C > Cry1F. Only pollen from Event 176 corn has been shown to adversely affect growth, fitness, and mortality of monarch butterflies (Losey *et al*. 1999, Jesse and Obrycki 2000, Stanley-Horn *et al*. 2001). However, this does not create a concern for monarchs since Event

176 corn comprises less than 2% of U.S. corn acreage and will no longer be sold after the 2003 growing season.

In the report of their two year study, Stanley-Horne *et al*. (2001) suggested that percent monarch adult emergence warranted further investigation. Also, the field studies recording that Bt11 or MON810 pollen had no effect on survival of monarch larvae for 14 to 22 days also needed further analysis. In addition, the authors also noted that these studies did not address chronic, long-term exposure of monarch larvae throughout their development cycle to determine the subtle effects of prolonged exposure to *Bt* toxin (Sears *et al*. 2001 and Stanley-Horne *et al*. 2001).

As a result of these conclusions, EPA included a statement in the 2001 EPA Registration Action Document for *Bt* corn that studies on long-term exposure of monarch larvae to *Bt* pollen should be considered. Researchers at the University of Maryland (Dively, *et al*. 2004) conducted such studies from 2001-2002, and the results were submitted jointly to EPA by Monsanto Company and Syngenta Seeds, Inc. in 2003. The study included the results of a risk assessment simulation model that projected, over the range of the Corn Belt, an increased risk of 0.6% in mortality to monarch larvae from prolonged exposure to *Bt* corn pollen. The increased risk to monarch larvae when considering all of North America was 0.3%. Based on these criteria the Agency concludes that, given the other natural and man-made hazards to the monarch populations which account for up to 90% annual monarch mortality without affecting the overall monarch abundance, a 0.3% increase in mortality due to Cry1Ab *Bt* corn pollen will not pose an unreasonable risks to the continued existence of monarch populations in North America. A full summary of the study is included below in subsection 4 (*Bt* pollen exposure and toxicity).

1) Monarch habitat

A baseline monarch population level cannot be reasonably developed. It is difficult to develop a baseline population level using current methodology and because the number of monarchs throughout the U.S. fluctuates between regions and years. There are several factors such as catastrophic weather (e.g., drought or floods) that may adversely affect monarch population size. However, monarch populations may recover from catastrophes as is evidenced by the large number of monarchs counted in 1994, the year after floods in the midwest. On the other hand, warm summers result in increased population size in North America and decreased numbers during cold summers. Among other factors that may affect monarch population size are: (1) overwintering site depletion, (2) number and fitness of monarchs that overwinter, (3) nectar availability to adults, (4) pathogens, parasites, parasitoids, and predators, (5) milkweed availability, (6) use of insecticides to control lepidopteran pests, and (7) accidents (e.g., collision with automobiles). Due to these factors, it is difficult to develop a baseline population size or to determine if *Bt* corn pollen was a contributing factor.

There have been several attempts made to determine monarch population levels. Swengel (1995) showed that from 1986-1994 there were significant changes in monarch counts including increases and decreases from five of eight year-pairs. Walton and Brower (2000) showed extreme variability in monarch counts in Cape May Point, NJ which is a major funnel point in

September and October for monarchs migrating to Mexico. In Cape May, the 1999 counts were seven times greater than in 1998 and almost twice as high as any year since 1992 when the census began. Monarch Watch has conducted annual surveys since 1993. Surveys from 1993-1999 are available online at www.monarchwatch.org.

Due to extreme annual swings in monarch population estimates, it is not reasonably possible to develop a baseline monarch butterfly population size. However, it is possible to continue surveys such as the one conducted by the Monarch Watch to identify sudden, drastic decreases in the number of monarchs in North America and its overwintering sites in Mexico.

The DCI addresses the potential of monarch exposure to *Bt* corn pollen in the field and whether pollen densities encountered present a risk to these butterflies. Monarch larvae potentially feed on 14 different species of milkweeds. Seven of these milkweed species are fed on by monarchs in the Corn Belt. Common milkweed (*Asclepias syriaca*) is the predominant species oviposited and fed on by monarchs. Whorled milkweed (*Asclepias verticillata*) may also be an important resource for monarchs (Harzler and Buhler 2000). Milkweed densities vary and typically depend on the management practices of the habitat.

Milkweeds can be found in a variety of habitats. However, non-agricultural areas are usually undisturbed supporting growth of more milkweeds. Surveys conducted in Ontario, Maryland, Indiana, Illinois, Iowa, Nebraska, and Kansas showed that more milkweeds occur near the corn field edge, in roadsides, or in non-agricultural areas than within corn fields (Harzler and Buhler 2000, Oberhauser *et al*. 2001). Roadsides that are mowed will have less milkweed than areas not mowed or tillage practices may affect densities in cultivated fields. It is difficult to control milkweed, particularly when reduced tillage is practiced, because milkweed reproduces vegetatively or by seed and is often found in clumps (Martin and Burnside 1984). Herbicides are generally not considered to be effective in controlling milkweeds. However, in some instances, "good" control of milkweeds may be provided by glyphosate, halosulfuron-methyl + dicamba (2,4-D), and nicosulfuron + dicamba.

Some milkweeds occur in and near corn fields, therefore, the proportion of the migrating monarch population that may encounter *Bt* corn fields was considered. There are potentially 105,174 square miles (2.73×10^7 hectares) of field corn grown in the U.S. that may provide breeding habitat for monarchs (USDA - NASS 1997). Of this 105,174 square miles, about 26,294 square miles consist of *Bt* corn fields that may provide breeding sites for monarchs. The edge of corn fields constitutes a very small area of potential monarch breeding habitat. Approximately 0.18% of monarch breeding sites may occur near corn field edges. This is equivalent to 0.11% of all land in this region. It can be concluded that the near edge (within 1 meter of the field edge) of *Bt* corn fields constitutes a negligible portion of monarch breeding habitat. Approximately 18% of monarch habitat in the central U.S. consists of corn fields (Taylor and Shields 2000) and current approximate acreage of *Bt* corn is equal to approximately 26,293 square miles (25% of total U.S. corn acreage) or 5.1% of monarch habitat. The information submitted to the Agency thus far suggests that 50% of monarchs probably pass through the Corn Belt (Taylor *et al*. 1999).

Monarchs feeding on milkweeds in and near *Bt* corn fields during anthesis will potentially be exposed to *Bt* pollen. Time to pollination varies among hybrids and regions and is determined according to growing degree units (GDU). Examples of the approximate number of GDU needed for pollination to occur in different regions are: (1) Fargo, ND = 1130; (2) Madison, WI = 1250; (3) Lincoln, NE = 1370; (4) Champaign, IL = 1390; (5) Salisbury, MD = 1400; and (6) Lubbock, TX = 1450. Individual corn tassels typically shed pollen for two to seven days (or longer) and silks on an ear are exposed to pollination for two to three days (Russell and Hallauer 1980, Ritchie *et al*. 1997). A field will shed pollen for up to 15 days depending upon microclimate (Russell and Hallauer 1980).

Corn pollen grains don't disperse far from its source because they are large (<90 to 100 microns). The majority of corn pollen stays within corn fields and only small quantities disperse beyond 5 meters from the field edge. However, pollen levels are higher further into the corn field (e.g., 147.5 grains/cm^2 were found 25 m into the field) than close to the edge (e.g., 55.5 grains/cm^2 were found 3 m into the field) (Pleasants *et al*. 2001). Raynor *et al*. (1972) found that 63% of corn pollen remained within fields, 88% settled within eight meters of the field edge, and 98% settled within 60 meters. They also determined that there was only 0.2% of pollen deposited at 60 m from the corn field edge. Pleasants *et al*. (2001) found pollen densities at corn field edges were 50% of the level found within the field and densities were greater on milkweed plants within rows than between rows.

It is difficult to report one specific quantity of corn pollen that will be deposited on milkweeds within corn fields and at varying distances from the field edge. Many factors influence pollen deposition and retention on milkweed leaves. Environmental factors such as rain and wind may increase the distance pollen will travel and may decrease the amount of pollen retained on leaves. Plant morphology such as leaf angle will also effect pollen deposition and retention. Upper leaves that are more upright and exposed to environmental factors retain less pollen than middle and lower leaves on the milkweed plant (Pleasants *et al*. 2001).

2) Corn pollen exposure

Pleasants *et al*. (2001) found that levels of corn pollen deposition on milkweed leaves are influenced by wind, wind direction, rainfall, plant architecture and the time period when pollen was sampled. In some instances, weather conditions such as thunderstorms and updrafts carry some pollen grains further than usual (Emberlin *et al*, 1999). However, wind, rain, and other environmental factors will probably remove most of the pollen deposited on milkweed leaves (Pleasants *et al*. 2001). Rainfall has been shown to remove most (86 - 92%) of the pollen from milkweed leaves, thus potentially reducing the length of monarch exposure to *Bt* pollen (Pleasants *et al*. 2001, Stanley-Horn *et al*. 2001). The level of exposure of monarch larvae to *Bt* pollen carried to milkweed plants on exoskeleton of adults is minimal. If pollen were to adhere to monarch adults and dislodge on milkweeds, quantities would not be great enough to adversely affect larvae feeding on these milkweeds. Since *Bt* Cry protein must be ingested and will not harm monarchs by contacting it's exoskeleton, there is minimal risk posed from monarchs transporting pollen among milkweed plants.

Monarchs will only be exposed to *Bt* while it remains biologically active in pollen. Microbial enzymes, secondary plant compounds, extremes in pH, ultraviolet light, wind and rain are known to degrade *Bt* proteins in microbial sprays. The insecticidal activity in *Bt* microbial sprays has been shown to break down rapidly for two days after application and is practically nonexistent four days post application (Gelernter, 1990). These factors also affect the insecticidal activity of *Bt* expressed in pollen. Head and Brown (1999) only found biological activity of MON 810 in fresh pollen. Laboratory assays showed that MON 810 activity was not detectable in pollen after seven days (Head and Brown 1999). The biological activity of *Bt* proteins probably decreases more rapidly in the field where it is exposed to elements such as ultraviolet light than in the laboratory. Therefore, the Cry protein may breakdown more rapidly than seven days under field conditions.

Monarch ovipositional and feeding behavior will also contribute to the level of milkweed pollen that the larvae will encounter. Surveys have shown that monarchs prefer to oviposit single eggs on the underside of milkweed leaves on young, tender tissue (Urquhart 1960, Borkin 1982, Pleasants *et al*. 2001). However, females may lay more than one egg per plant and may oviposit on the top of leaves, on stalks or flowers (Borkin 1982). The age of plant tissue is probably the most important influence on monarch ovipositional preference. Female monarchs prefer to oviposit on young tender plant tissue (Urquhart 1960, Borkn 1982). Neonate larvae begin feeding near the area where eggs were laid which is typically the underside of leaves. As larvae mature, they may feed through leaves, on top of leaves, or on leaf veins (Urquhart 1960).

Results vary regarding monarch preference, avoidance, or indifference to ovipositing on milkweeds in corn fields (Tschenn *et al*. 2001, Oberhauser *et al*. 2001). In the laboratory, Tschenn *et al*. (2001) showed that monarchs either do not show a preference for milkweeds with or without corn pollen dusted on them, or they avoid pollen dusted milkweeds. Field surveys conducted by Oberhauser *et al*. (2001) and Stanley-Horn *et al*. (2001) found monarch eggs on milkweeds dusted with pollen in and near corn fields. In some instances monarchs may prefer to oviposit on milkweeds occurring within corn fields (Oberhauser *et al*. 2001). Although milkweed densities are generally higher in nonagricultural habitats, surveys conducted in Minnesota, Wisconsin, and Iowa suggest that monarchs will oviposit in corn fields 45 to 107 times more often than in nonagricultural habitats (Oberhauser *et al*. 2001).

Oberhauser *et al*. (2001) and Pleasants *et al*. (2001) showed that monarchs do occur on milkweeds in the field during pollen shed. The Oberhauser *et al*. (2001) study showed considerable overlap between the peak of the migratory monarch generation and pollen shed in Minnesota and Ontario. In Iowa and Maryland, the final generation of monarchs peaked prior to pollen shed. Four different monarch breeding regions (east-central Minnesota and west-central Wisconsin, central Iowa, coastal Maryland, and southern Ontario) were monitored when monarchs were present.

TABLE 7. Temporal overlap of monarch larvae and corn pollination

State	% Overlap of Larvae & Anthesis	% Overlap of Migratory Gen. Larvae and Anthesis
Minnesota	20% to 68%	50%

State	% Overlap of Larvae & Anthesis	% Overlap of Migratory Gen. Larvae and Anthesis
Ontario	27% to 75%	50%
Maryland	0 to 36%	15%
Iowa	4% to 25%	15%
Wisconsin		50%

According to models developed by Calvin *et al*. (2000), overlap of monarch larval occurrence and corn pollination is negligible in southern and central parts of the Corn Belt, but there is up to 75% overlap in the northernmost area of the Corn Belt. This means that 0 to 5% of monarchs will be exposed to *Bt* corn pollen in most of the Corn Belt and 10% exposure will occur in the northern region. In general, the Calvin *et al*. (2000) model showed that the degree of co-occurrence generally increased as latitude or elevation increased.

Since monarch eggs and larvae were found on milkweed plants in the northern fields when pollen was present on leaves (Oberhauser *et al.* 2001) it can also be assumed that monarch larvae will consume both *Bt* or non-*Bt* pollen if it is encountered (Hellmich *et al.* 2001, Oberhauser *et al.* 2001). Laboratory (Hellmich *et al*. 2001, Tschenn *et al*. 2001) and field studies (Oberhauser *et al*. 2001) demonstrated that monarchs will not avoid feeding on plants dusted with *Bt* or non-*Bt* corn pollen. Since eggs and larvae were found on milkweed plants naturally dusted in corn pollen in the field, it appears that monarchs will not avoid pollen dusted plants nor do they avoid corn fields.

3) *Bt* Cry1Ab toxicity to monarchs

Since it has been established that monarch larvae can encounter and feed on *Bt* pollen in the field, it is important to know the *Bt* pollen toxicity level.

TABLE 8. LC_{50}s and the EC_{50}s (effect-eliciting concentration) for the various monarch larval stages fed purified trypsin resistant core of *Bt* Cry 1Ab proteins

Instar (N)	LC_{50} (95% C.I.) (ng Cry1Ab/mL treated artificial diet)	EC_{50} (95% C.I.) (ng Cry1Ab/mL treated artificial diet)
1^{st} (318)	3.29 (2.19-4.76)	0.76 (0.64-0.90)
2^{nd} - 3^{rd} (141)	35.1 (30-100)	9.60 (6.01-15.06)
3^{rd} - 4^{th} (125)	> 100 (-)	18.3 (9.4-40.3)

(Hellmich *et al*. 2001).

LC_{50}s for third and fourth instars were 30 times greater than first instars and second and third instar's LC_{50} was 11 time greater than first instars. The Cry1Ab no observable effect concentration (NOEC) was reported as ≤0.3 ng/mL diet (Hellmich *et al*. 2001).

In nature, monarchs are not expected to get uniformly distributed doses of *Bt* as is observed in the laboratory. Unlike feeding on diet in the laboratory, monarchs would probably ingest varied amounts of *Bt* in the field and also have the opportunity to avoid feeding on *Bt* altogether. *Bt*

activity in pollen is also expected to decline over time in the field. Therefore, levels of Cry1Ab ingested by monarchs in the field are expected to be lower than levels fed to them in the Hellmich *et al*. (2001) laboratory study.

4) *Bt* pollen exposure and toxicity

Cry 1Ab in pollen of the currently registered *Bt* field corn hybrids (MON 810 and Bt11) is only found in trace quantities (<0.09 µg/g dry wt. pollen). In order to determine the exposure of monarch larvae to *Bt* pollen on milkweed leaves, five independent surveys were conducted during the 2000 growing season in Iowa, Maryland, and Ontario (Table 9). The highest average corn pollen density monitored in the field was 586 grains/cm^2 found three meters inside a Bt11 sweet corn field (Stanley-Horn *et al.* 2001).

TABLE 9. Mean pollen density on milkweed leaves inside a cornfield (Pleasants *et al*, 2001)

Study	Anthesis level	Mean pollen density (cm^2)
Maryland 1999	Near peak	65.7
Iowa 2000b	100%	425.6
Iowa 2000c	Post anthesis (10days)	101.2
Iowa 2000d	100%	231.4
Ontario 2000	day11	97.7
Maryland 2000	day 9	161.3

The highest pollen densities were found in a *Bt* sweet corn field since sweet corn produces more pollen per plant than field corn. The highest level of pollen found averaged 504-586 grains/cm^2 and occurred on milkweeds located 3 m inside Bt11 sweet corn in Maryland (Stanley-Horn *et al.* 2001). There was no difference in densities of *Bt* and non-*Bt* pollen found on milkweed leaves (Stanley-Horn *et al.* 2001). In one corn field in Iowa, Pleasants *et al.* (2001) found a mean of 900 pollen grains/cm^2. However, first instar larvae feeding on milkweed leaves naturally dusted with pollen in the field resulted in no observable effects of MON 810 and Bt11 on survival and fitness of monarchs.

A study conducted by Hellmich *et al.* (2001) involved feeding first instar monarchs no pollen or known amounts of *Bt* (MON 810 and Bt11) and non-*Bt* pollen applied in the laboratory. Extremely high pollen levels (250 - 2000 grains/cm^2 for MON 810 and 150 - 4000 grains/cm^2 for Bt11) were fed to first instar monarch larvae in a controlled environment (since no pollen was removed due to environmental factors, these conditions are considered a worst case scenario) and resulted in no significant effects on larval weight (Hellmich *et al.* 2001). From this data, it can be concluded that the NOEL for MON 810 is >2000 grains/cm^2 and for Bt11 is >4000 grains/cm^2 which is greater than levels that occur under natural field conditions.

However, assuming a worst case scenario where 1000 pollen/cm^2 would show weight loss effects, the effect of *Bt* corn pollen on monarch larvae would still be minimal since these levels

are rare in the field, expected to occur at a 0.1% rate. In addition, monarch larvae exposed to sub-lethal concentrations of Cry1Ab protein have been shown to mature into healthy adults (Jesse & Obrycki, 2000).

TABLE 10. Frequency distribution of pollen deposition density on milkweed leaves (Pleasants *et al*, 2001):

Pollen density (cm^2)	Inside corn field	From edge of cornfield	
		0 meters	1 meter
0-100	0.625	0.833	0.900
100-200	0.190	0.093	0.062
200-300	0.091	0.033	0.022
300-400	0.037	0.017	0.066
400-500	0.018	0.008	0.002
500-600	0.010	0.007	0.002
600-700	0.009	0.002	0.001
700-800	0.004	0.002	0.000
800-900	0.004	0.003	0.001
900-1000	0.002	0.001	0.000
1000-1100	0.001	0.001	0.001

Therefore, it can be concluded that levels of MON 810 or Bt11 pollen toxic to monarch larvae do not occur under natural field conditions.

Stanley-Horn *et al.* (2001) studied the difference between first instar larvae feeding on Bt11 and on non-*Bt* pollen in the field (starting six days after initiation of pollen shed) for five days. The results showed no significant mortality, feeding, development, weight gain, % survival to pupation, days to pupation, pupal weight, emergence from pupae, adult weight and adult wing length. However, first instar monarchs feeding on milkweed sprayed with insecticides or subject to exposure from insecticide drift were adversely affected. There was 90% to 100% mortality of monarchs feeding on milkweeds collected from within the field and 21% to 45% mortality from plants outside the field. This study also suggests that *Bt* sweet corn may provide a safer habitat for monarchs than fields requiring insecticide applications (Stanley-Horn *et al*. 2001, Vlachos and Roegner 1997).

The 2001 EPA Registration Action document for *Bt* corn stated that studies on long term exposure of monarch larvae to *Bt* pollen should be considered due to the fact that monarch larvae hatching in corn fields may be exposed to *Bt* protein for periods of 12 days or longer. These studies were conducted from 2001-2002, and the results were submitted jointly by Monsanto Company and Syngenta Seeds, Inc. in 2003 (MRID 46162001) and later published in *Environmental Entomology* (Dively, *et al.* 2004). Effects on the monarch butterfly (*Danaus plexippus*) from continuous exposure of larvae to natural deposits of *Bt* and non-*Bt* corn pollen on milkweed (*Asclepias syriaca*) were measured in five studies using Cry1A(b)-expressing

hybrids (events BT11 and MON810) and non-transgenic corn in Maryland, Iowa, and Ontario, Canada. First instars were exposed beginning at 3 to 4 and 6 to 7 days after initial anthesis. Overall mean pollen densities during larval development in the first and second bioassays were 163 and 170 grains/cm^2, respectively. Pollen density on milkweed plants in the non-*Bt*, BT11, and MON810 hybrids averaged 163, 155, and 174 grains/cm^2, respectively, during the first bioassay and 172, 173, and 158 grains/cm^2, respectively, in the second bioassay. There were no statistically significant differences in pollen density between assays or among hybrid types. The number of anthers on the leaves was positively correlated with the amount of pollen deposited on the leaves.

The mean number of days for first instars to develop to eclosion was 22.7 in the laboratory reared cohorts, and 24.2 in the field reared cohorts. In the first bioassay, exposure to *Bt* pollen had a statistically significant effect on developmental time to pupation and to eclosion. Development to adult emergence was prolonged by 0.6 to 1.2 days over that for larvae exposed to non-*Bt* pollen. Results from the second bioassay were similar, with average delays of 0.7 to 2.4 days to pupation and 0.2 to 2.4 days to eclosion. For both bioassays combined, exposure to *Bt* pollen prolonged developmental time by an average of 1.8 days.

In the first bioassay, mean survival to pupation was 71.2%, 56.9%, and 50.6% for non-*Bt*, BT11, and MON810 pollen, respectively. Mean survival to eclosion was 66.7%, 51.3%, and 48.5%, respectively. There were no statistical differences between the BT11 and MON810 events. Overall, 25% fewer larvae exposed to *Bt* pollen survived to become adults. In the second bioassay, mean survival to pupation was 59.3, 50.4, and 44.4% for non-*Bt*, BT11, and MON810 pollen, respectively. Mean survival to eclosion was 58.6%, 47.8%, and 43.6%, respectively. Again, there were no statistical differences between the BT11 and MON810 events. Overall, 22% fewer larvae exposed to *Bt* pollen survived to become adults. For both bioassays combined, 23.7% fewer first instars exposed to *Bt* pollen reached the adult stage, compared to those exposed to non-*Bt* pollen. Overall survival of laboratory-reared larvae (66.1%) was significantly higher than field-reared larvae (44.6%), although natural mortality factors (e.g., weather, pathogens) probably accounted for the difference.

Exposure to *Bt* pollen during larval development decreased the weight of pupae and adults. The results were statistically significant for pupal weight in both bioassays and also for adult weight in the second bioassay. Weights of both pupae and adults reared from larvae exposed to *Bt* pollen were significantly reduced by an average of 5.5% compared to those exposed to non-*Bt* pollen. The adults from *Bt* pollen-exposed larvae also had slightly shorter wing length, but the difference was not statistically significant.

A risk assessment modeling system was used to estimate the proportion of second-generation monarchs that would be affected by exposure to *Bt* pollen (the first generation does not overlap with the period of anthesis in the corn belt). A table of parameter estimates of exposure risk and probability of harm for 15 U.S. Corn Belt states and Ontario was provided in MRID No. 46162001. The model results indicate that for the corn belt area, which represents 50% of the monarch breeding population, the risk to monarch larvae associated with long-term exposure to *Bt* corn pollen would be 0.6% additional mortality.

The Corn Belt constitutes 50% of the breeding population in North America. Monarchs outside the Corn Belt are relatively unaffected due to the low acreage of corn and a low percentage of overlap of monarch larvae with corn pollen shed. Therefore, when all of North America is considered, the risk to monarch larvae from long-term exposure to Bt11 and MON810 *Bt* corn pollen is an additional 0.3% mortality at the current adoption rates (37%) of Cry1Ab *Bt* corn. The OPP risk assessment process considers a >50% reduction in population (mortality) as posing a risk to the existence of a species (see Section 1: Tiered Hazard and Risk Assessment Process). Based on these criteria the Agency concludes that, given the other natural and man-made hazards to the monarch populations which account for up to 90 % annual monarch mortality without affecting the overall monarch abundance, a 0.3 % mortality due to Cry1Ab *Bt* corn pollen will not pose an unreasonable risks to the continued existence of monarch populations in North America.

5) *Bt* Cry1F toxicity to monarchs

A scientifically sound study submitted by Dow AgroSciences showed that Cry1F does not cause mortality to neonate monarch butterfly larvae when fed a 10,000 ng/mL diet dose. [Helmich, *et al* (2001) made the same observation at a 30,000 ng/mL dose level.] First instar larval weight and mortality were recorded after seven days of feeding. There was no mortality to monarchs fed 10,000 ng/mL diet, the highest rate tested. There was some growth inhibition at 10,000 ng/mL diet. Since pollen doses equivalent to 10,000 ng/mL diet are not likely to occur on milkweed leaves in nature, it can be concluded that Cry1F protein will not pose a risk to monarchs.

6) Conclusions:

MON 810 and Bt11 show relatively low toxicity to monarch larvae and the Cry1F protein has no detectable toxicity to monarch larvae. Overall, the available information indicates a very low probability of risk to monarchs in areas beyond the near edge of corn fields. Inside corn fields and at the near edge of corn fields there is low probability of monarch larvae encountering a toxic level of pollen for the *Bt* corn products covered by this risk assessment. Consideration of factors limiting exposure, such as relatively low pollen shed and monarch breeding overlap in much of the corn belt, the distribution of milkweed plants within corn fields compared to other milkweed habitats, the egg laying and feeding activity of monarch larvae, together with the low toxicity of the *Bt* corn products covered by this assessment indicate a low probability for adverse effects on monarch larvae.

The weight of evidence of data gathered to study the effects of *Bt* pollen on monarch larvae in the field indicate that milkweeds in corn fields to within 1 meter of cornfields are unlikely to be dusted with harmful levels of *Bt* pollen from the most widely planted corn varieties MON 810, Bt11 and TC1507.

Based on the review of the DCI data, in combination with data submitted since 2001, the Agency concludes that the current information on monarch toxicity and exposure does not give sufficient

cause for undue concern of widespread risks to monarch butterflies at this time.[11] EPA will continue to closely monitor the results from further monarch butterfly research as a part of its regulatory oversight of *Bt* products.

4. Horizontal Transfer of Transgenes from Bt Crops to Soil Organisms

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. Furthermore, several experiments (published in scientific journals), that were 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 the *Bt* toxins engineered into Cry1Ab and Cry1F corn are derived from soil-inhabiting bacteria, EPA has concluded that there is a low probability of risk from HGT of transgenes found in Cry1Ab- and Cry1F-producing corn.

5. Gene Flow and Weediness Potential

The movement of transgenes from the host plant into weeds has been a significant concern for EPA due to the possibility of novel exposures to the pesticidal substance. This concern has been considered for each of the *B.t.* plant-incorporated protectants currently registered and EPA believes that these concerns have been satisfactorily addressed. The Agency has determined that there is no significant risk of gene capture and expression of any *B.t.* endotoxin by wild or weedy relatives of corn, cotton (for the duration of the *Bt* cotton product registrations amended as of September 29, 2001), or potato in the U.S., its possessions or territories. In addition, the USDA/APHIS has made this same determination under its statutory authority under the Plant Pest Act. There is a possibility, however, of gene transfer from *B.t.* cotton to wild or feral cotton relatives in Hawaii, Florida and the Carribean. Where feral populations of sexually compatible cotton species exist in Florida and Hawaii, EPA has prohibited the sale or distribution of *B.t.* cotton in these areas. These containment measures prevent the movement of the registered *B.t.* endotoxin from *B.t.* cotton to wild or feral cotton relatives in Hawaii and Florida.

Under FIFRA, EPA has reviewed the potential for gene capture and expression of the *B.t.* endotoxins by wild or weedy relatives of corn, cotton and potatoes in the U.S., its possessions or

[11]The available data can be used to make an approximation that only 0.001% of the monarch population (1 in 100,000) may be exposed to sub-lethal amounts of Bt pollen in Bt corn fields (using the information that 50% of the monarchs go through the corn belt, that 18% of that habitat is corn, that 25% is Bt corn, that the maximum overlap of anthesis and larvae is 50% in the migratory population and that in a worst case scenario 0.1% of that population may encounter sub-lethal amounts of Bt pollen).

territories. *B.t.* plant-incorporated protectants that have been registered to date have been expressed in agronomic plant species that, for the most part, do not have a reasonable possibility of passing their traits to wild native plants. Feral species related to these crops, as found within the United States, cannot be pollinated by these crops (corn, potato and cotton) due to differences in chromosome number, phenology (*i.e.*, periodicity or timing of events within an organism's life cycle as related to climate, *e.g.*, flowering time) and habitat. The only exception, however, is the possibility of gene transfer from *B.t.* cotton to wild or feral cotton relatives in Hawaii, Florida and the Caribbean.

The FIFRA EPA Scientific Advisory Panel meeting held on October 18-20, 2000, further discussed the matter of gene flow and offered some issues for consideration in this matter. The panel agreed that the potential for gene transfer between corn (maize) and any receptive plants within the U.S., its possessions and territories was of limited probability and nearly risk free. Similarly, potatoes were seen as nearly risk free with regards to gene flow in that proximity of compatible wild relatives to this crop is insufficient to allow for cross pollination. Some concern was expressed, however, with respect to *B.t.* cotton grown in areas where wild relatives or feral populations of the crop are known to exist.

a. Gene Transfer - Gene Flow

Concern over the potential for species related to maize (*Zea mays* ssp. *mays*), such as *Tripsacum* species and the teosintes, as potential recipients of gene flow from genetically modified *Zea mays* indicated a need for review of what is known about this subject and to reevaluate the initial Agency assessments related to gene flow potential of *Zea mays*. Some *Zea* spp., such as the teosintes, are known to be interfertile with maize and are discussed as potential recipients of pollen directed gene flow from maize. This issue is of particular concern based upon the increased planting of genetically modified maize. Therefore, the Agency conducted a reevaluation in early 2000, the results of which are reported here.

b. *Zea mays* ssp. *mays* - Maize - General Biology

Zea mays is a wind-pollinated, monoecious, annual species with imperfect flowers. This means that spatially separate tassels (male flowers) and silks (female flowers) are found on the same plant, a feature that limits inbreeding. A large variety of types are known to exist (*e.g.*, dent, flint, flour, pop, sweet) and have been selected for specific seed characteristics through standard breeding techniques. Maize cultivars and landraces are known to be diploid (2n = 20) and interfertile to a large degree. However, some evidence for genetic incompatibility exists within the species (*e.g.*, popcorn x dent crosses; Mexican maize landraces x Chalco teosinte). *Zea mays* has been domesticated for its current use by selection of key agronomic characters, such as a non-shattering rachis, grain yield and resistance to pests. The origin of corn is thought to be in Mexico or Central America, based largely on archaeological evidence of early cob-like maize in indigenous cultures approximately 7200 years ago.

A recent study has indicated that cross-pollination of commercial maize cultivars at 100 ft downwind from the source of genetically modified maize was 1 % and this proportion declined exponentially to 0.1 % at 130 ft and further declined to 0.03 % at 160 ft. At 1000 ft, the farthest

distance measured, no cross-pollination was detected (Jemison and Vayda, 2000). For production of Foundation Seed, a distance of 660 ft has been generally required to ensure separation of pollen types. The relatively large size of corn pollen and its short viability period under most conditions preclude long distance transfer for purposes of outcrossing (Schoper, personal communication,1999). Under conditions of high temperature or low humidity, corn pollen may only survive for a matter of minutes. Under more favorable conditions in the field or with controlled handling in the laboratory, pollen life may be extended to several hours.

c. *Tripsacum* species - Gama Grass - General Biology

Close relatives of corn or maize are found in the genus *Tripsacum.* Sixteen species of *Tripsacum* are known worldwide and generally recognized by taxonomists and agrostologists; most of the 16 different *Tripsacum* species recognized are native to Mexico, Central and South America, but three occur within the U.S. In the Manual of Grasses of the United States, A. S. Hitchcock (revisions by Agnes Chase; 1971) reports the presence of three species of *Tripsacum* in the continental United States: *T. dactyloides*, *T. floridanum* and *T. lanceolatum*. Of these, *T. dactyloides*, Eastern Gama Grass, is the only species of widespread occurrence and of any agricultural importance. It is commonly grown as a forage grass and has been the subject of some agronomic improvement (*i.e.*, selection and classical breeding). *T. floridanum* is known from southern Florida and *T. lanceolatum* is present in the Mule Mountains of Arizona and possibly southern New Mexico.

For the species occurring in the United States, *T. floridanum* has a diploid chromosome number of 2n = 36 and is native to Southern Florida; *T. dactyloides* includes 2n = 36 forms which are native to the central and western U.S., and 2n = 72 forms which extend along the Eastern seaboard and along the Gulf Coast from Florida to Texas, but which have also been found in IL and KS; these latter forms may represent tetraploids (x = 9 or 18; Lambert, personal communication, 1999); and *T. lanceolatum* (2n = 72) which occurs in the Southwestern U.S. *Tripsacum* differs from corn in many respects, including chromosome number (*T. dactyloides* n = 18; *Zea mays* n = 10). Many species of *Tripsacum* can cross with *Zea*, or at least some accessions of each species can cross, but only with difficulty and the resulting hybrids are primarily male and female sterile (Duvick, personal communication, 1999; Galinat, 1988; Wilkes, 1967). *Tripsacum*/maize hybrids have not been observed in the field, but have been accomplished in the laboratory using special techniques under highly controlled conditions.

Eastern Gama Grass is considered by some to be an ancestor of *Zea mays* or cultivated maize (Mangelsdorf, 1947), while others dispute this (Galinat, 1983; Iltis, 1983; Beadle 1980), based largely on the disparity in chromosome number between the two species (maize n = 10; Gama Grass x = 9 or 18, with diploid, triploid and tetraploid races existing; 2n = 36 or 72), as well as radically different phenotypic appearance. Albeit with some difficulty, hybrids between the two species have been made (Mangelsdorf and Reeves, 1939; Chet DeWald, personal communication;1999). In most cases these progeny have been sterile or viable only by culturing with *in vitro* 'embryo rescue' techniques.

Even though some *Tripsacum* species occur in areas where maize is cultivated, gene

introgression from maize under natural conditions is highly unlikely, if not impossible (Beadle, 1980). Hybrids of *Tripsacum* species with *Zea mays* are difficult to obtain outside of the controlled conditions of laboratory and greenhouse. Seed obtained from such crosses are often sterile or progeny have greatly reduced fertility. Approximately 10 - 20% of maize-*Tripsacum* hybrids will set seed when backcrossed to maize, and none are able to withstand even the mildest winters. The only known case of a naturally occurring *Zea* - *Tripsacum* hybrid is a species native to Guatemala known as *Tripsacum andersonii*. It is 100% male and nearly 99% female sterile and is thought to have arisen from gene flow to teosinte, but the lineage is uncertain (Doebley, personal communication, 2000). *Zea mays* is not known to harbor properties that indicate it has weedy potential and, other than occasional volunteer plants in the previous season's corn field, maize is not considered as a weed in the U.S.

In a telephone conversation with Dr. Chester 'Chet' DeWald, USDA-ARS, Woodward, OK, a geneticist working on improvement of grasses, he stated that relatively few accessions of *T. dactyloides* will cross with maize and the majority of progeny are not fertile or viable even in those that do. In controlled crosses, if the female parent is maize, there is a greater likelihood of obtaining viable seed. When these hybrids have been backcrossed to maize in attempts to introgress *Tripsacum* genes for quality enhancement or disease resistance, the *Tripsacum* chromosomes are typically lost in successive generations. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the chromosomal complements of one of the parent species in subsequent generations.

Only recently has Dr. DeWald (or anyone else) succeeded in obtaining a true *Tripsacum* cytoplasm with a maize nuclear background. This was done by using gama grass as the female parent and maize as the male or pollen donor. Numerous accessions were tested and crosses made before this came to fruition. The *Tripsacum* derived mitochondrial chondrome and chloroplast plastome in these hybrids contribute to the seed qualities of the plants, but the nuclear genome appears to be totally maize in origin (DeWald *et al*., 1999).

Dr. DeWald concluded that the possibility of maize contributing genetic material to Eastern Gama Grass through random pollen flow in agricultural or natural situations is extremely remote based upon his experience trying to create hybrids under the best of conditions. He also felt that no other known grass species present in the continental U.S. would interbreed with commercial maize populations (*i.e.*, be recipients of pollen-directed gene flow). This is in agreement with Holm *et al*. (1979) who determined that none of the sexually compatible relatives of corn in the U.S. are considered to be serious, principal, or common weeds in the U.S.

d. *Zea* species - Teosintes - General Biology

Teosintes, specifically *Z. mays* ssp. *mexicana* (Schrader) Iltis, *Z. mays* ssp. *parviglumis* Iltis and Doebley, *Z. mays* ssp. *huehuetenangensis* (Iltis and Doebley) Doebley, *Z. luxurians* (Durieu and Ascherson) Bird, *Z. perennis* (Hitchc.) Reeves and Mangelsdorf and *Z. diploperennis* Iltis, Doebley and Guzman, have co-existed and co-evolved in close proximity to maize in the Americas over thousands of years; however, maize and teosinte maintain distinct genetic

constitutions despite sporadic introgression (Doebley, 1990).

The teosintes retain a reduced cob-like fruit / inflorescence that shatters more than cultivated maize, but still restricts the movement of seeds as compared to more widely dispersed weedy species. Hence, the dispersal of large numbers of seeds, as is typical of weeds, is not characteristic of teosintes or maize. In their native habitat, some teosintes have been observed to be spread by animals feeding on the plants. Teosintes and teosinte-maize hybrids do not survive even mild winters and could not propagate in the U.S. corn belt. Additionally, some types have strict day length requirements that preclude flowering within a normal season (***i.e.***, they would be induced to flower in November or December) and, hence, seed production under our temperate climate (Beadle, 1980; Iltis, personal communication; 2000; Wilkes, personal communication; 2000; Wilkes, 1967).

Since both teosinte and *Tripsacum* are included in botanical gardens in the U.S., the possibility exists (although unlikely) that exchange of genes could occur between corn and its wild relatives. EPA is not aware, however, of any such case being reported in the United States. Gene exchange between cultivated corn and transformed corn would be similar to what naturally occurs at the present time within cultivated corn hybrids and landraces. Plant architecture and reproductive capacity of the intercrossed plants will be similar to normal corn, and the chance that a weedy type of corn will result from gene flow with cultivated corn is extremely remote.

Like corn, *Zea mays* ssp. *mexicana* (annual teosinte) and *Zea diploperennis* (diploid perennial teosinte) have 10 pairs of chromosomes, are wind pollinated, and tend to outcross, but are highly variable species that are often genetically compatible and interfertile with corn, especially when maize acts as the female parent. *Zea perennis* (perennial teosinte) has 20 pairs of chromosomes and forms less stable hybrids with maize (Edwards *et al*., 1996; Magoja and Pischedda, 1994). Corn and compatible species of teosinte are capable of hybridization when in proximity to each other. In Mexico and Guatemala, teosintes exist as weeds around the margins of corn fields. The F_1 hybrids have been found to vary in their fertility and vigor. Those that are fertile are capable of backcrossing to corn. A few isolated populations of annual and perennial teosinte were said to exist in Florida and Texas, respectively (USDA-APHIS, 1997). The Florida populations were presumably an escape from previous use of *Z. mays* ssp. *mexicana* as a forage grass, but local botanists have not documented any natural populations of this species for approximately twenty-five years (Keith Bradley, personal communication, 2000; David Hall, personal communication, 2000; Richard Wunderlin, personal communication, 2000).

Consultation with botanists and agronomists familiar with Texas flora suggested that no teosinte populations exist in the state (Benz, personal communication, 2000; Read, personal communication, 2000; Orzell, personal communication, 2000; Wilson, personal communication, 2000). Further, given the day length characteristics of *Z. diploperennis*, it is highly unlikely a sustaining population would result from introduction of this species. *Z. mays* ssp. *mexicana, Z. mays* ssp. *parviglumis, Z. luxurians* and *Z. diploperennis* may cross with maize to produce fertile hybrids in many instances (Wilkes, 1967). None of these teosinte species have, however, been shown to be aggressive weeds in their native or introduced habitats (John Schoper, personal communication, 1999). Except for special plantings as noted above, teosinte is not present in the

U.S. or its territories. Its natural distribution is limited to Mexico, Honduras, Nicaragua, El Salvador and Guatemala.

Given the cultural and biological relationships of various teosinte species and cultivated maize over the previous two millennia, it would appear that significant gene exchange has occurred (based upon morphological characters) between these two groups of plants and that no weedy types have successfully evolved as a result. More recent cytogenetic, biochemical and molecular analyses have indicated that the degree of gene exchange is far less than previously thought (Doebley, 1984; Doebley *et al*., 1987; Kato, 1997a, 1997b; Smith *et al*., 1985). Partial and complete gametophytic incompatibility has been documented among cultivated maize, landraces and teosinte (Kermicle, 1997). The former is demonstrated by differential pollen growth and a skewed recovery of alleles linked to incompatibility genes. Complete incompatibility mechanisms serve to isolate a species or subspecies and are evidenced as pollen exclusion or non-functioning of pollen types on certain genotypes. Attempts to cross six collections of *Zea mays* ssp. *mexicana* with U.S. maize cultivars (W22, W23) yielded no or few seeds in five of the six groups (Kermicle and Allen, 1990).

Based on the ability of maize to hybridize with some teosintes, the suggestion of previous genetic exchange amongst these species over centuries, and their general growth habits, any introgression of genes into wild teosinte from *Zea mays* is not considered to be a significant agricultural or environmental risk. The growth habits of teosintes are such that the potential for serious weedy propagation and development is not biologically plausible in the United States.

e. Conclusions

The potential for pollen-directed gene flow from maize to Eastern Gama Grass is extremely remote. This is evidenced by the difficulty with which *Tripsacum dactyloides* x *Zea mays* hybrids are produced in structured breeding programs. Additionally, the genus does not represent any species considered as serious or pernicious weeds in the United States or its territories. Any introgression of genes into this species as a result of cross fertilization with genetically-modified maize 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 maize chromosomal complement in subsequent generations.

Many of the *Zea* species loosely referred to as "teosintes" will produce viable offspring when crossed with *Zea mays* ssp. *mays*. 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. 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 *Zea 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.

6. Impacts on Threatened or Endangered Species

Toxicity data show that the only endangered species of any potential concern are in the Lepidoptera and Coleoptera group. The majority of endangered species in these Orders has very restricted habitat range and do not feed on, or approach the *Bt* crop planting areas close enough to be exposed to toxic levels of *Bt* pollen. Examination of an overlay map showing the county level distribution of endangered lepidopteran species relative to corn production counties in the US shows that any potential concern regarding range overlap with corn production is restricted to the Karner blue butterfly. However, the Karner blue host plant, the wild lupine, does not occur in corn fields. Therefore it appears highly unlikely that significant numbers of lupine would occur within a few (two) meters of corn field edge, where the toxic levels of corn pollen may be present. Even using the conservative assumption that Karner blue larvae are relatively sensitive to all *Bt* proteins in all *Bt* corn events, the likelihood that the larvae would encounter sufficient grains of *Bt* corn pollen to exert toxicity is extremely remote. Also, relevant data and information indicate that the likelihood of wild lupines occurring adjacent to corn fields is low. Moreover, the overlap of the time of the year when corn pollen is shed with the times of the year when Karner blue larvae are likely to be present is limited.

An examination of the endangered bird and bat species shows that their habitats are mostly non-agricultural. Of those that do encroach on agricultural fields, none would rely on cotton or potato pests as a primary food source. Corn is not an issue, because the ECB is within the corn stalk and is not available for bird predation. Bats do not prey on larvae. They rely on flying insects. Taking all of these and other pertinent issues into consideration, it becomes apparent that reduction in the target pests of cotton and potatoes would not have an effect on the food source of endangered birds and bats. In the rare instances where these species may feed on the target pests, the reduction in the pest species will merely cause them to rely on other plentiful insects as a source of food. Submitted and published field data reviewed in this document show that a wide variety of insects is abundant in *Bt* crops as opposed to non-*Bt* crop fields when conventional insect pest control practices are used. Therefore the data show that *Bt* crops should actually be beneficial to bird and bat populations.

2010 Update: Current ecological effects data and EPA reviews of Cry1Ab and Cry1F support the Agency's determination that adverse effects will not occur to nontarget organisms. Due to a demonstrated lack of toxicity and/or exposure, no effects from Cry1Ab and Cry1F are anticipated for any nontarget species, including federally-listed threatened and endangered ("listed") lepidopteran and coleopteran species and their designated critical habitats. EPA has also determined that there are no indirect effects on endangered and threatened plant species, such as impacts on lepidopteran pollinators that are important and/or essential to an endangered or threatened plant. The Agency is therefore upholding its determination that the registered uses of Cry1Ab and Cry1F will have "No Effect," direct or indirect, on endangered or threatened terrestrial or aquatic species as listed by the U.S. Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS).

7. Potential Interaction Between Cry1Ab and Cry1F Proteins

(MRID 47677802)

A seven-day laboratory sensitive insect bioassay was conducted to determine if the combination PIP product 1507 x 59122 x MON810 has a synergistic effect in comparison to the individual parental events TC1507 (expressing Cry1F protein) and MON810 (expressing Cry1Ab protein) on target lepidopteran pests. The pests used in the bioassay were European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), fall armyworm (FAW, *Spodoptera furgiperda*), and corn earworm (CEW, *Helicoverpa zea*). The observed and expected larval mortality in the 1507 x 59122 x MON810 group were similar and mean larval weight of the survivors exposed to 1507 x 59122 x MON810 leaf tissue was not significantly different from the single parental events or the negative control of non-*Bt* maize. These results indicate that the Cry1F and Cry1Ab proteins act independently and do not have a synergistic or antagonistic effect on the target pests, other than an additive effect. Quantitative ELISA results also confirmed that the expression of each of the proteins in the combination PIP was not affected by the presence of the other protein. This study was classified acceptable.

8. Ecological Risk Assessment Conclusions

a. Direct Effects

In general, the reviewed publications, recent research data, and information submitted as a result of the data call in (DCI) provide a weight of evidence assessment indicating no unreasonable adverse effects of *Bt* Cry proteins expressed in plants to non-target wildlife or beneficial invertebrates, whether they are earthworms, springtails, parasites, predators, pollinators or soil microbial and invertebrate flora. Published field testing results and field test data submitted to EPA show minimal to undetectable to beneficial changes in the non-target insect populations. EPA is, however, continuing to participate in research and review the pertinent scientific literature for the purpose of reevaluating the Agency's Ecological Risk Assessment of the *Bt* crop registrations in the event that unexpected long range population, community or ecosystem effects are detected.

EPA believes that cultivation of transgenic plants expressing *Bt* Cry proteins may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, *Bt* crops require substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms. 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 plants expressing *Bt* Cry proteins is that the number of chemical insecticide applications for non-target pest control is reduced for crops with multiple pest problems.

b. 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 re-colonization 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.

c. Supplemental Data Needed

EPA has sufficient information to believe that there is no risk from the registered uses *Bt*11, MON810, TC1507 or TC6275 corn to non-target wildlife, aquatic and soil organisms and domesticated fowl and animals. 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. Similarly, 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).

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 OSCPP 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 OCSPP 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.

UPDATE (September 2010): Since the 2007 Rosi-Marshall et al. publication, numerous researchers have published peer-reviewed studies that identify issues with the scientific merit and relevance of the original caddis fly study (Swan et al. 2009, Jensen et al. 2010, summarized by Beachy et al. 2008, Parrott 2008, and Wolt and Peterson 2010). In response to comments received on the proposed terms and conditions for the extension of the 2010 expiring *Bt* corn registrations, EPA conducted a literature review of these recently published studies. Criticisms of the Rosi-Marshall et al. study included several findings: (1) adverse effects were not caused by toxicity of Cry1A but, rather, by other differences between plant test substances (Jensen et al. 2010); (2) the abundance of Trichoptera in streams containing residues of Cry1A was not reduced (Chambers et al. 2007); and (3) while post-harvest crop residue was identified as the most likely route of exposure (Carstens et al. 2010), aquatic exposure to biotech crops has been

shown to be limited temporally and spatially with low to negligible exposure concentrations of Cry proteins in post-harvest crop tissues (Swan et al. 2009, Griffiths et al. 2009, Jensen et al. 2010, Wolt and Peterson 2010, Carstens et al. 2010). In light of these results, EPA is waiving the requirement for additional aquatic invertebrate studies to assess hazard to aquatic shredder species for existing Cry protein PIP registrations.

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D. Insect Resistance Management

1. Introduction

Insect resistance management (IRM) is the term used to describe practices aimed at reducing the potential for insect pests to become resistant to a pesticide. *Bt* IRM is of great importance because of the threat insect resistance poses to the future use of *Bt* plant-incorporated protectants and *Bt* technology as a whole. Specific IRM strategies, such as the high dose/structured refuge strategy, will mitigate insect resistance to specific *Bt* proteins produced in corn. Academic

scientists, public interest groups, organic and other farmers have expressed concern that the widespread planting of these genetically transformed plants will hasten the development of resistance to pesticidal *Bt* endotoxins. Effective insect resistance management can reduce this risk of resistance development. This section provides EPA's scientific assessment of *Bt* corn plant-incorporated protectant IRM strategies by reviewing the data and information available to the Agency. The Agency has used this assessment, the report of the FIFRA SAP meeting on October 18, 2000, and all public comments in its development of its risk management decisions for *Bt* corn plant-incorporated protectants.

The following list will assist the reader with the acronyms for the insect pests discussed in this section.

Acronym	Common Name	Scientific Name	Crop
BCW	Black Cutworm	*Agrotis ipsilon* (Hufnagel)	corn
CEW	Corn Earworm	*Helicoverpa zea* (Boddie)	corn
CSB	Common Stalk Borer	*Papaipema nebris* (Guen.)	corn
ECB	European Corn Borer	*Ostrinia nubilalis* (Huebner)	corn
FAW	Fall Armyworm	*Spodoptera frugiperda* (J. E. Smith)	corn
SCSB	Southern Corn Stalk Borer	*Diatraea crambidoides* (Grote)	corn
SWCB	Southwestern Corn Borer	*Diatraea grandiosella* (Dyar)	corn

a. Elements of IRM Plans

To address the real concern of insect resistance to *Bt* proteins, EPA has imposed IRM requirements on registered *Bt* plant-pesticides. Sound IRM will prolong the life of *Bt* pesticides and adherence to the plans is to the advantage of growers, producers, researchers, and the American public. EPA considers the development of *Bt*-resistant insects to constitute an adverse environmental effect. EPA's strategy to address insect resistance to *Bt* is two-fold: 1) mitigate any significant potential for pest resistance development in the field by instituting IRM plans, and 2) better understand the mechanisms behind pest resistance.

Scientific experts believe that a high dose and the planting of a refuge (a portion of the total acreage using non-*Bt* seed) will delay the development of insect resistance to *Bt* crops by maintaining insect susceptibility. In addition to a high dose and structured refuge, IRM plans include additional field research on pest biology, refuge size and deployment, resistance monitoring for the development of resistance (and increased insect tolerance of the protein), grower education, a remedial action plan in case resistance is identified, annual reporting and communication. IRM plans will change as more scientific data become available.

Beginning with the first *Bt* plant-pesticide registration, the Agency has taken steps to manage insect resistance to *Bt* with IRM plans being an important part of the regulatory decision. The Agency identified (later confirmed by the 1995 SAP) seven elements that should be addressed in a *Bt* plant-incorporated protectant resistance management plan: 1) knowledge of pest biology and ecology; 2) appropriate dose expression strategy; 3) appropriate refuge; 4) resistance monitoring and a remedial action plan should resistance occur; 5) employment of integrated pest management (IPM); 6) communication and education strategies on use of the product; and 7) development of alternative modes of action. IRM plans also include grower education and measurement of the level of compliance. Because IRM plans change as more scientific data become available, EPA has also imposed research data requirements as part of the terms and conditions of registration. EPA has also made changes to IRM requirements as the science has evolved.

b. High Dose/Structured Refuge Strategy

The 1998 Science Advisory Panel Subpanel agreed with EPA that an appropriate resistance management strategy is necessary to mitigate the development of insect resistance to *Bt* proteins expressed in transgenic crop plants. The 1998 Subpanel recognized that resistance management programs should be based on the use of both a high dose of *Bt* and structured refuges designed to provide sufficient numbers of susceptible adult insects. The high dose/refuge strategy assumes that resistance to *Bt* is recessive and is conferred by a single locus with two alleles resulting in three genotypes: susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR). It also assumes that there will be a low initial resistance allele frequency and that there will be extensive random mating between resistant and susceptible adults. Under ideal circumstances, only rare RR individuals will survive a high dose produced by the *Bt* crop. Both SS and RS individuals will be susceptible to the *Bt* toxin. A structured refuge is a non-*Bt* portion of a grower's field or set of fields that provides for the production of susceptible (SS) insects that may randomly mate with rare resistant (RR) insects surviving the *Bt* crop to produce susceptible RS heterozygotes that will be killed by the *Bt* crop. This will remove resistant (R) alleles from the insect populations and delay the evolution of resistance. The 1998 and 2000 SAP Subpanels noted that insect resistance management strategies should also be sustainable and to the extent possible, strongly consider grower acceptance and logistical feasibility.

Although the high dose/refuge strategy is the preferred strategy for IRM, effective IRM is still possible even if the transformed plant does not express the *Bt* protein at a high dose for all economically-important target pests (e.g., by increasing refuge size). The lack of a high dose could allow partially resistant individuals (i.e., heterozygous insects with one resistance allele) to survive, thus increasing the frequency of resistance genes in an insect population. For this reason, numerous IRM researchers and expert groups have concurred that non-high dose *Bt* expression presents a substantial resistance risk relative to high dose expression (Roush 1994, Gould 1998, Onstad & Gould 1998, SAP 1998, ILSI 1998, UCS 1998, SAP 2001).

The 1998 SAP Subpanel defined (and the 2000 SAP Subpanel confirmed) a high dose as "25 times the protein concentration necessary to kill susceptible larvae." The logic for this approach is spelled out in the 1998 SAP report as well as in the scientific literature on insect resistance

management for *Bt* crops. In essence, *Bt* cultivars must produce a high enough toxin concentration to kill nearly all of the insects that are heterozygous for resistance. The Agency has adopted the 25X definition of high dose proposed by the 1998 SAP Subpanel.

The 1998 SAP Subpanel noted that a *Bt* plant-incorporated protectant could be considered to provide a high dose if verified by at least two of the following five approaches: 1) Serial dilution bioassay with artificial diet containing lyophilized tissues of *Bt* plants using tissues from non-*Bt* plants as controls; 2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar determined by quantitative ELISA or some more reliable technique; 3) Survey large numbers of commercial plants in the field to make sure that the cultivar is at the $LD_{99.9}$ or higher to assure that 95% of heterozygotes would be killed (see Andow & Hutchison 1998); 4) Similar to #3 above, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} value similar to field strains; and 5) Determine if a later larval instar of the targeted pest could be found with an LD_{50} that was about 25-fold higher than that of the neonate larvae. If so, the later stage could be tested on the *Bt* crop plants to determine if 95% or more of the later stage larvae were killed. The 2000 SAP concluded that the *Bt* corn PIPs registered at that time had *Bt* titers that would significantly exceed the 25X criteria for control European corn borer.

As an alternate definition for high dose, Caprio et al. (2000) recommend that a higher, 50-fold value be adopted (rather than 25-fold) because current empirical data suggest that a 25-fold dose may not be consistently high enough to cause high mortality among heterozygotes with known *Bt* resistance alleles. The 2000 SAP Subpanel did not recommend changing the existing 25-fold definition, but noted that the "25X" definition is imprecise, provisional, and may require modification as more knowledge becomes available about the inheritance of resistance. The Subpanel concluded that current *Bt* corn varieties have less than a 25-fold dose for CEW.

The size, placement, and management of the refuge is critical to the success of the high dose/structured refuge strategy to mitigate insect resistance to the *Bt* proteins produced in corn. The 1998 Subpanel defined structured refuges to "include all suitable non-*Bt* host plants for a targeted pest that are planted and managed by people. These refuges could be planted to offer refuges at the same time when the *Bt* crops are available to the pests or at times when the *Bt* crops are not available." The 1998 Subpanel suggested that a production of 500 susceptible adults in the refuge for every adult in the transgenic crop area (assuming a resistance allele frequency of 5×10^{-2}) would be a suitable goal. The placement and size of the structured refuge employed should be based on the current understanding of the pest biology data and the technology. The 1998 SAP Subpanel also recognized that refuges should be based on regional pest control issues. The 2000 SAP Subpanel echoed the 1998 SAP's recommendations that the refuge should produce 500:1 susceptible to resistant insects and that regional IRM working groups would be helpful in developing policies.

c. Predictive Models

EPA has used predictive models to compare IRM strategies for *Bt* crops. Because models cannot be validated without actual field resistance, models have limitations and the information gained

from the use of models is only a part of the weight of evidence used by EPA in assessing the risks of resistance development. It was the consensus of the 2000 SAP Subpanel that models were an important tool in determining appropriate *Bt* crop IRM strategies. They agreed that models were "the only scientifically rigorous way to integrate all of the biological information available, and that without these models, the Agency would have little scientific basis for choosing among alternative resistance management options." They also recommended that models must have an agreed upon time frame for resistance protection. For example, conventional growers may desire a maximum planning horizon of five years, while organic growers may desire an indefinite planning horizon. The Subpanel recommended that model design should be peer reviewed and parameters validated. Models should also include such factors as level of *Bt* crop adoption, level of compliance, economics, fitness costs of resistance, alternate hosts, spatial components, stochasticity, and pest population dynamics.

EPA's Office of Research and Development (ORD), National Risk Management Research Laboratory and Office of Pesticide Programs held a small expert group workshop in June, 2001 that focused on model design and parameter validation for *Bt* corn IRM. This workshop was the first in a series of public workshops intended to provide EPA with information on developing a standardized framework for evaluating *Bt* corn IRM. These workshops provided direction for the development of model verification and validation procedures (described in Glaser et al. 2009).

A number of models have been developed to assess resistance management for *Bt* cropping systems. The Agency's use of predictive models is also discussed in Matten & Reynolds (2003) and a draft report by ORD (2009).

d. Resistance Monitoring

The need for proactive resistance detection and monitoring is critical to the survival of *Bt* technology. The Agency mandates that registrants monitor for insect resistance (measurement of resistance-conferring alleles) to the *Bt* toxins as an important early warning sign to developing resistance in the field and whether IRM strategies are working. Grower participation (e.g., reports of unexpected damage) is also important for monitoring. Resistance monitoring is also important because it provides validation of biological parameters used in models. However, resistance detection/monitoring is a difficult and imprecise task. It requires both high sensitivity and accuracy. Good resistance monitoring should have well-established baseline susceptibility data prior to introduction of *Bt* crops. The chances of finding a resistant larva in a *Bt* crop depend on the level of pest pressure, the frequency of resistant individuals, the location and number of samples that are collected, and the sensitivity of the detection technique. Therefore, as the frequency of resistant individuals or the number of collected samples increases, the likelihood of locating a resistant individual increases (Roush & Miller 1986). If the phenotypic frequency of resistance is one in 1,000, then more than 3,000 individuals must be sampled to have a 95% probability of one resistant individual (Roush & Miller 1986). Current sampling strategies have a target of 100 to 200 individuals per location. Previous experience with conventional insecticides has shown that once resistant phenotypes are detected at a frequency >10%, control or crop failures are common (Roush & Miller 1986). Because of sampling

limitations and monitoring technique sensitivity, resistance could develop to *Bt* toxins prior to it being easily detected in the field.

The 2000 SAP Subpanel concluded that resistance monitoring programs should be peer reviewed and used to assess the success of current IRM plans. EPA's Office of Research and Development, National Risk Management Research Laboratory and Office of Pesticide Programs held a small expert group workshop in July, 2001 that focused on resistance monitoring plan design and detection techniques for *Bt* corn IRM.

Each of the following monitoring techniques described below have a number of advantages and disadvantages:

1) Grower Reports of Unexpected Damage

Growers can be encouraged to report any unsuspected control problems to a local technical expert. Toll-free telephone numbers and an Internet site can be provided by registrants to report any unusual control problems. A confirmed grower report of unexpected pest damage in a *Bt* crop may be a way to document a control failure and may be a useful monitoring system for determining the success or failure of existing resistance management strategies. However, once a grower detects a control failure, and resistance has been verified, the only available response may be to alter existing resistance management strategies.

2) Systematic Field Surveillance

Registrant sponsored surveys of grower *Bt* fields for damaged plants have been used to monitor resistance allele frequency of the development of resistance and gauge the geographic area where resistant populations exist. An in-field detection system (for quick determination of the presence or absence of Cry proteins in corn plants) has been developed for most *Bt* corn PIPs.

3) Discriminating Concentration Assay / Diagnostic Dose

The discriminating dose/diagnostic dose bioassay is currently required by the EPA. Discriminating dose bioassays are most useful when resistance is common or conferred by a dominant allele (resistance allele frequency >1%) (Andow & Alstad 1998). It should be considered as one of the central components of any monitoring plan, but other monitoring methods may have value in conjunction with the discriminating concentration assay.

Of the techniques available, the diagnostic dose has been the best developed and most thoroughly tested. Hawthorne et al. (2001) consider the diagnostic dose to be less expensive than in-field screens and the F_2 screen. It is best used when the frequency of resistance alleles is high ($>10^{-2}$) or the resistant allele is dominant. However, it is unclear (and likely pest-specific) whether resistance is carried by dominant or recessive alleles and what the frequency of resistance alleles are in pest populations. Measurement of low resistant allele frequencies ($<10^{-2}$) would not be possible using the diagnostic dose without extremely large sample sizes.

The October 2000 SAP Subpanel was asked whether the current resistance monitoring plans were adequate. They indicated that the diagnostic or discriminating dose technique could at best detect resistance when the resistance allele frequency has reached 1%. This is a level in which some field failure may be observed. At this lower level of precision, the least expensive methods are the discriminating dose assays (see U.S. EPA/USDA 1999, p. 47, Figure 3). Previous experience with conventional insecticides has shown that once resistant phenotypes are detected at a frequency >10%, control or crop failures are common (Roush & Miller 1986). If resistance is carried on a recessive allele, the frequency of individuals in a population that demonstrate resistance will equal the square of the allele frequency. For example, if the initial resistance allele frequency is one in 1,000, then one would need to assay more than a million larvae to find one homozygous resistant individual. Typically, discriminating dose assays are based on 100-300 larvae to detect resistance at a frequency of 1-3% (Roush & Miller 1986).

4) F_2 Screen

The F_2 screen may be a useful monitoring technique for *Bt* corn, especially for the detection of rare recessive resistant alleles. The technique also allows fewer samples to be collected to detect potential susceptibility shifts than the discriminating dose assay. The F_2 screen may be most useful to analyze populations that are expected to be at high risk to the development of resistance. Each isofemale line allows for characterization of four genomes, thus improving the sensitivity over the discriminating dose assay by 10-fold (Andow & Alstad 1998). The F_2 screen could be an effective method for detecting changes in the allele frequency of a recessive or partially recessive allele and can be used to verify some of the assumptions underlying high dose/refuge resistance management (Andow & Alstad 1998, Andow et al. 1998). If resistance alleles are found, they can be characterized to estimate the fitness of the genotypes, determine whether there is a cost of resistance, and enable predictions of the evolution of resistance. The F_2 screen is conducted by sampling mated females from natural populations, rearing the progeny of each female as an isofemale line and sib-mating her F_1 larvae using an appropriate screening procedure such as a discriminating concentration assay or *Bt* crop, and performing statistical analysis. Hawthorne et al. (2001) indicate that the F_2 screen is probably the only current method available to detect rare recessive alleles.

A number of the October, 2000 SAP Subpanel members indicated that the F_2 screen accompanied by field screening "could be very effective for detecting low frequencies of recessive and dominant resistance alleles." The F_2 screen can be a powerful method for detecting rare recessive alleles in natural populations. As described by Andow and Alstad (1998), it relies on inbreeding field-collected individuals so that all recessive alleles are expressed in the F_2 generation where they can be screened for the phenotype of interest. This method has been used to estimate the frequency of resistance to Cry toxins from *Bacillus thuringiensis* in ECB (Andow et al. 1998, 2000, Bourguet et al. submitted), *Scirpophaga incertulas* (Walker) (rice stem borer) (Bentur et al. 2000), and *Plutella xylostella* (L.) (diamondback moth) (Zhao et al. 2001).

Andow and Alstad (1998) also provide a statistical method for estimating the probability that the screen erroneously does not identify the targeted resistance allele. This is the probability of a

false negative, and its calculation is based on the probability of inheritance of the allele, the assumption that F_1 families mate randomly, and the probability that other mortality factors may interfere with the phenotypic evaluation of the F_2 individuals.

Current insect resistance management strategies assume that resistance alleles are initially rare. That is, it is assumed that *Bt* resistance alleles are $<10^{-3}$ for the high dose/refuge strategies currently used for Bt crops. Studies using the F_2 screen by Andow et al. (1998, 2000) and Andow and Alstad (1998, 1999) indicate that resistance alleles may be present at frequencies $<9 \times 10^{-3}$ in southern Minnesota and $<3.9 \times 10^{-3}$ in central Iowa. A F_2 screen of 1,200 isofemale lines of ECB collected in France and in the northern U.S. Corn Belt during 1999 and 2000 indicated that the frequency of resistance alleles in France was less than 1.27×10^{-3} with 95% certainty and in the U.S. was less than 1.24×10^{-3} with 95% certainty (Bourguet et al. 2001). These collective data support the assumption that the frequency of *Bt* resistance alleles in natural populations of ECB is less than 10^{-3}, validating one of the key assumption of the high dose/refuge strategy.

Using the F_2 screen would increase the probability of detecting rare resistant alleles and the threshold of detection would be lowered to <0.005. A sample of 100 female lines has a precision of ± 0.0025 for dominant alleles and ± 0.0025 for recessive resistance alleles. Leaving aside issues of accuracy, the theoretically best resolution of allele frequency is ± 0.0025 for dominant alleles and ± 0.05 for recessive alleles for a screen of 100 larvae using the discriminating dose (see Andow in U.S. EPA/USDA 1999, p. 42-43).

The time-frame to respond before control failures occur depends on the precision of monitoring and the recessivity/dominance of resistance. If the goal of resistance monitoring is to detect resistance at a low enough resistance allele frequency so that changes to the insect resistance management plan can be made to increase the longevity of the product and prevent field failure, then current resistance monitoring plans need refinement. The F_2 screen is one method of refinement that can detect and measure resistance at frequencies of ≤ 0.005 for approximately $5000 per site. This level of precision can provide seven to 12 years to respond with alternative resistance management tactics (see Andow in U.S. EPA/USDA 1999b, p.47, Figure 1b).

A potential obstacle to the F_2 screen is that it may be labor intensive and not suitable for routine screening purposes (Hawthorne et al. 2001). Andow has conducted a cost analysis for various monitoring techniques and has concluded that in general the F_2 screen is more expensive than other methods for detecting dominant resistant alleles when the resistance allele frequency is >0.01 (see Andow in U.S. EPA/USDA 1999b, p. 49, Figure 3). It is estimated that 750-1200 family lines must be screened to have a 95% probability of detecting a dominant resistance allele that is a frequency of 10^{-3} and would cost $13.90-19.70 per family line (Andow et al. 1998, 2000). However, for recessive alleles, Andow estimates that the F_2 screen is the least expensive method and can estimate resistance allele frequencies to a high level of precision (<0.005) for under $5,000 per location (see U.S. EPA/USDA 1999b, p. 41-49). Hawthorne et al. (2001), on the other hand, estimated the cost for each F_2 screen to be $14,000 to $20,000 per population. This, they conclude, would be too expensive for routine monitoring efforts, especially if there is

replication at each site. The area of cost and cost-effectiveness of the F_2 screen should be further evaluated.

Hawthorne et al. (2001) concluded that there is a need to further evaluate the precision and accuracy of the F_2 screen by using colonies with known frequencies of resistance alleles. Zhao et al. (2001) also came to this same conclusion. They validated the F_2 screen using a synthetic laboratory population of the diamondback moth (*Plutella xylostella* L.) for detecting the frequency of rare resistance alleles to Cry1Ac and Cry1C toxins of *Bt*. When using *Bt* broccoli as the diagnostic method, only one F_2 family was detected for Cry1Ac resistance and no family was detected for Cry1C resistance. Six families were detected for either Cry1Ac or Cry1C resistance using the diagnostic diet bioassay. Four F_2 families were confirmed to contain one copy of an allele resistant to Cry1Ac in the original single-pair matings and four other F_2 families contained an allele resistant to Cry1C. These results suggest that transgenic plants expressing a high level of a *Bt* toxin in a F_2 screen may underestimate the frequency of resistance alleles with false negatives or fail to detect true resistance alleles. The authors concluded that the diagnostic diet assay was a better F_2 screen method to detect resistance alleles, especially for the Cry1Ac resistance in diamondback moth. Zhao et al. (2001) conclude that further validation of the F_2 screen method for each insect-crop system should be conducted before the procedures used in the F_2 screen could be used routinely to detect rare *Bt* resistance alleles in field populations.

5) Screening Against Test Stocks

Gould et al. (1997) used a series of genetic crosses with test stocks of highly resistant tobacco budworm (*Heliothis virescens*; TBW) selected on Cry1Ac in the laboratory to estimate the resistance allele frequency in a natural population. This method can identify recessive or incompletely dominant resistance alleles from field-collected males. Using a colony of TBW that can survive on transgenic *Bt* cotton producing the Cry1Ac delta endotoxin, they crossed field-collected males with virgin-colony females so that all F_1 progeny would be heterozygous for resistance. By using an assay that discriminates between heterozygotes, they could establish which wild males carried a resistance allele. Using this allelic recovery method, Gould et al. (1997) estimated the resistance allele frequency to be 1.5×10^{-3}. This method is only useful when there are previously identified resistance alleles.

6) Sentinel *Bt*-Crop Field Plots

Venette et al. (2000) proposed the use of an in-field screen to examine resistance allele frequency. This method uses Bt sweet corn to screen for European corn borer and corn earworm that are resistant to the *Bt* protein. That is, the *Bt* crop is the discriminatory screen for resistant individuals. By sampling large numbers of *Bt*-expressing plants for live corn borer larvae, the frequency of resistance can be estimated and resistant individuals can be collected for documentation of resistance. For example, Venette et al. (2000) suggest that sampling ears (18-21 days post-silking stage) for European corn borer can increase sampling efficiency by two-orders of magnitude (over splitting stalks). Late-planted sentinel *Bt* sweet corn would provide a highly attractive oviposition site for females and reduce the number of plants required to attain an acceptable sample size. If the *Bt* sweet corn is planted at the appropriate time, larval attack

will cause extensive damage, and large areas of Bt sweet corn can be sampled rapidly by examining this damage. For example, if 10 resistant larvae are found in a sample of 5,000 *Bt* corn ears, and 50 larvae are recovered from 50 plants in the non-*Bt* field, then the expected phenotypic frequency of resistance would be 0.002. If potential resistant individuals or populations are identified in the field then they still must be brought to the laboratory so that resistance can be documented and quantified. Hawthorne et al. (2001) commented that the in-field screen coupled to a F_2 screen for verification of resistance might be an efficient method to detect resistance and capture resistance alleles especially in designated high-risk areas. The in-field screening method described by Venette et al. (2000) might be an alternative approach used for early detection of rare *Bt*-resistant phenotypes as well as an alternative method to estimate the initial frequency of resistance alleles.

There are potential problems with this method that must be addressed prior to its widespread adoption as discussed by Hawthorne et al. (2001). There is a high number of false positives that would reduce the efficiency and accuracy of resistance allele measurement. One source of false positives is the occurrence of weakly or non-expressing "off-type" plants among the sampled plants. Hawthorne et al. (2001) note that GeneCheck™ strips can be used to eliminate many of these "off-types." Another source might be surviving susceptible larvae that are incorrectly scored as resistant larvae because of larval movement between *Bt* and non-*Bt* off-types or weeds. A second problem is that there might not be sweet corn varieties that contain the same *Bt* genes as the field corn varieties. This would reduce the efficiency of sampling. Currently, there is only BT11 Cry1Ab field corn and sweet corn. As noted by Hawthorne et al. (2001), there are also additional concerns related to the large effort needed during harvest to complete an in-field screen. This type of effort limits its practicality.

e. Compliance with IRM requirements

Grower compliance with refuge and IRM requirements is a critical element for resistance management. Significant non-compliance with IRM among growers may increase the risk of resistance for *Bt* corn. However, it is not known what level of grower non-compliance will compromise the risk protection of current refuge requirements.

The Agency recognizes that compliance is a complex issue for *Bt* crops and IRM. There is currently disagreement as to the appropriate refuge size/deployment and the level of grower compliance necessary to achieve risk reduction. EPA considers the development of *Bt*-resistant insects to constitute an adverse environmental effect, therefore, IRM, and subsequently grower compliance, is very important. Optimally, refuge requirements would change over time as pest susceptibility changes. However, changes to refuge requirements are difficult to implement. Therefore, the Agency must set safe refuge requirements that preserve the pest(s) susceptibility and protect the benefits of *Bt* crops. Currently, the financial burden of implementing these refuge requirements is borne primarily by the growers. Increasing refuge size and/or limiting refuge deployment to better mitigate the risk of resistance is likely to increase costs to growers and result in a higher rate of grower non-compliance. Grower compliance with IRM strategies for current *Bt* crops is tied into the belief that new technologies, such as plants expressing multiple *Bt* toxins and other new synthetic insecticides, will reduce the risk of resistance.

To minimize the effects of non-compliance, it may be necessary to develop a broad compliance program as part of an IRM strategy. Ideally this program would include four major objectives: 1) an understanding of the effect of non-compliance on IRM; 2) identification of compliance mechanisms to maximize adoption of IRM requirements; 3) measurement of the level of compliance; and 4) establishment of an enforcement structure to ensure compliance and penalize non-compliance.

1) Effects of Non-Compliance on IRM

As a first step towards developing a compliance program, it is necessary to understand the impact of non-compliance on the development of pest resistance (i.e., the level of non-compliance that significantly increases the likelihood of resistance). In the past, many of the models that have been developed to evaluate refuge and resistance scenarios have assumed 100% compliance. However, based on existing surveys of grower compliance (discussed later in this section), it is unlikely that 100% compliance can be achieved. On the other hand, research and modeling work may show that some level of non-compliance can be tolerated without significantly increasing the risk of pest resistance. Models also tend to assume 100% adoption of the *Bt* technology. Compliance and adoption are both important factors that should be considered. Ultimately, many models may need to be updated to reflect some degree of non-compliance, so that the potential impact can be more thoroughly understood. Some later simulation models submitted to the Agency in support of *Bt* corn registrations have incorporated variable compliance percentages and alternate adoption scenarios.

2) Compliance Mechanisms

There have been a number of compliance mechanisms proposed by various parties (including the 2000 SAP Subpanel) to ensure grower conformity, reward compliance, and penalize non-compliance. These include such techniques as: grower contracts, grower certification tests, fines and other penalties, community refuge, sales incentives, crop insurance of the refuge, deposit/refund for planting refuge, databases of non-compliant growers, county/area-wide compliance goals and sales restrictions, intensified grower education, and grower audits. The 2000 SAP noted that, at present, there is little information on the relative effectiveness of different compliance options and that many mechanisms have both benefits and drawbacks. The potential efficacy of compliance mechanisms may depend on the perspective of the grower. For example, if non-compliance is the result of confusion over the requirements, increased education may be of value. However, if non-compliance is a willful act, then a punishment or incentive-based approach may be more appropriate (Hurley & Mitchell 2000). The 2000 SAP consensus was that compliance would be best managed through education and grower contracts, but also that sales incentives, refuge insurance, and refuge deposit/refund programs may have value if managed properly. Also, the 2000 SAP recognized that mechanisms that would reduce the cost of compliance will be more effective at improving compliance.

Mitchell et al. (2000) developed a model to evaluate crop (refuge) insurance and sales incentives as potential compliance mechanisms. The cost to growers (i.e., lost yield, higher inputs) to

adhere to IRM requirements can be an impediment to compliance. Therefore, by providing growers with incentives to reduce the cost of refuge mandates, compliance may be increased. Both insurance and sales incentives have the potential to reduce this cost of compliance to growers, although both have drawbacks as well. For refuge insurance to be profitable for private insurance companies, it would likely be too expensive for growers and would provide limited benefits. Sales incentives may be less costly to administer, but would require frequent, costly monitoring to ensure proper refuge implementation.

3) Measurements of Compliance

To assess the effectiveness of a compliance program, it is necessary to be able to accurately measure the level of grower compliance. The 2000 SAP noted several parties, other than the registrant alone, could verify compliance: 1) grower visits by industry, EPA, state authorities, USDA, or other third-parties; or 2) USDA/NASS or other third-party grower surveys. To date, compliance has been primarily measured through grower surveys conducted by industry or academics (e.g., Pilcher & Rice 1997, Rice & Pilcher 1999). However, the 2000 SAP indicated that while surveys such as these are useful for tracking grower attitudes, they are not always reliable for determining actual grower compliance. The format of the surveys (mail or phone interviews) may encourage non-compliant growers to misrepresent their actions or "cheat" in their responses. Without confirmatory visits to individual farms (i.e., audits), it is impossible to verify the accuracy of grower responses. The end result may be increased "false-positives," which may artificially inflate estimates of grower compliance. As such, actual non-compliance may be significantly higher than the survey results would suggest. To resolve this problem, the 2000 SAP suggested utilizing surveys created and conducted by independent parties to assess grower practices. In addition to this recommendation, it may be useful to conduct some on-farm visits for firsthand verification of compliance. Such visits could be performed as part of a survey process, to evaluate the accuracy of grower survey responses. The use of mapping systems, such as the Global Positioning System (GPS), may also prove useful for determining the size and position of *Bt* and non-*Bt* fields for compliance verification. The Arizona Cotton Research and Protection Council (ACRPC) has utilized GPS with Bt cotton grown in Arizona in conjunction with grower visits to assess the level of refuge compliance (Carrière et al. 2001).

4) Enforcement Structure / Penalties for Non-compliance

For a compliance program to be effective, a regulatory enforcement/compliance framework will be needed. Appropriate stakeholders and regulatory bodies will need to create clearly defined roles for compliance. At the present time, EPA's authority is over the product registrations and registrants but not individual growers. Registrants have been responsible for compliance at the grower level through the use of grower contracts. However, the 2000 SAP noted that EPA's reliance on industry to monitor and enforce compliance "was seen as a major problem." The SAP recommended that a third party compliance monitoring program should be developed. The compliance monitoring program should be accompanied by an appropriate enforcement program. Potential penalties for non-compliance might include: 1) sales restrictions at a county, state, regional, or national level; 2) sales prohibitions to specific growers; 3) registrant fines and

warnings; and 4) increased refuge for specific non-compliant growers (through grower contracts).

A compliance program has been developed for *Bt* corn as a term of registration. Details of this plan, including compliance surveys and enforcement mechanisms, are described in section D.2.b.7.

2. Corn

A summary of the Agency's risk assessment of insect resistance development and insect resistance management plans to mitigate resistance is provided in this section for *Bt* corn. Other Agency risk assessments of insect resistance management are found in A. Reynolds and R. Rose (OPP/BPPD) to M. Mendelsohn (OPP/BPPD), dated September 11, 2000. Subsequent information has been added to the Agency's risk assessment of insect resistance development and IRM plans following the October 18-20, 2000 SAP meeting as new data became available.

The Agency's IRM assessment focuses on Cry1Ab field corn, Cry1Ab sweet corn, and Cry1F field corn. EPA has used the best available scientific information in its IRM assessment and has updated its IRM position as additional information and data have become available.

In 1995, at the time of the initial registrations of *Bt* corn, there was no scientific consensus on the details of the IRM plans necessary for prevention of the development of resistance in the two primary target pests, ECB and CEW. At that time, the putative values for adequate refuge size ranged from 0% to 50% of non-*Bt* corn or other host plants per farm. While the minimum adequate refuge size or structure could not be determined until further research was conducted, it was thought that market penetration of these crops would be sufficiently slow that considerable non-*Bt* corn would remain to act as natural refuges while the additional research was conducted. Thus, the initial *Bt* corn registrants instituted voluntary IRM plans with the requirement that these registrants must submit a refuge strategy by April 1999. From 1995-1997, the registrants agreed to various voluntary refuge requirements in the Corn Belt (0% to 20%).

Since 1995, all *Bt* corn registrations have included a resistance monitoring plan for ECB and CEW that contained the following elements: 1) development of baseline susceptibility responses and a discriminating concentration to detect changes in sensitivity; 2) routine surveillance; and 3) remedial action if there is suspected resistance. One of the key purposes of resistance monitoring is to learn whether a field control failure resulted from resistance or other factors that might inhibit expression of the *Bt* delta-endotoxin. The extent and distribution of resistant populations can be mapped and alternative control strategies implemented in areas in which resistance has become prevalent. If monitoring techniques are sensitive enough to discriminate between resistant and susceptible individuals, it should be possible to detect field resistance before significant loss of efficacy and eliminate any resistant individuals using other control tactics. In addition, EPA mandated that all registrants must require customers to notify them of incidents of unexpected levels of target pest damage. Registrants are required to investigate these reports and identify the cause of the damage by local field sampling of the plant tissue and suspect insect populations followed by appropriate in vitro and *in planta* assays. Any confirmed incidents of

resistance are required to be reported to EPA. Based on these investigations, appropriate remedial action is required to mitigate pest resistance. These remedial actions include: informing customers and extension agents in the affected areas of pest resistance, increasing monitoring in the affected areas, implementing alternative means to reduce or control pest populations in the affected areas, implementing a structured refuge in the affected areas, and cessation of sales in the affected and bordering counties. All registrants have instructed growers to have regular surveillance programs and report any unexpected levels of target pest damage. Since 1995, the Agency is aware of no evidence of ECB, CEW or SWCB field resistance to any of the *Bt* proteins produced in corn (though populations of ECB have been found to be tolerant in laboratory assays – see section D.2.b.4 for information). In January 2000, the Agency required that the registrants provide a more detailed resistance monitoring plan that focused on ECB, CEW, and SWCB. The registrants provided the Agency with a revised monitoring plan in March 2000. This monitoring plan and subsequent updates are discussed in detail later in this section.

Based on the 1998 SAP Subpanel recommendations, the Agency began to institute mandatory refuge requirements on *Bt* field corn and popcorn products. In 1999, a coalition of *Bt* corn registrants (working with the National Corn Growers Association), the Agricultural Biotechnology Stewardship Technical Committee (ABSTC), approached EPA with a uniform IRM plan for their products. With some modifications to this plan, EPA put in place a consistent set of required refuge strategies for all *Bt* field corn products beginning with the 2000 growing season. These requirements greatly strengthened the IRM plan to mitigate ECB, CEW, and SWCB resistance to *Bt* proteins produced in field corn. Beginning with the 2000 growing season, EPA required a 20% non-*Bt* field corn refuge to be planted within ½ mile (<1/4 mile in areas where insecticides have been historically used to treat ECB and SWCB) (EPA letter to *Bt* corn registrants, 1/31/00). EPA also required a 50% non-*Bt* field corn (<½ mile, 1/4 mile preferred) refuge for *Bt* Cry1Ab field corn products in certain southern counties and states where most cotton is grown (EPA letter to *Bt* corn registrants, 1/31/00). The larger refuge was necessary to mitigate the development of resistance to *Bt* proteins in CEW populations feeding on both corn and cotton. These same refuge requirements were mandated for the Cry1F field corn products registered in May 2001.

a. Current Insect Resistance Management (IRM) Plans for *Bt* Corn

1) MON 810, BT11 (Cry1Ab) and TC 1507 (Cry1F) Field Corn

Registered MON 810, BT11, and TC 1507 products are known to produce a "high dose" for ECB based on the 25 x definition described by the 1998 SAP Subpanel (SAP 1998) and confirmed by the 2000 SAP Subpanel (SAP 2001). The terms and conditions for each of these registrations contain a complete description of the IRM requirements. Details are provided on the specific requirements for refuge (size and structure), resistance monitoring, remedial action, compliance assurance, grower education, and annual IRM reports. A summary of the refuge requirements (as amended in 2008 and 2009) for the Cry1Ab and Cry1F *Bt* corn plant-incorporated protectant is described in Table D1.

Table D1. Summary of Current *Bt* Field Corn Refuge Requirements

Active Ingredient	ECB Dosage	Refuge Size in Corn Belt	Refuge Size in Cotton Areas	Grower Agreement	Proximity	In-field strip refuges
MON 810, BT 11, & TC 1507	High dose	20% sprayed or unsprayed	50% sprayed or unsprayed	Required	< ½ mile of Bt field	At least 4 rows wide

2) BT11 Sweet Corn (Cry1Ab)

A key to understanding the resistance management issues with Attribute BT11 sweet corn is to appreciate the differences in the cultural practices of sweet corn versus field corn. Field corn is frequently grown in large blocks on farms of 500 - 1,000 acres. This results in large areas of field corn monoculture. Conversely, sweet corn is usually grown in blocks of 40 acres or less on farms that produce several crops that are also host plants for ECB and CEW.

In contrast to BT11 field corn, specific refuge requirements were not mandated for this *Bt* sweet corn product because sweet corn harvesting occurs before insects mature and reproduce. Sweet corn is harvested 18-21 days after silking while the plant has active photosynthesis. As a result, in transgenic sweet corn varieties, *Bt* protein production is high at the time of harvest. EPA mandated specific resistance monitoring requirements for ECB, CEW, and FAW, as well as sales reporting requirements. Syngenta is required through labeling and technical material to have growers destroy any Cry1Ab (BT11) sweet corn stalks that remain in the fields following harvest in accordance with local production practices. Stalk destruction is intended to reduce the possibility of any insects, including resistant insects, surviving to the next generation. The complete IRM requirements for sweet corn are detailed in the terms and conditions for Attribute corn (amended in December, 2008).

b. Analysis of the Risks Associated with Current IRM Plans and Alternatives

The risk that insect pests may become resistant to *Bt* plant-incorporated protectants and *Bt* microbial sprays has been acknowledged by many organizations and individuals including EPA's Scientific Advisory Panel (SAP) and Pesticide Program Dialogue Committee (PPDC). SAP meetings and reports in 1995, 1998, and 2000 have confirmed that EPA's approach and elements required in an insect resistance management plan are appropriate. EPA believes that pest biology and the dose of the *Bt* protein expressed in the various plant tissues influence the size and placement needed for an effective refuge. This section is a summary of the key elements of several options for IRM plans for corn and compares the level of risk of resistance development for each scenario. An additional Agency assessment of the IRM plan for *Bt* corn can be found in the Agency's memorandum, A. Reynolds and R. Rose (OPP/BPPD) to M. Mendelsohn (OPP/BPPD), dated September 11, 2000. IRM for TC 1507 was reviewed in R.Rose (OPP/BPPD) memorandum to M. Mendelsohn (OPP/BPPD) dated January 24, 2001. Subsequent information has been added to the Agency's risk assessment of insect resistance development following the October 18-20, 2000 SAP meeting and as new data became available.

The Agency's IRM program for *Bt* corn has also been described in Matten et al. (2004). This section has been updated (2010) to incorporate resistance monitoring, compliance, and other data submitted since 2001 for the Cry1Ab and Cry1F corn registrations.

1) Pest Biology

Knowledge of pest biology is critical for the development of effective IRM strategies and to increase confidence that the IRM plans will be effective at reducing the likelihood that insects will become resistant to *Bt* proteins.

a) European Corn Borer (Primary Target Pest)

European Corn Borer (ECB, *Ostrinia nubilalis*) is a major pest of corn throughout most of the United States. The pest has 1-4 generations per year, with univoltine (i.e., one generation per year) populations in the far North (i.e., all of North Dakota, northern South Dakota, northern Minnesota, and northern Wisconsin), bivoltine (i.e., two generations per year) populations throughout most of the Corn Belt, and multivoltine (3-4 generations) populations in the South (Mason et al. 1996). The February, 1998 SAP meeting on IRM identified a number of areas needing additional research including larval movement, adult movement, mating behavior, pre- and post-mating dispersal, ovipositional behavior, fitness, and overwintering habitat (SAP 1998). Since the first registrations of *Bt* corn hybrids in 1995, a significant amount of research has been undertaken in many of these areas, although additional work could enhance the knowledge base for this pest. A summary of key aspects of ECB biology that relate to IRM is presented below:

i. Larval Movement

ECB larvae are capable of significant, plant-to-plant movement within corn fields. Research conducted in non-transgenic corn showed that the vast majority of larvae do not move more than two plants within a row (Ross & Ostlie 1990). However, in transgenic corn, unpublished data (used in modeling work) from F. Gould (cited in Onstad & Gould 1998) indicates that approximately 98% of susceptible ECB neonates move away from plants containing *Bt*. Recent multi-year studies by Hellmich (1996, 1997, 1998) have attempted to quantify the extent of plant-to-plant larval movement. It was observed that 4th instar larvae were capable of movement up to six corn plants within a row and six corn plants across rows from a release point. Movement within a row was much more likely than movement across rows (not surprising, due to the fact that plants within are row are more likely to be "touching" as opposed to those across rows). In fact, the vast majority of across row movement was limited to one plant. This type of information has obvious implications for optimal refuge design. Larvae moving across *Bt* and non-*Bt* corn rows may be exposed to sublethal doses of protein, increasing the likelihood of resistance (Mallet & Porter 1992). Given the extent of ECB larval movement between plants, seed mixes have been determined to be an inferior refuge option (Mallet & Porter 1992, SAP 1998, Onstad & Gould 1998).

ii. Adult Movement

Information on movement of adult ECB (post-pupal eclosion) is necessary to determine appropriate proximity guidelines for refuges. Refuges must be established within the flight range of newly emerged adults to help ensure the potential for random mating. An extensive, multi-year project to investigate ECB adult dispersal has been undertaken by the University of Nebraska (Hunt et al. 1997, 1998a). Results from these mark and recapture studies (with newly emerged, pre-mated adults) showed that the majority of ECB adults did not disperse far from their emergence sites. The percentage recaptured was very low (< 1%) and the majority of those that were recaptured were caught within 1500 feet of the release site. Few moths were captured outside of 2000 feet. These results have specifically led to recommendations and guidelines for refuge proximity and deployment (discussed later in this document).

iii. Mating Behavior

In addition to patterns of adult movement, ECB mating behavior is an important consideration to insure random mating between susceptible and potentially resistant moths. In particular, it is important to determine where newly emerged females mate (i.e., near the site of emergence or after some dispersal).

It is well established that many ECB take advantage of aggregation sites (usually clusters of weeds or grasses) near corn fields for mating. Females typically mate the second night after pupal eclosion (Mason et al. 1996). One recent study suggested that it may be possible to manipulate aggregation sites to increase the likelihood of random mating between susceptible and potentially resistant ECB (Hellmich et al. 1998). Another recent study (mark/recapture studies with newly eclosed ECB) conducted by the University of Nebraska showed that relatively few unmated females moved out of the corn field from which they emerged as adults (Hunt et al. 1998b). This was especially true in irrigated (i.e., attractive) corn fields. In addition, a relatively high proportion of females captured close to the release point (within 10 feet) were mated. This work suggests that females mate very close to the point of emergence and that refuges may need to be placed very close to *Bt* fields (or as in-field refuges) to maximize the probability of random mating.

In terms of male mating behavior, a study by Showers et al. (2001) looked at male dispersal to locate mates. The study was carried out using mark-recapture techniques with pheromone-baited traps placed at 200, 800, 3200, and 6400 m from a release point. Results showed that males in search of mates were trapped more frequently at traps placed at 200 m from the release site. However, significant numbers were also trapped at 800 m or greater from the release site (Showers et al. 2001). Similar to Hunt et al., this work suggests that refuges may need to be placed relatively close to *Bt* fields to maximize random mating.

iv. Ovipositional Behavior

ECB ovipositional (egg-laying) behavior is important for refuge design. For instance, if oviposition within a corn field is not random, certain types of refuge (i.e., in-field strips) may not be effective.

After mating, which occurs primarily in aggregation sites, females move to find suitable corn hosts for oviposition. Most females will oviposit in corn fields near the aggregation sites, provided there are acceptable corn hosts. Oviposition begins after mating and occurs primarily at night. Eggs are laid in clusters of up to sixty eggs (one or more clusters is deposited per night) (Mason et al. 1996).

It is known that females generally prefer taller and more vigorous corn fields for oviposition (Beck 1987). This has implications for refuge design. To avoid potential host discrimination among ovipositing females, the non-*Bt* corn hybrid selected for refuge should similar to the *Bt* hybrid in terms of growth, maturity, yield, and management practices (i.e., planting date, weed management, and irrigation). It should be noted that research has shown no significant difference in ovipositional preferences between *Bt* and non-*Bt* corn (derived from the same inbred line) when phenological and management characteristics are similar (Orr & Landis 1997, Hellmich et al. 1999). Within a corn field suitable for egg laying, oviposition is thought to be random and not restricted to border rows near aggregation sites (Shelton et al. 1986, Calvin 1998).

v. Host Range

ECB is a polyphagous pest known to infest over 200 species of plants. Among the ECB plant hosts are a number of species of common weeds, which has led some to speculate that it may be possible for weeds to serve as an ECB refuge for *Bt* corn. In response to this, a number of recent research projects have investigated the feasibility of weeds as refuge. Studies conducted by Hellmich (1996, 1997, 1998) have shown that weeds are capable of producing ECB, although the numbers were variable and too inconsistent to be a reliable source of ECB refuge. This conclusion was also reached by the 1998 SAP Subpanel on IRM. In addition to weeds, a number of grain crops (e.g., wheat, sorghum, oats) have been investigated for potential as a *Bt* corn ECB refuge (Hellmich 1996, 1997, 1998, Mason et al. 1998). In these studies, small grain crops generally produced less ECB than corn (popcorn or field corn) and are unlikely to produce enough susceptible adult insects.

b) Corn Earworm

As was the case with ECB, the 1998 SAP identified a number of research areas that need additional work with corn earworm (CEW, *Helicoverpa zea*). In addition to increased knowledge regarding larval/adult movement, mating behavior, and ovipositional behavior, a better understanding of movement between corn/cotton and long distance migration is also needed (SAP 1998). Additional research regarding CEW biology has occurred since 1998. These data have been submitted as part of the annual research reports required as a condition of registration. The Agency has reviewed these data and has concluded that additional information would be useful for effective long-term improvements of IRM strategies to mitigate CEW resistance.

i. Host Range and Corn to Cotton Movement

CEW is a polyphagous insect (3-4 generations per year), feeding on a number of grain and vegetable crops in addition to weeds and other wild hosts. Typically, it is thought that CEW feeds on wild hosts and/or corn for two generations (first generation on whorl stage corn, second generation on ear stage corn). After corn senescence, CEW moves to other hosts, notably cotton, for 2-3 additional generations. By utilizing multiple hosts within the same growing season, CEW presents a challenge to *Bt* resistance management in that there is the potential for double exposure to *Bt* protein in both *Bt* corn and *Bt* cotton (potentially up to five generations of exposure in some regions).

Given the wide host range of CEW, it has been speculated that wild hosts (weeds) and other non-*Bt* crops (e.g., soybean) may be able to serve as refuge for CEW. However, research into the value of these alternate hosts as reliable producers of CEW is still lacking (1998 SAP).

ii. Overwintering Behavior

CEW are known to overwinter in the pupal stage. Although it is known that CEW migrate northward during the growing season to corn-growing regions (i.e., the U.S. Corn Belt and Canada), CEW typically are not capable of overwintering in these regions. Rather, CEW are known to overwinter in the South, often in cotton fields. Temperature, moisture, and cultivation practices are all thought to play some role in the overwintering survival of CEW (Caprio & Benedict 1996).

Overwintering is an important consideration for IRM--resistant insects must survive the winter to pass their resistance genes on to future generations. In the Corn Belt, for example, CEW incapable of overwintering should not pose a resistance threat. Given that different refuge strategies may be developed based upon where CEW is a resistance threat, accurate sampling data would help to precisely predict suitable CEW overwintering areas.

iii. Adult Movement and Migration

CEW is known to be a highly mobile pest, capable of significant long distance movement. Mark/recapture studies have shown that CEW moths are capable of dispersing distances ranging from 0.5 km (0.3 mi.) to 160 km (99 mi.) (some migration up to 750 km (466 mi.) was also noted) (Caprio & Benedict 1996). The general pattern of migration is a northward movement, following prevailing wind patterns, with moths originating in southern overwintering sites moving to corn-growing regions in the northern U.S. and Canada.

It has been assumed that CEW migration proceeds progressively northward through the course of the growing season. However, observations made by Dr. Fred Gould (N.C. State University) indicate that CEW may also move southward from corn-growing regions back to cotton regions in the South (described in remarks made at the 1999 EPA/USDA Workshop on *Bt* Crop Resistance Management in Cotton, Memphis, TN 8/26/99). If this is true (and more investigation is needed for confirmation of this effect), the result may be additional CEW exposure to *Bt* crops. In addition, the assumptions regarding CEW overwintering may need to be revisited--moths that were thought to be incapable of winter survival (and thus not a resistance

threat) may indeed be moving south to suitable overwintering sites.

Most CEW flight movement is local, rather than migratory. Heliothine moths move primarily at night, with post-eclosion moths typically flying short distances of less than 200 m (Caprio & Benedict 1996). However, as was indicated by the 1998 SAP, additional research would be useful, particularly as it pertains to CEW and optimal refuge design. On the other hand, given the long distance movements typical of CEW and the lack of high dose in *Bt* corn hybrids, the 2000 SAP noted that refuge placement for this pest is of less importance than with other pests (e.g., ECB) (SAP 2001).

iv. Mating/Ovipositional Behavior

Dr. Michael Caprio (entomologist, Mississippi State University) has indicated that there is significant localized mating among females (i.e., within 600 m (1969 ft.) of pupal eclosion), typically with males that emerged nearby or moved in prior to female eclosion (Caprio 1999). CEW females typically deposit eggs singly on hosts. A recent study (conducted in cotton fields) found that 20% of the eggs found from released CEW females were within 50-100 m (164-328 ft.) of the release point, indicating some localized oviposition. However, males were shown to be able to move over 350 m (1148 ft.) to mate with females (Caprio 2000). These data indicate that, in terms of CEW, refuges may not have to be embedded or immediately adjacent to a *Bt* field to be effective (although the data do not exclude these options). Additional research with mating and ovipositional behavior would provide useful information for CEW IRM.

v. Larval Movement

CEW larvae, particularly later instars, are capable of plant-to-plant movement. At the recommendation of the SAP (1998), EPA has not evaluated seed mixes as a viable refuge option for CEW.

c) Southwestern Corn Borer and Other Secondary Pests

Some southwestern corn borer (SWCB, *Diatraea grandiosella*) pest biology data have been provided as part of the annual research reports required as a condition of registration. However, there is still relatively limited information available and more data on SWCB pest biology would be beneficial to help develop IRM strategies for regions in which SWCB and ECB are both pests of economic concern. The 1998 SAP also noted the relative lack of information for SWCB, concluding that "[c]ritical research is needed for SWCB...including: short-term movement, long-distance migration, mating behavior relative to movement (i.e. does mating occur before or after migration)..." Because of this, it is unknown whether IRM strategies designed for ECB (another corn boring pest) will also function optimally for SWCB.

SWCB is an economic pest of corn in some areas (i.e., SW Kansas, SE Colorado, north Texas, west Oklahoma) and can require regular management. Like ECB, SWCB has 2-4 generations and similar feeding behavior. First generation larvae feed on whorl tissue before tunneling into stalks before pupation, while later generations feed on ear tissue before tunneling into stalks.

Females typically mate on the night of emergence and can lay 250-350 eggs (Davis 2000).

Research to investigate the movement patterns of SWCB has been initiated (Buschman et al. 1999). In this mark/recapture study, the following observations were made regarding SWCB from the 1999 data: 1) more males than females were captured at greater distances from the release point (similar to ECB); 2) most recaptures of SWCB were within 100 feet of the release site, although some were also noted at 1200 feet; and 3) the moth movement patterns for ECB and SWCB appear to be similar in most regards. Given these results, it is likely that this part of the IRM strategy (refuge proximity guidelines established for ECB) will also be applicable to SWCB. However, the 1999 results were hampered by low SWCB numbers available for testing and the authors have indicated that this work will continue during the 2000 season.

Research for other secondary pests (e.g., black cutworm, fall armyworm, southern corn stalk borer, others) is also lacking and could be useful for specific regions in which these pests may pose an additional concern. However, the 1998 SAP indicated that CEW and SWCB should have the highest priority for biology research among the secondary corn pests.

2) High Dose

A high level of *Bt* protein expression (termed "high dose") is considered to be an essential aspect of high dose/structure refuge strategy to mitigate the risk of *Bt* resistance. The lack of a high dose could allow partially resistant (i.e., heterozygous insects with one resistance allele) to survive, thus increasing the frequency of resistance genes in an insect population. For this reason, numerous IRM researchers and expert groups have concurred that non-high dose *Bt* expression presents a substantial resistance risk relative to high dose expression (Roush 1994, Gould 1998, Onstad & Gould 1998, SAP 1998, ILSI 1998, UCS 1998). To mitigate the additional resistance risk of a non-high dose *Bt* corn product, alternate refuge strategies (i.e., larger refuges) may need to be developed.

The 1998 and 2000 SAPs defined high dose as "25 times the protein concentration necessary to kill susceptible larvae" and provided five techniques to verify high dose (defined earlier in this document). However, the 2000 SAP noted that this definition is imprecise, provisional, and may require modification as more knowledge becomes available about the inheritance of resistance (SAP 2001). It is also important to consider protein expression over the course of the growing season as some *Bt* corn hybrids may not maintain a steady level of protein expression over the season. The 1998 SAP noted these concerns indicating that the "toxin concentration encountered by the pest" should be the true measure.

Among the currently registered *Bt* corn products, most have been evaluated to determine high dose (via the 1998 SAP verification techniques) for ECB (the primary target pest). It is likely that BT11, MON 810, and TC 1507 corn have a high dose for ECB. It is also known that none of the currently registered *Bt* corn products expresses a high dose for CEW (CEW is known to be less susceptible to *Bt* proteins than other targeted lepidopteran pests). High dose evaluations for other secondary pests (i.e., SWCB, FAW, etc.) have been sporadic. Ideally, high dose could be evaluated for all susceptible pests, so that appropriate resistance management strategies could be

developed. However, verification of the high dose using the 1998 SAP Subpanel techniques may be best directed at the major target pests of *Bt* corn (ECB, CEW, and SWCB), due to the fact that these pests play a larger role in the formulation of IRM strategies. Below, each registered *Bt* corn product is discussed individually in regard to high dose (as defined by the 1998 SAP) for each of the labeled target pests. It is not expected that label claims of "control" or "suppression" for individual target pests are indicative of high dose.

a) Syngenta BT11 Cry1Ab Corn

According to grower guides, BT11 corn is targeted against ECB (claims of "control"), SWCB ("control"), CEW ("control" of 1st generation, "suppression" of 2nd gen.), FAW ("suppression"), and SCSB ("suppression").

Syngenta has not submitted any data to the Agency to confirm high dose, via the 1998 SAP guidelines, for any of the targeted pests. However, the Agency is able to conclude that BT11 probably produces a season-long high dose for ECB based on the review of all available data submitted to the Agency. Submitted studies have shown consistent control of ECB from the whorl stage to kernel maturity (VanDuyn et al. 1997, Catangui & Berg 1998). BT11 has also been shown to be effective against late instar ECB (Walker et al. 2000).

For CEW, several submitted studies suggest that BT11 does not contain a season-long high dose. These studies revealed excellent control of first generation CEW on whorl stage BT11 but also showed significant survival of second generation CEW on BT11 corn ears (Dively & Horner 1997, VanDuyn et al. 1997). However, in both studies, surviving second generation CEW showed fitness costs (i.e., reduced weight and delayed developmental time). Other research has shown similar results (VanDuyn et al. 1998).

For SWCB, no information on the potential for high dose has been submitted to the Agency. For FAW, one submitted study with BT11 showed good control during whorl stage, but significant infestation during ear stage (Benedict et al. 1998). It is therefore unlikely that BT11 contains a full season high dose for FAW. For SCSB, one study with a limited data set has been submitted, showing good control (VanDuyn 1998). With additional data, it may be possible to confirm whether BT11 contains a high dose for SCSB.

b) Monsanto MON 810 Cry1Ab Corn

According to grower guides and product labels, MON 810 is targeted against ECB (claim of "control"), SWCB ("control"), SCSB ("control"), CEW ("suppression"), CSB ("suppression"), and FAW ("suppression").

For ECB, Monsanto has submitted information to verify (with the 1998 SAP guidelines) that MON 810 expresses a high dose (reviewed by EPA, R.Rose/S.Matten memo to M.Mendelsohn, 5/30/99). SAP techniques #2, 3, and 5 were utilized to confirm the high dose expression.

For SCSB, submitted research showed that MON 810 provided good control versus non-*Bt* corn

(VanDuyn et al. 1997, VanDuyn 1998, VanDuyn et al. 1998), although there was not enough information (due to low pest pressure in the tests) to determine if there is a high dose expression. With additional data, it may be possible to determine whether there is a high dose expression for control of SCSB.

For CEW, submitted studies have shown significant larval survival on MON 810 corn, particularly in ear stage corn (Dively et al. 1997, Dively & Horner 1997, VanDuyn et al. 1997, Benedict et al. 1998, VanDuyn et al. 1998). Therefore, it is unlikely that MON 810 expresses a season-long high dose for CEW. For FAW, MON 810 was found to have good whorl stage control, but significant ear infestation later in the season (Benedict et al. 1998). Given this, and the known lower sensitivity of FAW to Cry1A proteins, it is unlikely that MON 810 has a season-long high dose for FAW. High dose has not been verified for SWCB or CSB with the 1998 SAP techniques. With additional data, it may be possible to verify whether there is a high dose expression for control of SWCB or CSB.

c) Pioneer and Dow TC 1507 Cry1F Field Corn

TC 1507 is targeted against ECB, BCW, FAW, and SWCB (label claims "control" of these pests).

For ECB, data have been submitted to demonstrate high dose (using the 1998 SAP criteria - techniques #4 and #5) (MRID# 451311-01; reviewed in R.Rose memo to M.Mendelsohn, 1/24/01).

Other submitted data showed that TC 1507 provided good protection against SWCB and FAW, although insufficient information was submitted to determine high dose (MRID# 450201-14; reviewed in R.Rose memo to M.Mendelsohn, 1/24/01). This same data also showed some damage to TC 1507 plants from CEW and BCW. It is unlikely that TC 1507 expresses a high dose for these pests.

d) Syngenta Attribute Cry1Ab Sweet Corn

Attribute sweet corn is targeted against ECB, CEW, and FAW. Attribute contains the same *Bt* gene as the BT11 hybrid.

For ECB, like BT11, it is probable that Attribute sweet corn expresses a high dose, although it has not been verified with the SAP criteria. Research submitted to EPA specifically for Bt sweet corn has shown virtually no survival of ECB (Dively & Linduska 1998).

For FAW and CEW, it is less likely that *Bt* sweet corn will express a high dose. Several submitted studies have shown (limited) FAW and CEW survival and damage on Attribute *Bt* sweet corn (Dively & Linduska 1998, Whalen & Spellman 1999, Lynch et al. 1999).

The current knowledge base for high dose expression is summarized in the following table.

Table D2. High Dose Summary

HYBRID	SEASON-LONG HIGH DOSE FOR CORN PESTS					
	ECB	CEW	SWCB	FAW	SCSB	CSB
BT11	Probable	NO	Unknown	NO	Unknown	Unknown *
Bt Sweet Corn (BT11)	Probable	NO	Unknown*	NO	Unknown *	Unknown *
MON 810	YES	NO	Unknown	NO	Unknown	Unknown
TC 1507	YES	NO	Unknown	Unknown	Unknown *	Unknown *

YES = high dose verified with 1998 SAP recommended techniques; NO = information indicates that no high dose is likely; Probable = information indicates high dose likely (but not verified by SAP guidelines); Unknown = no or insufficient information available for high dose determination; * = untargeted pest

3) Refuge

The February 1998 and October 2000 FIFRA SAP Subpanels agreed that a high dose/refuge strategy is necessary to mitigate target insect resistance to *Bt* field corn (SAP 1998, 2001). A structured refuge should be planted and managed to produce 500 insects susceptible to *Bt* for every one potentially resistant insect. Refuge options should address regional differences and varying levels of the dose of *Bt* in the crop that effect refuge management as well as the need for feasibility and flexibility for the growers. However, if there is not a high dose for the primary target pests, the risk of resistance increases. Larger refuges, increased monitoring, and possible sales restrictions may be used to mitigate some or all of this risk.

a) Deployment of Refuges

There have been a number of approaches proposed for the optimal design of refuges for *Bt* corn. These include external blocks, in-field strips, seed mixes, temporal refuge strategies, and non-corn hosts. A number of research projects have been undertaken to identify the most appropriate refuge design.

i. Hosts for the Refuge

Non-*Bt* field corn should provide the best refuge to increase the probability that susceptible insects will mate with potentially resistant ECB from the *Bt* corn. Non-*Bt* corn hybrids used as refuges should be selected for growth, maturity, fertility, irrigation, weed management, planting date, and yield traits similar to the *Bt* corn hybrid. Hybrids that are not agronomically similar may result in different developmental times in corn pests that could lead to assortive (non-random) mating between plants in refuge and *Bt* fields.

Research has shown that temporal and alternate host, non-corn refuges (e.g., weeds, oats, alfalfa, soybeans) are inadequate strategies (Rice et al. 1997, Ostlie et al. 1997b, Calvin et al. 1997, Mason et al. 1998, Hellmich 1998). In addition, non-*Bt* popcorn may also be viable as refuge for *Bt* corn (Hellmich 1998).

ii. Seed Mixes vs. In-Field Strips vs. External Blocks

The NC-205 group has recommended three options for refuge placement relative to *Bt* corn: blocks planted adjacent to fields, blocks planted within fields, or strips planted within fields (Ostlie et al. 1997). In general, refuges may be deployed as external blocks on the edges or headlands of fields or as strips within the *Bt* corn field.

Research has shown that ECB larvae are capable of moving up to six corn plants within or between rows with the majority of movement occurring within a single row. Later instar (4th and 5th) ECB are more likely to move within rows than between rows (Hellmich 1998). This is a cause for concern because heterozygous (partially resistant) ECB larvae may begin feeding on *Bt* plants, then move to non-*Bt* plants (if planted nearby) to complete development, thus defeating the high dose strategy and increasing the risk of resistance. For this reason, seed mixes (refuge created by mixing seed in the hopper) have been discouraged as possible ECB refuges (Mallet & Porter 1992, Buschman et al. 1997).

Buschman et al. (1997) suggested that the within field refuge is the ideal strategy for an IRM program. Since the ECB larvae tend to move within rows, the authors suggest intact corn rows as an acceptable refuge. Narrow (filling one or two planter boxes with non-*Bt* corn seed) or wide strips (filling the entire planter with non-*Bt* seed) may be used as in-field refuges. Data indicate that in-field strips may provide the best opportunity for ECB produced in *Bt* corn to mate with ECB from non-*Bt* corn. Since preliminary data suggests that the refuge should be within 100 rows of the *Bt* corn, Buschman et al. (1997) recommended alternating strips of 96 rows of non-*Bt* corn and 192 rows of *Bt* corn. This would result in a 33% refuge that is within 100 rows of the *Bt* corn.

In-field strips (planted as complete rows) should extend the full length of the field and include a minimum of six rows planted with non-*Bt* corn alternating with a *Bt* corn hybrid. NC-205 has recommended planting six to 12 rows of non-*Bt* corn when implementing the in-field strip refuge strategy (NC 205 Supplement 1998). The 2000 SAP also agreed that, due to larval movement, wider refuge strips ($\geq$ 6 rows) are superior to narrower strips, although planter sizes may restrict strip sizes for some smaller growers (SAP 2001). In-field strips may offer the greatest potential to ensure random mating between susceptible and resistant adults because they can maximize adult genetic mixing. Modeling indicates that strips of at least six rows wide are as effective for ECB IRM as adjacent blocks when a 20% refuge is used (Onstad & Guse 1999). However, strips that are only two rows wide might be as effective as blocks, but may be more risky than either blocks or wider strips given our incomplete understanding of differences in survival between susceptible borers and heterozygotes (Onstad & Gould 1998).

Given the concerns with larval movement and need for random mating, either external blocks or in-field strips (across the entire field, at least 6 rows wide) are the refuge designs which may provide the most reduction in risk of resistance development. Research indicates that random mating is most likely to occur with in-field strip refuges.

iii. Proximity

The issue of refuge proximity is a critical variable for resistance management. Refuges must be located so that the potential for random mating between susceptible moths (from the refuge) and possible resistant survivors (from the *Bt* field) is maximized. Therefore, pest flight behavior is a critical variable to consider when discussing refuge proximity. Refuges planted as external blocks should be adjacent or in close proximity to the *Bt* corn field (Onstad & Gould 1998, Ostlie et al. 1997b). NC-205 initially recommended that refuges should be planted within ½ sections (320 acres) (NC-205 Supplement 1998). Subsequently, the recommendation was revised to specify that non-*Bt* corn refuges should be placed within 1/2 mile of the *Bt* field (1/4 mile "would be even better") (Ortman 1999).

Hunt et al. (1997) completed a study which suggests that the majority of ECB do not disperse far from their pupal emergence sites. According to this mark-recapture study, the majority of ECB may not disperse more than 1500 to 2000 feet. A majority (70-98%) of recaptured ECB were trapped within 1500 feet of the release point. However, in an addendum to the 1997 study, the authors caution that the 1500 foot distance does not necessarily represent the maximum dispersal distance for ECB (Hunt et al. 1998a).

Another mark-recapture ECB project was devoted to within-field movement of emerging ECB (in particular unmated females) (Hunt et al. 1998b). Relatively few unmated females were recaptured (10 over the entire experiment), although the majority of those were found within 85 ft of the release point. This suggests that unmated females may not disperse far from the point of pupal eclosion (this was especially true in the irrigated field). In addition, a relatively high proportion of mated females (31%) in irrigated fields were trapped within 10 feet of the release point, suggesting that mating occurred very close to the point of emergence. Both of these observations indicate that many emerging ECB females may not disperse outside of their field of origin. With respect to resistance management and refuge proximity, these results suggest that refuges should be placed in close proximity to *Bt* corn fields (or as in-field refuge) to increase the chance of random mating (especially for irrigated fields).

In terms of male ECB dispersal, another mark-recapture study by Showers et al. (2001) showed that males dispersing in search of mates may move significant distances (> 800 m). However, a greater percentage of males were trapped at closer distances (200 m) to the release point. Based on this research, the authors suggest that, in terms of male movement, the current refuge proximity guidelines of ½ mile should be adequate to ensure mating between susceptible moths and any resistant survivors from the *Bt* field.

While it is clear that ECB dispersal decreases further from pupal emergence points, the quantitative dispersal behavior of ECB has not been fully determined. However, in terms of optimal refuge placement, it is critical that refuge proximity be selected to maximize the potential for random mating. Based on Hunt et al. data, the closer the refuge is to the *Bt* corn, the lower the risk of resistance. Since the greatest number of ECB were captured within 1500 feet of the field and most females may mate within ten feet of the field, placing refuges as close to the *Bt* fields as possible should increase the chance of random mating and decrease the risk of resistance. The proximity requirement for *Bt* corn refuge was initially established as ½ mile (1/4

mile in areas where insecticides have been historically used to treat ECB and SWCB) (EPA letter to *Bt* corn registrants, 1/31/00). The 2000 SAP agreed with this guideline, stating that "...refuges should be located no further than a half mile (within 1/4 mile if possible) from the *Bt* corn field" (SAP 2001).

In 2008 and 2009, the *Bt* corn registrations were amended to simplify the distance proximity requirement to read "external refuges must be planted within ½ mile" (see EPA review in A. Reynolds memo to M. Mendelsohn, 7/9/08).

iv. Temporal and Spatial Refuge

The use of temporal and spatial mosaics has received some attention as alternate strategies to structured refuge to delay resistance. A temporal refuge, in theory, would manipulate the life cycle of ECB by having the *Bt* portion of the crop planted at a time in which it would be most attractive to ECB. For example, *Bt* corn fields would be planted several weeks before conventional corn. Because ECB are thought to preferentially oviposit on taller corn plants, the hope is that the *Bt* corn will be infested instead of the shorter, less attractive conventional corn. However, there are indications from experts in the field that temporal refuges are an inferior alternative to structured refuges (SAP 1998). Research has shown that planting date cannot be used to accurately predict and manipulate ECB oviposition rates (Calvin et al. 1997, Rice et al. 1997, Ostlie et al. 1997b, Calvin 1998). Local climatic effects on corn phenology make planting date a difficult variable to manipulate to manage ECB. Additional studies will have to be conducted under a broad range of conditions to fully answer this question. In addition, a temporal mosaic may lead to assortive mating in which resistant moths from the *Bt* crop mate with each other because their developmental time differs from susceptible moths emerging from the refuge (Gould 1994).

Spatial mosaics involve the planting of two separate *Bt* corn events with different modes of action. The idea is that insect populations will be exposed to multiple proteins, reducing the likelihood of resistance to any one protein. However, because many of the registered *Bt* corn products only express one protein and the primary pests of corn (ECB, CEW, SWCB) generally remain on the same plant throughout the larval feeding stages, individual insects will be exposed to only one of the proteins. In the absence of structured refuges producing susceptible insects, resistance may still have the potential to develop in such a system as it would in a single protein monoculture.

v. CEW North to South Movement and Refuge Issues

It is known that during the growing season CEW move northward from southern overwintering sites to corn-growing regions in the Corn Belt. However, as discussed in the pest biology section (D.2.b.1.b.iii), observations of CEW north to south migration (from corn-growing regions to cotton-growing regions) have been noted. Although more research is needed for confirmation, this phenomenon could result in additional exposure to *Bt* crops and increased selection pressure for CEW resistance. This effect is compounded by the fact that neither *Bt* cotton nor any registered single trait *Bt* corn event contains a high dose for CEW. As such, it may be necessary

to consider additional mitigation measures for CEW.

In considering this issue, the 2000 SAP indicated that CEW refuge is best considered on a regional scale (instead of structured refuge on an individual farm basis) due to the long distance movements typical of this pest (i.e., refuge proximity is not as important for CEW). According to the SAP, a 20% refuge (per farm) would be adequate for CEW, provided the amount of *Bt* corn in the region does not exceed 50% of the total corn crop. If the regional *Bt* corn crop exceed 50%, however, additional structured refuge may be necessary (SAP 2001). However, the SAP did not define what a "region" should be (i.e., county, state, or other division).

Based on the last available acreage data for *Bt* corn, it should be noted that a number of counties in the Corn Belt exceed the 50% threshold recognized by the 2000 SAP. Because of this, there may be additional risk for CEW resistance. As a condition of registration (EPA letters to *Bt* corn registrants, October 15, 2001), *Bt* corn registrants were required to investigate CEW north-south movement as it relates to resistance management and submit a report to EPA. To accomplish this task, the Agricultural Stewardship Technical Committee (ABSTC, a consortium representing *Bt* corn registrants) conducted 1) a review of studies on the extent of CEW north-south migration and 2) computer modeling to evaluate the effect of north-south migration on the risk of CEW adaptation to *Bt* corn and *Bt* cotton in a mixed cropping system. This information was submitted to the Agency in a report dated May 8, 2003 (no MRID number).

Fitt (1989) reviewed evidence for long-range migration of CEW in North America. Other noctuids, black cutworm (*Agrotis ipsilon*) and fall armyworm (*Spodoptera frugiperda*) have also been shown to migrate from southern overwintering areas to the Corn Belt. Long distance migration in all of these insects was associated with passage in weather fronts. Showers (1997) provided evidence for the return migration of *A. ipsilon* on southerly air mass flows in the fall from Iowa to Louisiana and Texas. Pair et al. (1987) presented evidence that CEW return migration can occur from north Texas to the Lower Rio Grande Valley.

Gould et al. (2002) provided indirect evidence for migration of CEW moths from the other corn-growing regions to the cotton-growing regions. Data were collected from two locations – Bossier Parish, Louisiana and College Station, Texas. The timing and extent of reverse migration appears to vary considerably from year to year (Gould et al. 2002, figs. 2, 3, and 4). Gould et al. (2002) pointed out that at the time of apparent reverse migration, there is very little vegetation that is capable of supporting larval development in Texas. This means that it is unlikely that any migrating moths (from the north) can contribute to the local over-wintering population. However, if the moths can move further south into more tropical areas in which they would contribute to the local over-wintering population then their genes could contribute to the persisting population. There may be locally suitable host plants in cotton-growing regions (other than in the examined area of Texas) such that a migrating population could be important.

ABSTC noted that Gould et al. (2002) did not address the point that cotton in the areas investigated is treated for CEW either by *Bt* PIPs, applied chemical insecticides, or both. *Bt* PIPs and insecticides are very effective at reducing boll damage and CEW larval survival by 80% or more. This means that the number of moths produced in cotton would be relatively small

compared to the number of eggs laid in cotton. The converse situation is true for Midwest corn, up to 25% of which is *Bt* corn, which is seldom if ever treated for CEW. ABSTC suggested that care should be taken when interpreting the Gould et al. (2002) data that the relative importance of selection in the Corn Belt versus the Cotton Belt not be overstated.

For the second part of the analysis, ABSTC used a computer model to quantify how migration may impact adaptation rates under a range of different circumstances. The spatially-explicit model of CEW adaptation to corn and cotton (Storer et al. 2003) was modified to incorporate south-north migration in the spring and north-south migration in the summer. Effectively, two models were run in parallel for the summer generations: one for the cotton-growing region and one for the corn-growing region. The Storer et al. (2003) model focused on CEW adaptation in eastern North-Carolina; however, alternations to the adaptation risk in that region are likely to be quantitatively similar to the alternations to the risk in other regions. The scenarios modeled were based on the available data and the conclusions of Gould et al. (2002).

The results of the modeling showed no significant interaction between the percent of the late summer adult CEW population in the south that is made up of immigrants and the date at which return migrants actually return. This means that even with 50-60% CEW migrating from the north (as inferred by Gould et al. 2002) and producing pupae, there was no effect of return migration on the resistance gene frequency.

There was, however, a weak interaction between the percentage of *Bt* corn planted in the north and return migration. At 80% *Bt* corn in the north, return migration increased the 15-year gene frequency from $2.2e^{-3}$ to $2.4e^{-3}$, an increase in adaptation rate of 2%. At 30% *Bt* corn in the north, return migration decreased the 15-year gene frequency from $2.3e^{-3}$ to $2.1e^{-3}$, a decrease in adaptation rate of 4%. Thus, even with extreme adoption rates of *Bt* corn of 80%, return migration had very little effect on the CEW adaptation rate in the south.

There was a trend for return migration to slow resistance evolution when investigating the percentage of insects moving north. This is due to the returning population having a lower resistance (r) allele frequency than the resident population. The main decrease in adaptation rate due to return migration was 5%.

Two of the parameters had significant main effects on the 15-year r-allele frequency: increasing the percentage of *Bt* corn or *Bt* cotton in the south increased the 15-year r-allele frequency. These effects are expected because the percentage of *Bt* corn and *Bt* cotton determine the intensity of selection exerted on the insects feeding in these two crops. At the highest level of *Bt* cotton deployment simulated (95%), selection in the south is most intense and return migrants are expected to reduce the adaptation rate by introducing a population that has experienced lower selection. However, this model suggests that even under this scenario, return migration would not significantly affect the rate of adaptation.

EPA reviewed ABSTC's report (S. Matten memo to M. Mendelsohn, 3/23/04) and agreed with ABSTC's analysis and conclusions. Based on the modeling studies submitted by ABSTC parameterized using the data in Gould et al. (2002), CEW reverse migration has no significant

impact (0.05<P) on CEW adaptation to *Bt* crops. Under current levels of *Bt* crop deployment (30% *Bt* corn and 60% *Bt* cotton), return migration is expected to slow adaptation by about four percent (4%). If *Bt* corn reached its maximum allowable level of 80% adoption in the Corn Belt, reverse migration would only increase the rate of adaptation by about two percent (2%) if *Bt* cotton was at 60% adoption (the low end of the range investigated). Even if extreme parameters were used in the model, reverse migration would be predicted to have a 10% impact on CEW adaptation. However, it is extremely unlikely that all of these parameter conditions would be met in the field year after year. Modeling studies indicated that the percentage of *Bt* cotton and *Bt* corn in the south significantly increased the 15-year r-allele frequency, but return migration did not significantly affect the rate of adaptation even when *Bt* cotton was at 95% adoption.

b) Refuge Options

This analysis of refuge options pertains only to single toxin PIPs expressing Cry1Ab or Cry1F. Refuge options for pyramided or stacked products containing these (and other) toxins are not discussed here.

i. High Dose Events; **MON 810, BT11, TC 1507 (Field Corn)**

Non-Cotton Growing Regions That Don't Spray Insecticides on a Regular Basis (e.g., Corn Belt)

This region encompasses most of the Corn Belt east of the High Plains. The original USDA NC-205 refuge recommendations included a 20-30% untreated structured refuge or a 40% refuge that could be treated with non-*Bt* insecticides (Ostlie et al. 1997a). In the case of ECB, the primary pest of corn for most of the U.S., it is known that on average less than 10% of growers use insecticide treatment to control this pest (National Center for Food and Agriculture Policy 1999). Due to the fact that many growers do not regularly treat for ECB, NC-205 modified their position in a May 24, 1999 letter to Dr. Janet Andersen (Director, BPPD). In this letter, NC-205 amended their recommendation to a 20% non-*Bt* corn refuge that may be treated with insecticides and should be deployed within 1/2 mile (1/4 mile is better) of the *Bt* corn. Specific recommendations in the letter were: "1) insecticide treatment of refuges should be based on scouting and accepted economic thresholds, 2) treatment should be with a product that does not contain *Bt* or Cry toxin, 3) records should be kept of treated refuges and shared with the EPA, 4) the potential impact of sprayed refuges should be monitored closely and evaluated annually, and 5) monitoring for resistance should be most intense in higher risk areas, for example where refuges are treated with insecticides" (Ortman 1999).

Since most growers (>90%) do not typically treat field corn with insecticides to control ECB, a refuge of 20% non-*Bt* corn that may be sprayed with non-*Bt* insecticides if ECB densities exceed economic thresholds should be viable for the Corn Belt. Refuges can be treated as needed to control lepidopteran stalk-boring insects with non-*Bt* insecticides or other appropriate IPM practices. Insecticide use should be based on scouting using economic thresholds as part of an IPM program.

Non-Cotton Growing Regions That Spray Insecticides on a Regular Basis (e.g., the High Plains

for SWCB)

NC-205 (1998) noted that there are some areas that regularly require insecticide treatment (e.g., the High Plains for SWCB or spider mites) and that separate refuge strategies may be needed for these regions. This is because highly effective insecticides may significantly reduce the number of susceptible adults emerging from the refuge. In a May 1999 letter sent to Dr. Andersen (BPPD Division Director), NC-205 stated: "A refuge management strategy that is more conservative than the one applied across the greater Corn Belt, yet less restrictive than the one proposed for areas growing both corn and cotton, may be most appropriate in the heavily treated areas jointly infested with SWCB and ECB" (Ortman 1999). The size of the refuge is based on the amount of non-*Bt* corn needed to produce 500 susceptible insects for every resistant insect. When insecticide sprays are used on the refuge, fewer susceptible insects are produced and the refuge area may need to be larger to produce the 500:1 ratio.

Entomologists from Kansas State University (Dr. Randy Higgins, Dr. Lawrence Buschman, and Dr. Phillip Sloderbeck) have indicated that the frequent use of highly effective insecticides in areas that are co-infested with both SWCB and ECB is the issue of concern rather than the mere presence of SWCB. Using highly effective insecticides in these areas will decrease the number of susceptible insects emerging from the refuge and reduce refuge efficacy (Buschman and Sloderbeck 1999; Higgins 1999). The 2000 SAP rationalized that a 20% refuge treated with an insecticide with high efficacy (>90% kill) will be equivalent to a 2% unsprayed refuge (SAP 2001). As a result of the Agency's new IRM requirements for *Bt* corn products for the year 2000, areas that are routinely treated with insecticides were specifically identified by the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) in a letter to Dr. Janet Andersen dated March 31, 2000. This area includes counties in southwest Kansas, southeast Colorado, and the Texas/Oklahoma Panhandle.

After reviewing the insecticide issue, the 2000 SAP concluded that insecticide use may negatively impact IRM if only the refuge (and not the *Bt* crop) is treated. The panel did not, however, reach a consensus on whether additional measures would be needed to mitigate the potential risk. Some panel members felt that additional refuge is needed in these areas, while others thought that current refuge requirements (20%) are adequate and would help maintain compliance. Another potential mitigation alternative proposed by the panel was to restrict insecticide use to allow for only those treatments that provide <70% kill. The panel also noted that additional information would be needed to define these areas and that the NC-205 group was looking at the issue and was planning to survey grower insecticide use practices (SAP 2001).

To address concerns about risks to IRM strategies, registrants of *Bt* field corn were required to investigate the potential impact of insecticide use on the effectiveness on non-*Bt* corn refuges as a condition of registration (EPA letters to *Bt* corn registrants, 10/15/01). A protocol was required to be submitted by March 15, 2002, followed by an interim report in 2003 and the final report in 2004. ABSTC submitted a protocol which was accepted by the Agency on May 8, 2002 (EPA letter to ABSTC). An interim report was subsequently submitted on May 8, 2003 and the final report was submitted on May 7, 2004 (no MRID number was assigned).

ABSTC employed two separate techniques to address the issue of insecticide use in refuges. The first was to conduct a survey of crop consultants to assess insecticide use in regions known to frequently treat for corn borers while the second method was to utilize models to simulate the effects of insecticide use on the effectiveness of refuges.

For the survey (conducted in 2002), a total of 220 questionnaires were mailed to crop consultants in three states where insecticides are frequently used (Nebraska, Kansas, and Colorado) with a response rate of 45% (96 total). Responses were divided by reporting regions and survey state quarters as follows: Southwest (SW Kansas, SE Kansas, and SE Colorado), West (NW Kansas, SW Nebraska, and NW Nebraska), and East (NE Kansas, SE Nebraska, and NE Nebraska). The southwest region experiences regular control issues with ECB, SWCB, and spider mites. The west region experiences ECB and spider mite infestations (but little SWCB), while the east region primarily has ECB control issues (and little SWCB or spider mite problems).

The results showed that the vast majority of corn grown in all three regions is irrigated with consultants overseeing an average of 9,978 acres. Consultants manage most of the corn in all three regions. The percentage of *Bt* corn acreage was lowest in the west region (31%) and higher in the southwest (47% irrigated, 29% non-irrigated) and highest in the east (54%). Insecticide use patterns were as follows:

> *Early Whorl Stage:* Insecticides were applied on early whorl stage, irrigated corn on 3.4% of Bt corn acreage and 7.5% of non-*Bt* corn acreage during the 2002 season. On non-irrigated corn, insecticide use was slightly less: 1.9% (*Bt*) and 3.8% (non-*Bt*). Treatments were applied on more acreage in the east region than the southwest or west regions, although the differences between regions were not significant. The historical (1998-2002) patterns were similar with 0.7% irrigated *Bt* corn, 9.6% irrigated non-*Bt* corn, 1.2% non-irrigated *Bt* corn, and 3.6% of non-irrigated, non-*Bt* corn acreage treated (the east region reported the highest percentage of treated acres).
>
> *Late Whorl Stage:* Insecticide use was significantly higher on irrigated, late-whorl stage corn. Overall, 26.7% of *Bt* corn and 44.9% of non-*Bt* corn acres were treated during 2002, with the highest insecticide use in the southwest region. For non-irrigated corn, insecticide use was much less: 1.5% *Bt* acreage and 1.0% non-*Bt* acreage. The survey authors attributed the lower insecticide use in non-irrigated corn to drought conditions. Of the treated acreage, a minority was treated exclusively for corn borers (most reported treating for both borers and other pests or exclusively for other pests) and single treatments were more common than repeated applications. The historical (1998-2002) patterns reflected the 2002 data, with 15.4% irrigated *Bt* corn, 31.2% irrigated non-*Bt* corn, 2.0% non-irrigated *Bt* corn, and 9.2% of non-irrigated, non-*Bt* corn acreage treated (the southwest region reported the highest percentage of treated acres). In both the 2002 and historical survey data, consultants generally reported treating small (i.e., <50%) percentages of their total acreage (most claimed to have treated <30%). However, the number of consultants who described treating 90-100% of their total acreage was highest in the southwest region.

In the second part of ABSTC's analysis of the effects of insecticide use on *Bt* corn refuges, the data generated in the crop consultant survey was incorporated into two resistance management models. ABSTC indicated that two models were used: Guse et al. (2002), which includes variables for irrigation and pest biology (dispersal) and Onstad et al. (2002) which allows for the evaluation of insecticide use in refuges. However, data were reported only for the Onstad model in the submission.

Data regarding insecticide efficacy and use frequency was obtained from the consultant survey and applied to the models. In terms of efficacy, a value of 70% was assigned based on the lower effectiveness of insecticides on corn-boring lepidoptera. In practice, eggs and neonate larvae are more likely to be exposed and killed by insecticide treatments, while later instar larvae frequently lodge themselves inside corn plants and escape exposure. For insecticide use frequency, a value of 0.25 was assigned (i.e., 25% of refuges treated). This value was derived from the consultant survey which indicated that less than 50% of non-*Bt* corn is treated and that the majority of those treatments are targeted at second generation corn borers. Therefore, with 50% of non-*Bt* corn acres treated for the second generation (i.e., 50% of the annual borer generations), the total net effect is 25% treatment of the functional refuge within a growing season. Non-irrigated corn was not included in the simulations due to the infrequent use of insecticides on those acres.

For dose expression, the definitions used by Onstad et al. (2002) for theoretical high dose (recessive resistance) and practical high dose (partially recessive resistance) were included in the simulation. Refuge sizes were set between 20 and 50% (the 50% value was derived from the total amount of non-*Bt* corn planted in the survey regions). Biological parameters for ECB and SWCB dispersal were also incorporated into the models.

Simulations were run for both SWCB and ECB. For SWCB, the Onstad model predicted that normal patterns of insecticide use (i.e., those identified in the crop consultant survey) would have no impact on refuge effectiveness. Effects were noted only if resistance was assumed to be dominant or insecticides were extremely effective (i.e., 99.9%), scenarios that are extremely unlikely for SWCB and *Bt* corn.

For ECB, the picture was somewhat more complicated. When the Onstad model was run with parameters discussed above (spray frequency = 0.25, insecticide efficacy = 70%), the time to resistance was reduced by a third (30 years to 20 years). However, the use of irrigated corn was a key factor in the simulations run in the Onstad model. ECB dispersal is more limited in irrigated corn than dryland corn, as demonstrated by Hunt et al. (2001). When the reduced ECB dispersal characteristic of irrigated fields was incorporated into the model, refuge treatment had no impact on resistance over a 100 year period. This revised simulation also utilized more conservative values for spray frequency (50% - all refuges treated for second generation ECB) and spray efficacy (90%).

EPA reviewed the data submitted by ABSTC (A. Reynolds memo to M. Mendelsohn, 7/6/04) on the IRM impacts of insecticides in refuges. Based on the crop consultant survey and simulation model (Onstad et al. 2002), it is unlikely that refuges for SWCB will be negatively affected by

insecticide use practices. For ECB, the model predicted that sprays on non-irrigated corn could decrease the time to resistance by a third. However, according to the consultant survey, non-irrigated corn is rarely treated (<10% of acreage) in the survey regions. For irrigated corn (more likely to be treated), detrimental effects on refuge from insecticide treatments were not observed, largely due to increased ECB dispersal. Overall, it is likely that any impact on potential ECB resistance from insecticide use in "high spray" regions will be small or negligible.

ii. High Dose (MON 810, BT11, and TC 1507) Field Corn Events in Cotton-Growing Regions

As part of their April 1999 and January 2000 submissions, the NCGA/Industry Coalition requested growers be required to plant a minimum of 20% non-*Bt* corn in the northern portion of the corn/cotton region. The northern corn/cotton region corresponds to northern Arkansas, Missouri Bootheel, northern Texas, and the states of North Carolina, Oklahoma, Tennessee and Virginia. A minimum 50% refuge of non-*Bt* corn was suggested for the southern portion of the corn/cotton-growing region. The southern corn/cotton region corresponds to the entire states of Alabama, Florida, Georgia, Louisiana, Mississippi, South Carolina, as well as southern Texas and southern Arkansas.

Cotton-growing regions represent a higher risk for resistance due to the potential double exposure of CEW to both *Bt* corn (Cry1Ab, Cry1F) and *Bt* cotton (Cry1Ac) during the same growing season. Dr. Mike Caprio (Mississippi State University) developed a corn-cotton ecosystem model for resistance evolution in CEW to *Bt*-endotoxins expressed in plants to examine the movement of CEW between corn, cotton, soybean, and other wild hosts (Caprio 1997). In the model, the presence of *Bt* cotton (160 fields) and the ratio of *Bt* corn/non-*Bt* corn fields (120 total fields) are important factors. As the ratio of non-*Bt* corn decreases relative to *Bt* corn, the time to resistance also decreases; meaning that less non-*Bt* corn planted as a refuge results in quicker resistance. This effect was most pronounced when the percent of *Bt* to non-*Bt* corn exceeded 50%. Caprio's model suggests that even without cross-resistance as a variable, a sizable proportion of non-*Bt* corn (at least 50%) should be planted with *Bt* corn in *Bt* cotton growing regions to avoid the quick evolution of resistance. The years to resistance are also impacted by the percent of Bt cotton relative to Bt corn. A second model, developed by Storer et al. (1999), has also examined CEW resistance in corn/cotton regions (represented by eastern North Carolina). This model showed that resistance can develop rapidly when the percentage of *Bt* cotton is high relative to *Bt* corn (which is true for some northern cotton growing regions), underscoring the need for robust refuge in these regions.

In terms of the proposed "northern cotton-growing region," a significant increase in *Bt* cotton in these areas has been observed over the past several growing seasons. From 1996 to 1999, the percent Bollgard acreage increased in North Carolina from 3% to 19% (total increase: 250,000 acres), in Oklahoma from 7% to 20% (total increase: 57,773 acres), in Tennessee from 2% to 68% (total increase: 380,000 acres), and in Virginia from 1% to 7% (total increase: 6,214 acres) (MRID# 450294-01). This shows that the *Bt* cotton acreage cannot be predicted accurately and may not be an appropriate justification for reduced refuge.

Dr. Fred Gould (North Carolina State University) has also identified resistance risk issues in southern cotton growing regions (described in remarks made at the 1999 EPA/USDA Workshop on *Bt* Crop Resistance Management in Cotton, Memphis, TN 8/26/99). According to Dr. Gould, CEW are thought to feed on corn in Mexico in the early spring before moving to cotton in the southern U.S. and ultimately corn in more northern areas. If these CEW diapause in the northern areas and die over the winter, they pose no resistance problem. However, some indirect evidence has indicated that at least some CEW move from northern areas to southern cotton growing regions to overwinter. CEW that move from the north to south to overwinter could be exposed for four generations or more to *Bt* crop hosts. Along these lines, data were developed by ABSTC to address this concern and are discussed in the previous section. Based on the modeling studies submitted by ABSTC parameterized using the data in Gould et al. (2002), CEW reverse migration has no significant impact on CEW adaptation to *Bt* crops.

Drs. Caprio, Van Duyn, and Gould recommend a minimum of a 50% non-*Bt* corn refuge that may be treated only as necessary with non-*Bt* insecticides is needed in all cotton-growing regions to reduce the risk of resistance. Smaller refuges may present a greater risk and may result in a more rapid evolution of resistance. Since cotton is a preferred overwintering site for CEW, post-harvest plowing of *Bt* cotton fields to destroy potentially overwintering CEW pupae may also be an effective tool to decrease the risk of resistance, but further research is necessary.

iii. Non-High Dose Events

Non-Cotton Growing Regions That Do Not Spray Insecticides on a Regular Basis (e.g., Corn Belt)

As indicated earlier, there are no specific non-high dose products for ECB that have been considered in this scientific assessment. It is also clear that a high dose/refuge strategy is preferred for IRM with *Bt* crops. However, an assessment of non-high dose is included here to provide a comprehensive review of all possibilities.

Research regarding refuge size for non-high dose *Bt* events is limited. In general, non-high dose *Bt* corn hybrids pose a higher risk (approximately five times higher) of resistance than high dose events (Onstad & Gould 1998). The International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/HESI) recommended larger refuges (e.g., 40% unsprayed in the North) for non-high dose or high risk varieties (ILSI 1998). The Union of Concerned Scientists (UCS) also suggested that a separate resistance management strategy should be developed for varieties that do not meet the high dose refuge strategy. UCS recommended a 50% refuge that should not be sprayed with insecticides for *Bt* corn varieties that do not contain a high dose (UCS 1998).

For non-high dose events, larger refuges may be necessary (Gould 1998, ILSI 1998, UCS 1998). Based on the ILSI and UCS reports, at least a 40% unsprayed refuge in non-cotton growing regions (Corn Belt) would be needed to mitigate the threat of resistance. According to the National Center for Food and Agriculture Policy (1999), the percent insecticide use for ECB control in U.S. field corn is on average < 10%. Since most refuges will not be routinely sprayed

and some growers need the option of spraying if pests reach economic injury levels, mandating an unsprayed refuge should not be necessary. The risk of insect resistance to the non-high dose events could also be limited by restricting sales (e.g., a total sales cap or in areas where ECB are univoltine). Since ECB exposure to *Bt* is limited in areas where there is one generation per year, restricting the use of non-high dose events to these areas will likely decrease the risk of resistance.

Non-Cotton Growing Regions That Spray Insecticides on a Regular Basis (e.g., the High Plains for SWCB)

Non-high dose plants have an increased risk of insect resistance which is compounded if the refuge is sprayed with insecticides. The ILSI panel has recommended larger refuges for non-high dose or "high" risk *Bt* corn varieties. For areas where the refuge will be sprayed with insecticides, the ILSI recommended an 80% non-*Bt* corn refuge (ILSI 1999). Since there may be an increased risk of resistance in areas that are routinely sprayed with insecticides, restricting sales of non-high dose events could reduce the risk. In addition to planting restrictions, larger refuges (e.g., the ILSI Panel's recommended 80% insecticide treatable refuge) are an option that could be implemented to mitigate the risk of resistance.

4) Monitoring

a) Monitoring Strategies

A monitoring program for *Bt* corn is useful to evaluate the effectiveness of resistance management programs. Detecting shifts in the frequency of resistance genes through resistance monitoring can be an aggressive method to detect the onset of resistance before widespread crop failure occurs.

In general, resistance monitoring plans should include a detailed sampling strategy for all pests susceptible to the expressed *Bt* proteins regardless of whether they are stated on the label. For *Bt* field corn and sweet corn, the susceptible pests would include, but are not limited to: ECB, SWCB, and CEW. To be effective, the monitoring for resistance should be undertaken in areas where the pests are known to regularly overwinter. For FAW and BCW (target pests of TC 1507 Cry1F corn), resistance monitoring is less of a concern. These secondary corn pests overwinter in the south (FAW overwinters only in south Texas, south Florida, and the Caribbean) and migrate north during the growing season. Both FAW and BCW are also polyphagous insects that feed on a variety of other crops and weeds and corn is not necessarily a primary host for these pests. Therefore, resistance to *Bt* corn is not likely and a specific resistance monitoring plan should not be necessary. However, if large amounts of *Bt* corn (particularly Cry1F corn targeting FAW) were to be planted in areas in which FAW overwinters (e.g., >1000 acres), selection pressure for resistance may increase and a resistance monitoring plan could be warranted. Other secondary corn pests such as SCSB and CSB may also need to be monitored (on a case-by-case basis), as these pests may be of local or regional significance.

The resistance monitoring plan should not be tied to specific sales thresholds but be based on

sampling areas in which selection pressure for ECB resistance development is the greatest. Samples should be distributed throughout all corn-growing areas but can be concentrated in higher resistance risk areas (SAP 1998, 2001).

Dr. Blair Siegfried (entomologist, University of Nebraska) has indicated that at least 100 or more insects, with a target of 500-1000 insects, should be collected per location (noted at the June 18, 1999 EPA/USDA *Bt* Crop Insect Resistance Management Workshop in Chicago, IL). Sampling locations should be selected to reflect all crop production practices and should be separated by a sufficient distance to reflect distinct populations. More intensively planted *Bt* corn areas in which selection pressure is expected to be higher should also be targeted.

The utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan. The following monitoring techniques can be considered as part of a tiered approach to monitoring: 1) Grower reports of unexpected damage; 2) Systematic field surveying of *Bt* corn; 3) Discriminating concentration assay; 4) F_2 screen; 5) Screening against resistant colonies; and 6) Sentinel *Bt*-crop field plots. These techniques were discussed in detail in the Introduction (section D.1).

b) Agricultural Biotechnology Stewardship Technical Committee's (ABSTC) Tiered Approach

i. 2000 ABSTC Monitoring Plan

In response to requirements detailed in Agency letters to *Bt* corn registrants (12/20/99 and 1/31/00), the ABSTC submitted (March 31, 2000) a refined *Bt* field corn resistance monitoring plan for ECB, SWCB, and CEW for the 2000 growing season. The ABSTC plan was designed to concentrate resistance monitoring in areas where *Bt* corn market penetration is highest as well as areas with the highest insecticide use. The plan included the identification of counties growing more than 50,000 acres of field corn (*Bt* and non-*Bt*) to focus monitoring efforts. ABSTC's plan was intended to detect resistance when it reaches 1-5% (a level that may allow for detection of resistance before field failures occur). Four corn-growing regions were identified with monitoring for each pest to occur in the regions in which the pests are prevalent. ABSTC proposed a sampling goal of 4-6 locations in Regions I and III and 2-3 locations in Regions II and IV. When possible, at least 200 first or second flight adults (100 females), 100 second flight egg masses, or 100 diapausing larvae per site were to be collected in each region, though insect population levels may limit the number collected. It should be noted that the ABSTC plan applied to both Cry1Ab (MON 810 and BT 11) and Cry1F (TC 1507) *Bt* field corn hybrids.

The October, 2000 SAP concluded that it did not have enough detailed information to adequately evaluate the current resistance monitoring plans. The SAP Subpanel suggested that there be a "careful peer review to assess the adequacy of all *Bt* resistance monitoring programs."

A number of the October, 2000 SAP members indicated that the F_2 screen accompanied by field screening "could be very effective for detecting low frequencies of recessive and dominant resistance alleles." The F_2 screen can be a powerful method for detecting rare recessive alleles in

natural populations and is described in detail in the Introduction (section D.1.d.4).
The time-frame to respond before control failures occur depends on the precision of monitoring and the recessivity or dominance of resistance. If the goal of resistance monitoring is to detect resistance at a low enough resistance allele frequency so that changes to the insect resistance management plan can be made to increase the longevity of the product and prevent field failure, then the ABSTC resistance monitoring plan needs further consideration. The F_2 screen can detect and measure resistance at frequencies of less than or equal to 0.005 for approximately $5000 per site. This level of precision can provide seven to 12 years to respond with alternative resistance management tactics (see U.S. EPA/USDA 1999, p.47, Figure 1b). Hawthorne et al. (2001) concluded that there is a need to further evaluate the precision and accuracy of the F_2 screen by using colonies with known frequencies of resistance alleles. Zhao et al. (2001) also came to this same conclusion.

The October, 2000 SAP Subpanel indicated that the diagnostic or discriminating dose technique could at best, detect resistance when the resistance allele frequency has reached 1%. This is a level in which some field failure may be observed. At this lower level of precision, the least expensive methods are the discriminating dose assays (see U.S. EPA/USDA 1999, p. 47, Figure 1b).

One performance standard to consider is that a resistance monitoring plan could be designed so that there is at least a 95% confidence level in detecting resistance and that there is also a 95% confidence level that resistance will not go undetected. The chance of finding a resistant larva in a *Bt* crop depends on the level of pest pressure, the frequency of resistant individuals, and the number of samples that are collected. Therefore, as the frequency of resistant individuals or the number of collected samples increases, the likelihood of locating a resistant individual increases (Roush & Miller 1986). If the phenotypic frequency of resistance is one in 1,000, then more than 3,000 individuals must be sampled to have a 95% probability of one resistant individual (Roush & Miller 1986). The ABSTC strategy proposes to detect resistance alleles once they reach a frequency of one in 100. This level of detection may not be low enough to detect resistance alleles prior to some field failure. Previous experience with conventional insecticides has shown than once resistant phenotypes are detected at a frequency >10%, control or crop failures are common (Roush & Miller 1986). Using the F_2 screen could increase the probability of detecting rare resistant alleles, so that the threshold of detection would be lowered to <0.005 or 50-fold more sensitive than the diagnostic or discriminating dose assay.

The October, 2000 SAP agreed that sampling efforts must be concentrated in areas of high risk in which high usage of a *Bt* crop would be used as an interim definition. This is also the same recommendation made by the February, 1998 SAP (SAP 1998). The 2000 ABSTC resistance monitoring plan identified those counties that are >50% *Bt* corn sales with at least 50,000 acres of *Bt* and non-*Bt* corn. Based on the 1999 sales data, there were approximately 40-50 counties that exceeded this level of market penetration. Most of these counties were located in Minnesota, Iowa, and South Dakota (Region I as defined by the ABSTC plan). The ABSTC resistance monitoring plan had a goal of 4-6 sampling locations in Region I. The October, 2000 SAP Subpanel indicated that it would be difficult to determine how many areas of high risk should be sampled, but that genetic differentiation of insect samples over large transects could

help answer that question. Further evaluation of the ABSTC's sampling strategy including statistical analysis and detection sensitivity is recommended.

ii. 2003 Revised ABSTC Monitoring Plan

As part of the terms of conditions of the amended 2001 registrations for Cry1Ab and Cry1F corn PIPs, registrants were required to submit a revised resistance monitoring plan by 2003 (see part V - "*Bt* Corn Confirmatory Data and Terms and Conditions of the Amendment" in this BRAD). To accomplish this, the registration terms specified that "ABSTC will convene an advisory panel of academic experts from NC-205, USDA, and EPA to examine the current monitoring program and methodology and to consider enhancements to the current monitoring program for implementation in 2002." In addition, the terms mandate that registrants must follow up on reports of unexpected pest damage in the field for the major target pests (ECB, SWCB, and CEW).

Pursuant to these conditions, ABSTC convened a meeting in May, 2002 with representatives from industry, academia, USDA, and EPA to discuss refinements for *Bt* corn resistance monitoring. Based on these discussions, a revised monitoring plan ("Updated Monitoring Plan for *Bt* Corn" - no MRID number was assigned) was submitted on January 31, 2003 by ABSTC. This plan is similar in substance to the plan developed by ABSTC in 2000 and is based on two tiers of monitoring: reports of unexpected damage from growers and random population sampling.

The sampling strategy utilizes the geographic regions that were identified in the original 2000 ABSTC monitoring plan. For ECB and SWCB, four regions have been identified in corn-growing areas. A map of these regions was provided in ABSTC's submission and is attached to the end of this review. For CEW, monitoring is to occur in the south (10-12 locations), primarily in areas with the greatest *Bt* cotton plantings.

For ECB/SWCB monitoring, Region 1 (ECB collections only) is defined as southwest Minnesota, eastern South Dakota, southeast North Dakota, and northwest Iowa. The target will be to sample 4-6 ECB populations from Region 1. Region 2 (ECB and SWCB collections) covers southwest Kansas and the Texas/Oklahoma panhandle. From this region, 4-6 ECB populations and 3-4 SWCB (at least one from Texas) will be targeted for sampling. Region 3 (ECB only) consists of central/southeastern Iowa and north-central Illinois (target sampling of 4-6 ECB populations). Region 4 (SWCB only) is focused on the Missouri Bootheel, western Kentucky, western Tennessee, and the southern tip of Illinois (target sampling of 3-4 SWCB populations). Individual sampling sites are to be determined by pest population size and will be taken at distances greater than ½ mile from *Bt* cornfields to minimize the effects of "elevated resistance gene frequencies misrepresentative of the population average."

For each insect population to be sampled, a target of 200 larvae, 200 adults, 100 mated females, or 100 egg masses will be collected for an overall goal of 400 genomes per population (egg masses are assumed to have at least 4 genomes). In the event of low pest numbers, the minimum population sizes to be collected will be 50 larvae, 50 adults, 25 mated females, or 25 egg masses.

The specific life stage to be sampled will depend on the insect and the practicality of the collection method. For example, pheromone traps may prove to be the most efficient way to sample CEW. Most collections will occur at the peak flights of second or later generations (first generation flights may not produce sufficient numbers for sampling).

To conduct the resistance assays, neonates are needed. Therefore, all collected larvae and (unmated) adults will be reared and mated in the laboratory to obtain F_1 progeny. Neonates from collected egg masses will be directly used in the assays. In the event of small samples, populations may be reared for multiple generations to obtain sufficient numbers of neonates for testing.

The primary bioassays to be utilized to detect potential increases in resistance allele frequency will be assessments of baseline susceptibility (LC_{50}) and the discriminating dose assay. The discriminating dose assay utilizes artificial diet with a high dose (LC_{99} or EC_{99}) of *Bt* toxin such that only "resistant" insects will be detected – i.e., homozygous resistant (for recessive resistance) or heterozygous resistant (for dominant/incomplete recessive resistance). Discriminating dose assays will be conducted with 500 neonates in four replicates. Based on the number of genomes to be sampled (400 per population), the assays are designed to detect resistance allele frequencies at 1) 0.01 for high dose *Bt* corn and incomplete recessive resistance (probability of detecting a resistance allele in 400 genomes = 98.2%); 2) 0.002 for a moderate dose and dominant resistance (probability of detecting a resistance allele = 55.0%); 3) 0.075 for recessive resistance (probability of detecting homozygous resistance = 88.0%, assuming mating of collected insects). Scenarios 1 and 3 apply to ECB and SWCB (high dose in *Bt* corn, resistance is likely to be recessive or incompletely recessive) and scenario 2 applies to CEW (non-high dose, resistance likely to be dominant). The resistance detection thresholds are based on an analysis of population survival and a time to resistance of 5 generations (2 ½ growing seasons) after resistance allele detection. The data generated from the assays may be combined for populations within a region or across multiple years to increase the statistical power of the resistance allele estimates.

The Agency reviewed ABSTC's revised 2003 plan (A. Reynolds memo to M. Mendelsohn, 2/4/04) and determined that it was adequate to address the terms and conditions of the amended *Bt* corn registrations (as of 10/15/01). However, questions remain as to the overall effectiveness of the monitoring strategy for suspected or confirmed resistance in that it is unknown whether any monitoring plan can sufficiently sample all corn-growing regions to proactively detect resistance before field failure. Further, the review recommended that ABSTC continue to research and develop techniques to improve the sensitivity and precision of the current monitoring strategy. Such techniques could include improved (larger) sampling and development of the F_2 screen and/or DNA markers. Greater sensitivity could allow for more opportunities to adjust the IRM plan to mitigate potential resistance.

iii. 2008 Revisions to the Monitoring Program

In 2008, ABSTC amended the *Bt* corn registrations to make minor adjustments to the resistance monitoring program. Specifically, changes were requested in the sampling program for ECB,

CEW, and SWCB as well as adjustments to the procedures to confirm resistance and mitigate resistance. ABSTC's submission (MRID# 474070-01) contains a complete text of the revised monitoring strategy.

In terms of sampling, ABSTC proposed to maintain the overall numerical targets for each of the monitored corn pests. For ECB, at least 12 populations will be collected annually, six for SWCB, and 10 for CEW. Additionally, the overall target for each population of 400 genomes (consisting of egg masses, larvae, adults, or mated females) with a minimum of 100 genomes will remain the same. As with the past sampling strategy, the goal will be to sample in areas with greater potential for resistance development (i.e., areas with large proportions of *Bt* corn adoption and pest pressure). However, ABSTC proposed to remove the specific sampling regions that had been established for ECB and SWCB monitoring. Four regions had been established for different parts of the Corn Belt including two regions for ECB sampling, one region for SWCB sampling, and one region for both ECB and SWCB collections. Rather, the collections of populations will be taken from areas of "regional importance of the insect species as a pest," but without specifically-defined sampling zones. ABSTC also proposed to be able to modify the sampling program "based on changes in pest importance and/or the adoption levels of lepidopteran-resistant *Bt* corn."

Should resistance monitoring bioassays detect a population with low susceptibility to *Bt*, a series of follow-up steps are to be initiated. These steps follow the procedures in the previously-developed 2003 ABSTC monitoring strategy and include the following elements: 1) Confirmation that the resistance is heritable; 2) Confirmation that the resistance will be observed in the field (i.e., on live *Bt* corn plants); 3) Determination of the nature of resistance (dominant, recessive); 4) Estimation of the resistance allele frequency; 5) Analysis of whether the resistance allele frequency is increasing; 6) Determination of the geographic extent of the resistance allele distribution; and 7) Design of a remedial action plan if the resistance allele frequency is spreading. ABSTC's new proposal included caveats that if the trait is found to be either not heritable or incapable of conferring the ability to survive on *Bt* plant tissue (i.e., #1 and 2 above), that the registrants will cease further investigative activity since the observed tolerance is not relevant for *Bt* corn in the field. In addition, ABSTC indicated that the analytical work will be initiated "as soon as practical" after low sensitivity is noted in the bioassays.

EPA reviewed ABSTC's proposal (A. Reynolds memo to M. Mendelsohn, 7/9/08) and agreed with ABSTC on the need for flexibility: 1) to identify areas (i.e., outside of the four previous collection regions) in which *Bt* corn adoption may be high or increasing; and 2) to be able to sample in areas with known high pest pressure. Both of these scenarios may be indicative of high selection pressure for resistance and would warrant sampling for resistance monitoring. Overall, the minimum number of populations collected would remain the same (12 for ECB, 10 for CEW, and 6 for SWCB). However, the review cautioned that samples should not be "bunched" in only one or two states. While it may be convenient to group samples, the collections that are taken should be representative of corn-growing regions in the U.S. Therefore, it was recommended that a caveat be added to the sampling plan to ensure that pest populations are collected from multiple corn-growing states reflective of different geographies and agronomic conditions.

ABSTC's 2008 revised monitoring plan keeps largely intact the follow-up investigations for populations with low sensitivity to *Bt* toxin(s). The Agency believes these procedures are a crucial component of the overall monitoring program and are needed to verify potential field resistance. On multiple occasions in the past (ECB collections in Kandiyohi, MN, Hamilton County, IA, and Jefferson County, NE) these follow-up steps have been implemented to assess populations demonstrating tolerance to Cry1Ab and/or Cry1F in laboratory bioassays (see discussions in the next section). However, it has also been recommended that ABSTC initiate the investigative work "as soon as possible" after low sensitivity is detected, rather than "as soon as practical" as proposed by ABSTC. Confirmation of resistance can be an important, time-sensitive process that should be completed quickly enough to ensure effective implementation of remedial action plans (if warranted).

c) Monitoring Results

EPA currently mandates that both baseline susceptibility and a discriminating concentration assay be employed for certain primary target pests including ECB, SECB and CEW. Baseline susceptibility data have been collected for each labeled/target pest although consideration should be given for all potentially susceptible pests (e.g., BCW, FAW, SCSB) with focus on major economic pests. This information is essential to managing resistance in pest populations, especially in assessing whether a field control failure was due to actual resistance or other factors affecting expression of the *Bt* protein. These baseline data are helpful in documenting the extent and distribution of resistant populations. Continued monitoring efforts are needed to provide the Agency with standardized information to determine whether resistance is developing to the registered Bt toxins. ABSTC has submitted annual reports to EPA on resistance monitoring results for ECB, CEW, and SWCB with Cry1Ab and Cry1F as required by the terms of registration. The results of these monitoring reports are detailed below and are complete through the 2008 growing season.

i. ECB

Cry1Ab

Dr. Blair Siegfried (University of Nebraska) has coordinated a standardized monitoring program for ECB (since 1995) and CEW (through 2000) involving LC_{50} susceptibility determinations and diagnostic concentration (LC_{99}) bioassays to determine susceptibility levels to *Bt* corn. In terms of baseline susceptibility (LC_{50}), bioassays have been conducted for ECB (Siegfried et al. 1999a, Siegfried & Spencer 2000) and CEW (Siegfried et al. 2000, see CEW discussion in the next section). For 1999, ECB were collected from 14 separate sites and F_1 and/or F_2 generations were bioassayed to determine LC_{50}s. Bioassays utilized dilutions of purified Cry1Ab obtained from *Bt kurstaki* strain HD1-9 (provided by Novartis) spread on artificial diet. Neonate larvae were exposed to the diet less than 24 hours after hatching and mortality and larval weight were recorded seven days later. For 2000, 13 ECB populations were sampled using similar procedures with formulated Cry1Ab protein (CellCap, provided by Dow/Mycogen). This CellCap source of Cry1Ab was used for ECB monitoring from 2000 to 2003. ECB are more sensitive to the

CellCap Cry1Ab formulation, therefore, susceptibility results during this time period are not directly comparable with those from 1995-1999. Starting in 2004, a new source of Cry1Ab toxin was used in the assays, which has been used in each subsequent year of testing. The new toxin (obtained from Monsanto) was purified, trypsin-resistant Cry1Ab core protein and produced results similar to the purified toxin used from 1995-1999. Monitoring results for ECB through the 2008 season are displayed in Table D3 and show no significant change in ECB susceptibility (LC_{50}) to Cry1Ab over 13 years of testing (1995 - 2008).

Table D3. Mean Susceptibility of ECB to Cry1Ab from 1995 to 2008 (created from data in Siegfried et al. 1999a; Siegfried & Spencer 2000, 2001a, 2001b, 2002a, 2003a; Siegfried et al. annual reports 2005 to 2009)

Year	LC_{50} (ng Cry1Ab/cm^2) ± SEM	Disc. Dose (% mortality)
1995	4.34 ± 0.68	---
1996	6.25 ± 1.25	> 99
1997	2.12 ± 0.53	> 99
1998	2.57 ± 0.28	100
1999	4.01 ± 0.49	99.9 - 100
2000[1]	(0.12 - 0.49)	99.7 - 100
2001[1]	(0.14 - 1.34) [2]	97.8 - 100 [3]
2002[1]	(0.18 - 0.47)	99.1 - 100
2003[1]	(0.06 - 0.21)	99.6 - 100
2004	(1.74 - 19.86) [4]	50.7 - 100 [4]
2005	(1.33 – 4.45)	99.4 - 100
2006	(1.18 - 4.28)	99.5 - 100
2007	(1.70 - 4.26)	100
2008	(1.49 - 2.94)	100

[1] Data collected for 2000 - 2003 were obtained using a different Cry1Ab formulation (CellCap) that is more toxic to ECB. As such, results from these years are not directly comparable with results from other years. LC_{50} values for 2000 - 2008 are given as a range (without SEM).
[2] 2001 included one outlier population with an LC_{50} of 1.34 ng/cm^2. The range for the other populations was 0.14 - 0.46 ng/cm^2.
[3] Three populations from 2001 had < 99% mortality at the diagnostic dose.
[4] 2004 included one tolerant population with an LC_{50} of 19.86 ng/cm^2 and a 50.68% mortality at the diagnostic concentration. The other tested populations had LC_{50} values of 1.74 - 5.24 ng/cm^2 and discriminating dose mortalities > 99.6%.

For 1999 diagnostic concentration analysis (LC_{99}), baseline susceptibility studies conducted by Marçon et al. (2000) were used to determine the discriminating concentration for ECB. These tests with the discriminating concentrations were conducted in a similar manner to the bioassays to determine LC_{50} values. For 2000, a new discriminating dose (10 ng/cm^2) was established for the CellCap Cry1Ab formulation. The results (for both 1999 and 2000 populations) showed nearly 100% mortality for ECB at the discriminating dose (LC_{99}) (Siegfried et al. 1999a,

Siegfried & Spencer 2000). For the change in toxin source during 2004, a revised concentration (LC_{99}) was calculated (55 ng/cm^2 Cry1Ab) based on dose/response assays done for ECB populations from Nebraska. This diagnostic concentration has been used subsequently for ECB monitoring. Results of the diagnostic assays through the 2008 season are summarized in Table D3.

Since 2001, there have been two ECB populations that showed less than expected mortality (i.e. < 99%) on the diagnostic concentration. Survival on diagnostic concentrations can be a sign of some degree of resistance to the toxin and triggers follow-up investigations. In 2001, one population from Kandiyohi, MN was shown to have 98.4% mortality in the discriminating dose assay. The second population was collected from Hamilton County, IA and was shown to have only 50.68% mortality to the Cry1Ab discriminating concentration (this population also demonstrated high tolerance to Cry1F – see the discussion in the next section). Procedures for follow-up testing included the following: 1) Rearing additional generations from the populations (F_2 and F_3) for further discriminating dose testing; 2) Pooling and rearing survivors from the discriminating dose assays for additional testing (F_3); 3) Testing with leaf discs (F_3); 4) Continued selection of survivors at the diagnostic concentration (F_4 - F_7); and 5) Testing on whorl stage *Bt* corn (F_7).

For the Kandiyohi population, F_2 larvae were retested at the diagnostic concentration and showed 99.9% mortality. F_3 larvae showed significant survival (48.8%) on *Bt* leaf discs, but those survivors were shown to have very low weights relative to the control group (non-*Bt* corn leaf discs). The colony was also tested on whole, whorl stage MON 810 plants in a greenhouse test. Individual corn plants were infested with Kandiyohi egg masses (F_7, each generation selected with a diagnostic concentration) and were assessed for feeding damage and larval survival after three weeks. The *Bt* corn plants were found to be undamaged and no larval survival was noted (there was no difference between the Kandiyohi strain and a control strain). Given the results of the follow-up testing, successfully conducted within the allotted two year time frame, the Kandiyohi population was determined not to be field resistant (reviewed in A. Reynolds memo to M. Mendelsohn, 2/4/04). Further, resistance monitoring in the Kandiyohi area in the following years (2002 - 2004) did not reveal any instances of decreased ECB susceptibility to Cry1Ab.

For the Hamilton County, IA population, the original colony was reared for additional generations and subjected to diagnostic and dose response bioassays with Cry1Ab protein (described in MRID# 468749-01). Surviving larvae from the initial Cry1Ab diagnostic assays were separately pooled and reared as independent colonies. These "survivor colonies" were subjected to purified protein, leaf disc, and on-plant assays. Results of the bioassays indicated that the Hamilton ECB population continued to exhibit significantly lower susceptibility to the Cry1Ab protein compared to the non-resistant control colonies. Mortality at the diagnostic concentration was 15.2 and 11.9 for the F_7 and F_8 generations, respectively. However, the F_6 and F_8 generation from this colony exhibited no survival on leaf discs obtained from Cry1Ab expressing plants compared with high rates of survival on non-expressing leaf discs from non-*Bt* isoline plants. Separately, the Cry1Ab survivor colony showed intermediate (47.9%) survival and severe stunting on leaf discs from Cry1F-expressing plants when tested at the F_6 generation.

In the last set of tests, the survivor colony was unable to develop on Cry1Ab-expressing whorl- or reproductive-stage plants – only one live (severely stunted) larva was found among the 10 Cry1Ab-expressing plants infested with the colony.

Three new populations of ECB were collected from Hamilton Co. in 2005 and results from these assays indicated that the newly sampled populations did not have reduced susceptibility to Cry1Ab compared to control colonies. Further collections taken from Hamilton in 2006, 2007, and 2008 have also shown no signs of elevated tolerance to Cry1Ab and no incidents of field level failure have been identified in the region. Given these results, the Hamilton, IA 2004 population was determined not to be field relevant despite the high survival noted in the diagnostic concentration assay (Hamilton, IA data were reviewed in 1) A. Reynolds memo to M. Mendelsohn, 1/23/06; 2) T. Milofsky memo to M. Mendelsohn, 3/12/07; 3) A. Reynolds memo to M. Mendelsohn, 4/2/08; and 4) S. Borges memo to M. Mendelsohn, 4/8/09).

Cry1F

Resistance monitoring for Cry1F and ECB was initiated with the 2000 season. Similar to Cry1Ab, the program for Cry1F has been coordinated by Dr. Blair Siegfried (University of Nebraska) since the initial 2000 season. The sampling strategy and bioassays used for Cry1Ab (i.e., baseline susceptibility and diagnostic concentrations) are also employed for Cry1F.

Baseline susceptibility (LC_{50}) results for ECB ranged from 0.17 µg Cry1F/g diet (1st instar) to 10.67 µg Cry1F/g diet (4th instar) (MRID# 453077-01; reviewed in R.Rose memo to M.Mendelsohn, 1/24/01). As a condition of registration, Bt corn registrants were required to develop a diagnostic concentration for ECB, CEW, and SWCB. ABSTC's monitoring submissions from the 2001 and 2002 growing seasons reported testing and validation of a ECB diagnostic concentration of 60 ng/cm^2 Cry1F. This concentration has been used subsequently for ECB Cry1F monitoring and adequately addressed the condition of registration.

ABSTC uses the same sampled ECB populations for both Cry1Ab and Cry1F monitoring. To illustrate, in 2001 ECB collections were made from populations at 10 locations from four states, including Nebraska (2 locations), Illinois (4 locations), Iowa (2 locations), and Minnesota (2 locations). In 2002, samples were obtained from populations at 14 locations from six states. Similar numbers of populations from locations in the Corn Belt have also been sampled in succeeding years. The toxin used in the assays since 2004 has been purified and truncated Cry1F protein obtained from recombinant *Pseudomonas fluorescens* (provided from Dow AgroSciences). A summary of the susceptibility (LC_{50}) and diagnostic concentration assay data through the 2008 growing season is detailed in Table D4 below.

Table D4. Monitoring Results for ECB and Cry1F Corn from 2000 to 2008 (Siegfried & Spencer 2001c, 2002b, 2003b; Siegfried et al. annual reports 2005 to 2009)

Year	LC_{50} range (ng Cry1F/cm^2)	Disc. Dose (% mortality range)
2000	2.35 - 6.26	98.66 - 100
2001	1.23 - 3.68	97.47 - 100
2002	1.04 - 5.32	99.50 - 100

Year	LC_{50} range (ng Cry1F/cm^2)	Disc. Dose (% mortality range)
2003	2.33 - 8.45	99.10 - 100
2004 [1,2]	3.76 - 48.35	48.35 - 100
2005 [1]	2.96 – 13.8	99.2-100
2006 [1,3]	1.59 - 9.81	97.89 - 100
2007 [1]	2.06 – 8.00	100
2008 [1]	3.78 - 6.72	99.23 - 100

[1] A new source of Cry1F toxin (purified and truncated toxin obtained from recombinant *Pseudomonas fluorescens*) has been used since the 2004 bioassays

[2] 2004 included one tolerant population with an LC_{50} of 45.42 ng/cm^2 and a 48.35% mortality at the diagnostic concentration. The other tested populations had LC_{50} values of 3.76 - 9.73 ng/cm^2 and discriminating dose mortalities > 96.47%.

[3] One population collected in 2006 had < 99% mortality at the diagnostic concentration.

As with the Cry1Ab monitoring, two populations of ECB have shown greater than expected survival (>1%) in diagnostic concentration tests. The first was the same population collected in Hamilton County, IA during the 2004 season that showed high tolerance to Cry1Ab (discussed in the next section below). A second population sampled in Jefferson County, Nebraska, was observed to have somewhat reduced susceptibility to Cry1F in 2006.

ECB collected from the Jefferson County population were observed to have lower than expected mortality (97.89%) to the Cry1F diagnostic concentration (expected mortality > 99%). ABSTC initiated a follow-up investigation on this population (using the same procedures described in the Cry1Ab section above). This testing suggested some heritability of the trait (32.8% of F_2 progeny survived at the same diagnostic concentration). However, low survival (10%) with growth stunting was noted on separate tests with Cry1F plant tissue and testing with F_3 larvae on a higher (10x) diagnostic concentration produced 100% mortality. Given the low level of tolerance displayed by the Jefferson County population, it is unlikely that the trait detected in the monitoring assays could have conferred field resistance. Despite the 2006 data, Jefferson County was not sampled by ABSTC in 2007 or 2008. This area should be included as a collection site for Cry1F bioassays in subsequent growing seasons and monitored for unexpected pest damage.

Hamilton County, IA Cry1F-Tolerant ECB Population

The Hamilton County (HC) population collected in 2004 exhibited substantially higher tolerance to Cry1F than the other tested ECB populations from that year with a Cry1F LC_{50} of 45.42 ng/cm^2 (compared to a range of 3.76 - 9.73 ng/cm^2 for the other sampled populations). Additionally, the population had only 48.35% mortality on the Cry1F diagnostic concentration. Follow-up testing (conducted by Dow AgroSciences and described in MRID# 466958-01 and 470112-01) focused on four major objectives: 1) The level and heritability of the potential resistance; 2) Survival on *Bt* (Cry1F) expressing plants; 3) The genetics of the potential resistance; and 4) The frequency of resistance in field populations from Hamilton County.

Heritability testing was initially conducted on F_7 and F_8 generation of the HC ECB colony. Bioassays were performed with artificial diet and a diagnostic concentration of 60 ng Cry1F/cm^2 (based on the LC_{99} of ECB), similar to the procedures used for the routine annual resistance monitoring of *Bt* corn. When exposed to this concentration in the trial, few F_7 (2.7%) or F_8 (0.9%) larvae were killed. These reported mortalities were substantially lower than the 48.3% mortality that was observed with the F_3 generation in the original monitoring work. With such high survival, the investigators were unable to generate a dose-response curve to calculate LC_{50} or EC_{50} values. Subsequent bioassays were conducted on F_{13} and F_{18} generations using higher diagnostic concentrations of Cry1F. F_{13} larvae (previously unexposed to Cry1F since the F_3 generation) were exposed to a diagnostic concentration of 600 ng Cry1F/cm^2 (10x the standard concentration used for monitoring). These larvae also demonstrated high tolerance to Cry1F, with only 4.1% mortality (compared with 3.9% mortality in an unexposed control group). A final test of the F_{18} generation (used as a control group for the reciprocal cross test described later in this review) was conducted at concentrations up to 12,000 ng Cry1F/cm^2 (200-fold greater than the standard diagnostic concentration). No detectable mortality or growth inhibition was observed at even the highest 12,000 ng Cry1F/cm^2 concentration. Considering the extremely low mortality (< 5%) to the diagnostic concentrations observed in the generations (F_7 - F_{18}) after initial (F_3) selection, Dow concluded that the Cry1F tolerance trait was heritable in the HC ECB colony. Overall, tested ECB exhibited a resistance to Cry1F levels that exceeded 2,000 to 6,000 times the LC_{50} for susceptible ECB.

Dow also investigated the ability of the HC ECB colony to survive on Herculex *Bt* corn plants. Pest populations showing tolerance to *Bt* toxins are generally not considered "resistant" unless they are able to develop into adults on *Bt* expressing plants. Without the ability to survive on *Bt* plants, tolerant populations will not be able to reproduce and will not proliferate in the field. Prior to conducting the whole plant assays, Dow challenged the HC population on leaf disks taken from Herculex (Cry1F) and non-expressing isoline plants. The results showed that 79.2% of the HC ECB survived after four days exposure to the Cry1F leaf disks (survival on the non-*Bt* leaf disks was 90.6%). Larval weights of the HC colony averaged 0.42 mg for those on Cry1F disks and 0.71 mg for the non-*Bt* disks. By comparison, the unselected control colony had no survival on the Cry1F disks and 97.8% on the non-*Bt* leaf disks.

Whole plant assays were also conducted in greenhouse settings and were done with different generations (F_7-F_8 and F_{11}) from the HC ECB colony. In the first set of assays, F_7 and F_8 generation larvae were infested (30-40 per plant) on whorl and ear stage corn plants (Cry1F and isoline). With whorl stage corn, the HC ECB (F_7) were found in approximately equal numbers on both Cry1F and isoline corn plants, although the overall number of larvae recovered was small (<0.5 per plant). In contrast, high numbers of larvae from the control colony were recovered on isoline corn (>3 per plant), but only one larvae was found on Cry1F corn. HC larvae collected on Cry1F plants weighed less on average than those from isoline plants (~ 30 mg Cry1F vs. ~ 50 mg isoline). For reproductive stage corn (i.e., ear stage), similar numbers of larvae from the HC colony (F_8) were recovered on both Cry1F and isoline corn, although unlike the whorl stage results high numbers were recovered for both treatments (~ 7 per plant). Similar to the whorl stage corn test, the recovered HC larvae from Cry1F ear stage corn weighed less than those collected from the isoline corn (~ 40 mg Cry1F vs ~ 60 mg isoline). High survival

was noted for the control colony (~ 9 per plant) on isoline corn, while few larvae (<1 per plant) were found on the Cry1F corn plants. The investigators noted that the experiment was "inconclusive" because the surviving larvae were not allowed to fully develop into pupae and adults.

A second whole plant test was performed on the F_{11} and F_{12} generations from the HC colony. As with the first test, whorl and ear stage Cry1F corn plants were infested with 30 neonates per plant from the HC colony or an unselected control group. After three weeks, the plants were assessed for damage and living larvae were collected (from diapause) and for ear stage corn ultimately reared to adults. The results of this experiment showed that on whorl stage corn, few HC F_{11} larvae were recovered from either Cry1F or isoline corn. A total of four HC larvae were recovered from isoline corn and only one larva from Cry1F plants. This compared with 1.4 larvae/plant from the control colony on isoline corn (no control larvae were recovered on Cry1F plants). Cry1F corn plants showed some damage from HC feeding (2.3 avg. Guthrie score) though not to the extent of the isoline plants (4.5). In comparison, the unselected control group had Guthrie ratings of 5.3 on isoline corn and 1.0 (no visible feeding) on Cry1F plants. For ear stage corn, equal numbers of F_{12} HC larvae (68 total) were recovered on Cry1F and isoline corn, while only three larvae from the control colony were found on Cry1F corn (114 were found on isoline corn). Of the 68 HC larvae found on Cry1F corn, 45 completed diapause, 36 pupated, and 26 emerged as adults (38% of the total recovered larvae). Fewer HC larvae completed development on isoline corn (38% diapause, 31% pupae, and 28% eclosion) than on the Cry1F hybrids.

Based on the results from the greenhouse studies, Dow surmised that the Cry1F tolerance trait in the HC population allows larvae to grow and complete development on Herculex Cry1F corn plants. Although, it was noted that there are differences between greenhouse and field environments, the company concluded that the ability of HC larvae to survive Cry1F in the field is a "strong possibility."

The third goal of the HC studies was to determine the genetic structure of the Cry1F resistance trait. Diet bioassays and crosses with known susceptible (laboratory) ECB colonies were utilized to evaluate dominance/recessiveness of the trait and potential sex linkage. Crosses were conducted with HC (F_{13} and F_{18} generations) and control (Cry1F-susceptible) ECB colonies to produce F_1 larvae for the bioassays. Four groups were established: 1) HC; 2) HC male x control female; 3) HC female x control male; and 4) control. The F_{13} generation HC was crossed with a laboratory control colony, while the F_{18} were bred with a field-collected control colony. Progeny obtained from the crosses were then exposed to a range of Cry1F concentrations in diet bioassays (up to a maximum of 12,000 ng/cm^2). Both sets of crosses resulted in similar responses to Cry1F: HC ECB crossed with the susceptible colonies remained sensitive to the toxin while the uncrossed HC group was highly tolerant to Cry1F. The dose response curves of the two crosses (HC ♂ x CC ♀ and HC ♀ x CC ♂) were similar to that of the uncrossed control colony, although the crosses showed some increased ability to tolerate Cry1F relative to the control group. An EC_{50} or LC_{50} for the uncrossed HC groups could not be calculated because no mortality or growth inhibition was observed at the highest test concentration (12,000 ng/cm^2). Given the

results from the reciprocal crosses, Dow concluded that the Cry1F tolerance trait in the HC colony is recessive but autosomal and not sex-linked.

The final step in Dow's follow-up investigations of the Cry1F tolerant ECB from HC was an estimation of the prevalence of the trait in field populations from the county. To accomplish this objective, Dow (via ABSTC) conducted ECB sampling of the HC area during the 2005 growing season as part of the annual resistance monitoring work. Three populations were collected directly from HC (543 total ECB) and their F_1 progeny were screened against the standard Cry1F diagnostic concentration (60 ng/cm^2). None of the tested HC progeny survived the diagnostic Cry1F concentration, indicating that the Cry1F tolerance trait was below the level of detection. Subsequent sampling in 2006, 2007, and 2008 has also failed to produce any survivors to the diagnostic concentration and there have been no reports of field failures in this area.

EPA reviewed the HC data (A. Reynolds memo to M. Mendelsohn, 8/7/07) and concluded that the collected 2004 HC colony was resistant to the Cry1F toxin. The colony met all of the major criteria for resistance: the trait was heritable (determined to be a single recessive gene), the trait conferred survival to high levels of Cry1F, and ECB with the trait were capable of developing to adults on Cry1F-expressing corn plants. This appeared to be the first documented case of pest resistance to a high dose *Bt* toxin in corn; though it should also be noted that no documented cases of field failure or resistance with ECB have been detected in HC or elsewhere. Given that the sampling strategy for the annual resistance monitoring program covers only a small portion of corn-growing regions, it is possible that such traits are relatively common. Other cases may remain undetected if significant field-level effects are not observed.

Dow contends that because the trait was not detected in 2005 (and beyond), the resistance allele frequency was below the level of concern and a remedial action plan was not needed. To verify this conclusion, additional monitoring in HC was recommended during subsequent growing seasons. ABSTC has conducted monitoring in this county since 2005 (through 2008, the last season tabulated in this document) with no incidents of increased tolerance or field failure to Cry1F corn. Additional EPA reviews of HC monitoring data can be found in 1) T. Milofsky memo to M. Mendeloshn, 3/12/07; 2) A. Reynolds memo to M. Mendelsohn, 4/2/08; and 3) S. Borges memo to M. Mendelsohn, 4/8/09).

ii. CEW

Cry1Ab

Since the 2001 season, resistance monitoring assays have been conducted by Custom Bio-Products (Maxwell, IA). Similar to ECB, monitoring is based on susceptibility bioassays and diagnostic concentration tests.

For CEW, baseline susceptibility (LC_{50}) values to Cry1Ab ranged from 70.3 ng/cm^2 (lab colony) to 221.3 ng/cm^2 (field colony) (Siegfried et al. 2000). A separate diagnostic concentration analysis (using similar methods to those used for ECB) was conducted for CEW (using a dose of 6600 ng/cm^2), which showed nearly 100% mortality (Siegfried et al. 1999b). This diagnostic

dose has been revised since this initial assessment, largely due to the fact that CEW is less sensitive to Cry1Ab then other lepidoptera. The insect frequently exhibits highly variable responses to the toxin, such that it has been difficult to determine a reliable diagnostic concentration based on an LC_{99}. Monitoring in 2001-2 used a concentration of 40 ng Cry1Ab/cm^2. In 2004, ABSTC increased the Cry1Ab diagnostic concentrations to 80 ng/cm^2 for the CEW monitoring. Despite this increase, a number of populations have continued to survive at levels greater than 1%.

The Cry1Ab toxin used in the diet bioassays has been provided by Dow AgroSciences since 2001. CEW are collected as larvae, pupae, or eggs from field sites and shipped to Custom Bio-Products for establishment of test colonies. Bioassays are conducted with neonate larvae from the established colonies. Baseline susceptibility (LC_{50} and EC_{50}) and diagnostic concentration bioassays are performed in accordance with the methods used by Siegfried et al. (2000).

The results of LC_{50} and diagnostic concentration assays are summarized in Table D5 below. Susceptibility data (LC_{50} ranges) have been reasonably consistent over the tested growing seasons and support the conclusion that there has been no documented CEW field resistance to Cry1Ab. The range of LC_{50} values from 2007 was less varied and closer to the higher end of the ranges from previous years, though this could be an artifact of the small number of populations that were assayed that year. Four populations in 2008 also exhibited LC_{50} values on the high end of the historical range (i.e., > 8.0 ng Cry1Ab/cm^2), though most of the populations collected during this season had LC_{50}s of < 4.5 ng/cm^2. In addition to the monitoring assays, ABSTC has reported that no incidents of field failure or unexpected CEW damage have been observed through the 2008 growing season. The diagnostic concentration that has been used since 2004 (80 ng Cry1Ab/cm^2) also appears to have remained functional (i.e., mortality > 99% in susceptible CEW colonies) and is an improvement over the previously used concentration of 40 ng Cry1Ab/cm^2, though several populations in 2008 had slightly less than 99% mortality.

Table D5. Monitoring Results for CEW and Cry1Ab and Cry1F Corn from 2001 - 2008 (Custom Bio-Products annual reports 2002 through 2009)

	Cry1Ab		Cry1F	
Year	LC_{50} range (ng Cry1Ab/cm^2)	Disc. Dose (% mortality range) [a]	LC_{50} range (ng Cry1F/cm^2)	Disc. Dose (% mortality range) [b]
2001	1.37 - 4.98	≥ 99	89.68 - 2301.48	94 - 97
2002	1.09 - 8.08	97.77 - 99.85	269.45 - 2151.99	85.27 - 100
2003	2.35 - 8.94	90.8 - 100	671.39 - 1366.61	82.9 - 99.0
2004	0.15 - 8.93	99.4 - 100	45.30 - 2828.28	99.1 - 100
2005	1.68 - 11.45	97.5 - 100	306.21 - 2804.42	62.2 - 96.1
2006	0.73 - 8.40	99.6 - 100	65.29 - 806.90	87.5 - 100
2007	6.00 - 7.99	99.4 - 100	1129.12 - 1555.88	48.7 - 99.6
2008	0.78 - 10.55	96.7 - 100	87.10 - 2259.55	69.0 - 100

[a] Cry1Ab discriminating concentration for 2001-3 = 40 ng Cry1Ab/cm^2; for 2004 - 2007, concentration = 80 ng Cry1Ab/cm^2
[b] Cry1F discriminating concentration for 2001 = 5000 ng Cry1F/cm^2; for 2002-3, concentration = 7500 ng Cry1F/cm^2; for 2004, concentration = 54,675 ng Cry1F/cm^2; for 2005-8, high concentration = 4,000 ng Cry1F/cm^2

Cry1F

Resistance monitoring for Cry1F and CEW has also been conducted by Custom Bio-Products using the same paradigm for Cry1Ab. Both the susceptibility and diagnostic concentration testing with Cry1F have shown broad variability in response by CEW. In general, the pest has been less susceptible to Cry1F than Cry1Ab. As such, it has proven difficult to determine a reliable diagnostic concentration (based on a LC_{99}) for year-to-year screening of field populations.

The initial diagnostic concentration for monitoring in 2001 was set 5000 ng Cry1F/cm^2 but was increased in 2002 to 7500 ng Cry1F/cm^2, based on the results from 2001. However, even the higher concentration failed to consistently produce mortalities > 99% in collected populations indicating that the concentration was actually less than the LC_{99} for CEW. In 2004, the dose was further increased to 54,675 ng/cm^2, a level at which all sampled populations had >99% mortality. However, in 2005-2008 testing the concentration was reduced to 4000 ng/cm^2 which has frequently resulted in mortality below 99% in sampled populations (Table D5). ABSTC has indicated that the revised test concentration was based on the LC_{50} (instead of the LC_{99}) given that TC 1507 (Cry1F) corn does not cause 99% mortality to CEW. BPPD notes that a diagnostic concentration based on a LC_{50} is unlikely to function as an effective screening tool for possible resistant individuals. It remains unclear the level of survival (if any) that would be needed to trigger follow-up testing for resistance determination. In fact, no follow-up testing has been reported for any of the populations in the diagnostic assays, regardless of the level of survival. In light of these results, it is apparent that 4,000 ng/cm^2 is not acting as a functional diagnostic concentration (i.e., a benchmark that triggers investigation of potential resistance) and has limited value as a monitoring tool. It has been recommended that ABSTC either reestablish the higher test concentration (54,675 ng/cm^2) or work to develop an effective alternate technique for monitoring CEW in future testing so that potentially resistant individuals can be identified.

The results of Cry1F monitoring for CEW are summarized in Table D5 above. Because of the high variability in LC_{50} ranges, it is difficult to discern year-to-year trends, though there does not appear to be a significant decrease (if any) in the susceptibility of CEW to Cry1F. Additionally, no incidents of field failure due to CEW damage to Cry1F corn have been reported to the Agency.

iii. SWCB

Cry1Ab and Cry1F

Additional monitoring work has been done with SWCB. Based on collections from 1998 and 1999, a study was conducted by Trisyono and Chippendale (1999) to determine SWCB susceptibility to Cry1Ab and establish a diagnostic concentration. A bioassay was conducted

that established a diagnostic concentration for SWCB of 110 ng Cry1Ab protein/g diet. Susceptibility data (LC_{50}s and EC_{50}s), determined after 7 and 14 days of exposure to Cry1Ab, are summarized in Table D6 below. SWCB monitoring has been conducted since the 2000 growing season by Dr. Qisheng Song at the University of Missouri using similar methodology (Song et al. 2000) to obtain susceptibility data (LC_{50}s and EC_{50}s) and perform diagnostic concentration assays. A diagnostic concentration assay was performed (7 day test dose = 0.35 µg Cry1Ab/g diet, 14 day test dose = 5 µg Cry1Ab/g diet), which resulted in 100% mortality for all tested populations. The 5 µg Cry1Ab/g concentration has been used as the diagnostic dose since 2000.

For Cry1F, baseline susceptibility assays provided an estimate for the SWCB LC_{50} of 0.70 µg Cry1F/cm^2 diet (MRID# 450201-01; reviewed in R. Rose memo to M. Mendelsohn, 1/24/01). Annual Cry1F monitoring formally started with the 2001 growing season. The 2001 SWCB susceptibility tests resulted in a LC_{50} range of 3.69 - 5.73 µg Cry1F/ml (7 day exposure) and 0.79 - 1.63 µg Cry1F/ml (14 day exposure) for field populations; a lab colony was also tested (7-day LC_{50} = 27.20 µg Cry1F/ml; 14-day LC_{50} = 7.05 µg Cry1F/ml). Discriminating doses of 216.4 µg Cry1F/g (7 days) and 68.6 µg Cry1F/g (14 days) were used in the initial testing and produced 100% mortalities for both exposure periods. The 14 day diagnostic dose 68.6 µg/g has been used for SWCB monitoring since 2001.

SWCB monitoring data are summarized in Table D6 below. Taken together, the SWCB monitoring results show that, to date, no appreciable increase in susceptibility has resulted from exposure to the Cry1Ab or Cry1F toxins. Also, the laboratory colonies evaluated as control groups have been less susceptible to Cry1Ab than the field collected populations. Furthermore, there have been no survivors to the 14 day diagnostic concentration in any of the years tested or reports of unexpected pest damage in Cry1F corn due to SWCB. It is noted that the results from 1998 and 1999 indicated that a bioassay using growth inhibition is more sensitive than one based on larval mortality. Trisyono and Chippendale (1999) suggested that bioassays based on growth inhibition rather than larval mortality may have greater benefits because they require a smaller amount of *Bt* protein, sublethal effects can be observed, the time of observation is flexible (weight gain is being compared to a control), and variation may be minimized.

Table D6. SWCB Susceptibility (LC_{50}) to Cry1Ab and Cry1F from 1998 to 2008 (Trisyono and Chippendale 1999; Song et al. annual reports 2000 to 2009)

Year	Cry1Ab		Cry1F	
	LC_{50} (µg Cry1Ab/ml diet) - field populations	Discriminating Dose (5 µg Cry1Ab/g) (percent mortality)	LC_{50} (µg Cry1F/g diet) - field populations	Discriminating Dose (68.6 µg Cry1F/g) (percent mortality)
1998[1]	7-day: 0.22 - 1.09 14-day: 0.04 - 0.09	--	–	--
1999[1]	7-day: 0.07 - 0.17 14-day: 0.02 - 0.05	--	–	–
2000	7-day: 0.08 - 0.15 14-day: 0.04 - 0.09	100	--	--
2001	7-day: 0.09 - 0.22 14-day: 0.05 - 0.12	100	7-day: 3.69 - 5.73 14-day: 0.79 - 1.63	100

Year	Cry1Ab		Cry1F	
	LC_{50} (µg Cry1Ab/ml diet) - field populations	Discriminating Dose (5 µg Cry1Ab/g) (percent mortality)	LC_{50} (µg Cry1F/g diet) - field populations	Discriminating Dose (68.6 µg Cry1F/g) (percent mortality)
2002	14-day: 0.07 - 0.12	100	14-day: 1.04 - 2.14	100
2003	14-day: 0.06 - 0.21	100	14-day: 0.98 - 2.34	100
2004	14-day: 0.10 - 0.28	100	14 day: 0.87 - 2.57	100
2005	14-day: 0.07 - 0.41	100	14-day: 2.02 - 6.04	100
2006	14-day: 0.06 - 0.28	100	14-day: 1.64 - 3.18	100
2007	14-day: 0.08 - 0.25	100	14-day: 1.90 - 4.03	100
2008	14-day: 0.12 - 0.47	100	14-day: 2.48 - 4.29	100

[1] The units for the 1998 and 1999 data are µg Cry1Ab/g diet for LC_{50} values.

iv. FAW

There is also a monitoring program for FAW as part of the *Bt* sweet corn registration. The status of this program is described in the *Bt* sweet corn section (D.2.b.10).

FAW Resistance to Cry1F in Puerto Rico

Separately, Dow AgroSciences and Pioneer Hi-Bred reported to EPA that unexpected *Spodoptera frugiperda* (fall armyworm) damage had occurred on Cry1F TC1507 maize (also known as Herculex® Insect Protection Maize) in Puerto Rico during the 2006 season. Damage was reported in corn fields by three customers as well as in Dow (Mycogen Seeds) research plots on the island (detailed in MRID# 471760-01).

Dow AgroSciences' personnel performed follow-up testing on the Puerto Rican populations. The following conclusions were reached from these investigations:

- Testing using ELISA lateral flow membrane strips confirmed that the TC1507 maize expressed the Cry1F protein.
- Testing confirmed that the unexpected damage was caused by FAW larvae feeding on TC1507 maize (company expert).
- Testing of the susceptibility to the Cry1F protein indicated that the two FAW larval populations collected from VEH Farms (a commercial farm) and Mycogen Research Farm (SI) had significantly lower susceptibility to the Cry1Fa protein than the control, Benzon colony. The screening level assessment of the Cry1Fa-sensitivity of F_1 progeny from the field-collected larvae at 10,000 ng Cry1F/cm^2 revealed no significant mortality of either Puerto Rico collection on the Cry1F-treated diet. Testing of the F_2 progeny did not show a significant concentration response to Cry1F, nor did these progeny show a significant reduction in growth. The resistance ratio was >167, but an exact level could not be calculated for the Puerto Rico populations because the LC_{50}s could not be

determined.

A specific remedial action plan for FAW resistance was not required under the terms and conditions of the Cry1F *Bt* (TC1507) maize registrations (EPA registration numbers 68467-2 and 29964-3) because FAW is considered to be a secondary pest. Therefore, Dow AgroSciences and Pioneer Hi-Bred formulated a specific remedial action plan designed to reduce the selection pressure for FAW resistance to TC1507 maize. This plan is analogous to that required by EPA for the primary target pests of TC1507 maize (ECB, SWCB, and CEW). The following steps were and are being taken by the registrants following confirmation of FAW resistance Puerto Rico (described in a letter from Dow AgroSciences to EPA, dated April 23, 2009):

- Existing customers were notified and recommended to use insecticides (as needed) to control FAW damage on existing TC1507 commercial fields.
- The two registrants took steps to discontinue commercial sales of TC1507 maize in Puerto Rico by terminating grower agreements and revising product use guides to prohibit plantings in Puerto Rico.
- FAW populations were managed in maize research and production fields in Puerto Rico with insecticides.
- Before any commercial reintroduction of TC1507 (Cry1F) maize in Puerto Rico, Dow/Pioneer will confirm the susceptibility of FAW. Dow's letter indicated that there are currently no plans to reintroduce Cry1F corn to Puerto Rico.
- Additional FAW sampling was conducted during 2007 and 2008 in Puerto Rico, which also showed similar tolerance to Cry1F.

Several unique factors likely contributed to the development of Cry1F-resistant FAW in Puerto Rico. These factors include:

- An island setting provides an isolated ecosystem that reduces insect migration;
- A tropical climate allows for year-round maize production and year-round insect pressure;
- TC1507 maize was heavily adopted in Puerto Rico for highly effective control of FAW (a major pest on the island);
- More than 50 FAW generations were continually exposed to Cry1F maize (TC1507) after its introduction into Puerto Rico in 2003 through 2006; and
- A high population and drought conditions in 2006 funneled a large percentage of insects through irrigated TC1507 maize (FAW larvae migrate from declining quality host plants).

There are a number of limiting factors that reduce the likelihood of FAW resistance in the continental U.S. FAW is a migratory seasonal pest. The species cannot develop at temperatures below 50°F and, as a tropical species, do not diapause. FAW only survive winter in the extreme south of Texas and Florida and thus, selection for Cry1F-resistance in maize-growing areas exerts no long-term selection pressure. In the continental U.S., there are only one or two generations per year on maize. Alternate host crops, which include primarily grasses, are abundant. FAW is not a key target pest of *Bt* maize, except for *Bt* sweet corn. Both Cry1F- and

Cry1Ab-expressing maize are planted in the continental U.S. and this market mix reduces the selection pressure on either protein. Finally, there have been no reports of FAW performance problems on TC1507 maize in the continental U.S.

Based on the results of the screening level bioassay and concentration-dependent bioassay, EPA concluded that the unexpected performance failures observed in 2006 in Puerto Rico were due to Cry1F-resistant FAW feeding on TC1507 maize (reviewed in S. Matten memo to M. Mendelsohn, 8/24/07). This represented the first documented case of field failure associated with insect resistance to a *Bt* crop. Since FAW is the most important pest of maize in Puerto Rico, Herculex® I (TC1507) Insect Protection Maize cannot be used effectively on the island.

5) Remedial Action

Remedial action plans are a potential response measure should resistance develop to *Bt* crops. Since resistance may develop in "localized" pest populations, it may be possible to contain the resistance outbreak before it becomes widespread. A specific remedial action plan should clearly indicate what actions the registrant will take in cases of "suspected" resistance (i.e., unexpected damage) and "confirmed" resistance. The remedial action plan can also include appropriate adaptations for regional variation and the inclusion of appropriate stakeholders. To fully mitigate resistance, a critical element of any remedial action plan should be that once pest resistance is confirmed, sales of all *Bt* corn hybrids that express a similar protein or a protein in which cross-resistance potential has been demonstrated would be ceased in the affected region.

A remedial action plan was proposed by ABSTC for *Bt* corn (applicable to MON 810, BT11, and TC 1507) consisting of two elements: 1) Strategies for unexpected damage; and 2) Strategies for confirmed resistance. Both components are discussed below.

a) Actions to be Taken if Unexpected Levels of Insect Damage Occur

ABSTC proposed a strategy for unexpected pest damage in *Bt* corn in the "Industry Insect Resistance Management for Cry1A Plant-Expressed Protectants in Field Corn" (submitted 4/19/99). The language of the ABSTC plan is as follows:

> *"Customers (growers and seed distributors) will be instructed to contact the registrant or authorized distributor if incidents of unexpected levels of target insect damage occur during use of the registrant's Bt corn products. Registrants (or their authorized distributors) will investigate and identify the cause for this damage by local field sampling of plant tissue from corn hybrids that contain the Bt corn plant-expressed protectant and sampling of local pest populations, followed by appropriate in vitro and in planta assays. Upon confirmation by immunoassay that the plants contain the appropriate Cry1A/Cry1F protein, bioassays will be conducted to determine whether the collected insect population exhibits a resistant phenotype.*
>
> *Where available and validated for a target pest species, a discriminating concentration assay will be employed to define a confirmed instance of resistance. For other target*

pests, until such time that a discriminating concentration assay is established and validated, registrants will utilize the following to define a confirmed instance of insect resistance:

Progeny from the sampled pest population will be considered resistant if they exhibit BOTH of the following characteristics in bioassays initiated with neonates:

1. An LC_{50} in a standard diet bioassay (incorporating the appropriate Cry1A/Cry1F protein) that exceeds the upper limit of the 95% confidence interval of the mean historical LC_{50} for susceptible pest populations, as established by the ongoing baseline monitoring program.

2. > 30% survival and > 25% leaf area damaged in a five-day bioassay using the appropriate Cry1A/Cry1F-positive leaf tissue under controlled laboratory conditions.

Based upon continued experience and research, this working definition of confirmed resistance may warrant further refinement. In the event that the registrants find it appropriate to alter the criteria specified in the working definition, the registrants will obtain Agency approval in establishing a more suitable definition."

In the January 31, 2000 letter to *Bt* corn registrants, the Agency agreed with this strategy and the working definition of "confirmed resistance." The letter also clarified the Agency's interpretation of "suspected" resistance to be:

"...in the case of reported product failure, that corn in question has been confirmed to be *Bt* corn, that the seed used had the proper percentage of corn expressing *Bt* protein, that the relevant plant tissues are expressing the expected level of *Bt* protein, that it has been ruled out that species not susceptible to the protein could be responsible for the damage, that no climatic or cultural reasons could be responsible for the damage, and that other reasonable causes for the observed product failure have been ruled out. The Agency does not interpret 'suspected resistance' to mean grower reports of possible control failures, nor does the Agency intend that extensive field studies and testing to fully scientifically confirm insect resistance be completed before responsive measures are undertaken."

Two other elements that could further mitigate the risk of resistance in the event of unexpected damage (i.e., these measures could be undertaken while the cause of the suspected resistance is investigated) are:

1) The immediate use of alternate control measures to control the pest suspected of resistance to *Bt* corn in the affected region.

2) The destruction of crop residues in the affected region immediately after harvest (i.e., within one month) with a technique appropriate for local production practices to

minimize the possibility of resistant insects overwintering and contributing to the next season's pest population.

A panelist on the 2000 SAP also noted that given the logistics of monitoring, it may take two years from resistance detection to remedial action plan implementation. During this period of "suspected" resistance, the panelist noted that increasing refuge size could help to prolong susceptibility (SAP 2001).

b) Remedial Measures in Confirmed Cases of Insect Resistance

In cases of "confirmed" resistance (as defined in section a) above), ABSTC proposed the following strategy for *Bt* corn hybrids:

"The registrant will report all instances of confirmed pest resistance, as defined above, to the Agency within 30 days. Upon identification of a confirmed instance of resistance, registrants will take the following immediate mitigation measures:

1. Notify customers and extension agents in the affected area,

2. Recommend to customers and extension agents in the affected area the use of alternative control measures to reduce or control the local target pest population, and

3. Where appropriate, recommend to customers and extension agents in the affected area that crop residues be incorporated into the soil following harvest, to minimize the possibility of overwintering insects.

Within 90 days of a confirmed instance of pest resistance, as defined above, registrants will:

1. Notify the Agency of the immediate mitigation measures that were implemented,

2. Submit to the Agency a proposed long-term resistance management action plan for the affected area,

3. Work closely with the Agency in assuring that an appropriate long-term resistance management action plan for the affected area is implemented, and

4. Implement an action plan that is approved by EPA and that consists of some or all the following elements, as warranted:

a. Informing customers and extension agents in the affected area of pest resistance,

b. Increasing monitoring in the affected area, and ensuring that local target pest populations are sampled on an annual basis,

c. Recommending alternative measures to reduce or control target pest populations in the affected area,

d. Implementing intensified local IRM measures in the affected area based on the latest research results. The implementation of such measures will be coordinated by the Agency with other registrants; and

e. If the above elements are not effective in mitigating resistance, registrants will voluntarily cease sale of all Bt corn hybrids subject to the Industry IRM Plan in the county experiencing loss of product efficacy and in the bordering counties until an effective local management plan approved by EPA has been implemented. During the voluntary suspension period, registrants may sell and distribute in these counties only after obtaining EPA approval to study resistance management in those counties. The implementation of such a strategy will be coordinated by the Agency with other registrants and stakeholders.

If EPA agrees that an effective local resistance management plan has been implemented which mitigates resistance, the registrants can resume sales in the affected county(ies).”

The Agency agreed with this strategy for confirmed resistance, with the condition that once resistance has been confirmed, the sale and distribution of *Bt* corn in the affected counties must be halted until an EPA-approved mitigation plan is in place. In addition, *Bt* corn registrants assumed responsibility for resistance mitigation actions (EPA letter to *Bt* corn registrants, 1/31/00).

In addition to the remedial strategy for confirmed resistance developed by ABSTC, the following elements could further mitigate the risk of resistance development:

1) Immediate suspension of the sale of *Bt* corn hybrids expressing the same or similar *Bt* protein (i.e. same mode of action, cross-resistant varieties) as the suspected *Bt* corn hybrid harboring the resistant population in the affected region (this was mandated in the 1/31/00 letter).

2) The mandatory use of alternate control measures and post-harvest crop residue destruction in the affected region (the ABSTC plan “recommends” these measures).

3) For mitigation of resistance in the growing season(s) following a confirmed resistance incident(s), use of the following procedures:

a) Maintenance of the sales suspension of all *Bt* corn hybrids (with the same protein or similar *Bt* proteins as the *Bt* corn hybrids with the resistant population)

in the affected region, which would remain in place until resistance has been determined to have returned to acceptable levels.

b) The development and use of alternative resistance management strategies for controlling the resistant pest(s) on corn in the affected region.

c) Notification of all relevant personnel (e.g., growers, consultants, extension agents, seed distributors, processors, university cooperators, and state/federal authorities) in the affected region of the resistance situation.

d) Intensified monitoring and surveillance in the affected region(s) for resistance and definition of the boundaries of the affected region. These studies could also include assays to track the decline of resistance in the field and determine the potential for cross-resistance in the resistant population.

In discussing remedial action, the 2000 SAP suggested that eradication of a resistance gene (as part of a remedial action plan) may prove to be too difficult. Rather, a plan based on slowing the spread of resistance genes (and possibly causing their decline) may prove more practical. As part of a plan to slow resistance genes, the SAP suggested the following elements: 1) Education of growers/crop consultants to look for unexpected pest damage; 2) Monitoring for plant damage, pest susceptibility, and resistance allele frequency (with rapid verification and alternate control strategies for verified resistance); 3) Sales suspensions of the affected product in the region until it can be shown that the product's benefits will outweigh its risks; 4) Continual monitoring to determine the effectiveness of the remedial action plan; and 5) An assessment of how the resistance problem occurred (SAP 2001).

c) 2008 Revisions to the Remedial Action Plan

In 2008, ABSTC amended the remedial action plan to adjust the process for confirming resistance (reviewed in A. Reynolds memo to M. Mendelsohn, 7/9/08). Under the revised plan, to confirm resistance a pest population must demonstrate: 1) 30% survival and commensurate insect feeding in a bioassay representative of field exposure to *Bt* corn for ECB and SWCB only (CEW was removed); 2) survival on a laboratory diagnostic concentration that demonstrates a genetic basis for the tolerance and a resistance allele frequency ≥ 0.1; 3) a LC_{50} in a standardized laboratory bioassay that exceeds the upper 95% LC_{50} confidence interval for a susceptible population. For the first standard, ABSTC's revised plan removed CEW, removed the explicit requirement to test on *Bt* corn plant tissue, and eliminated criteria for > 25% leaf feeding. Steps two and three are essentially the same as in the original remedial action plan. The revised plan maintained the existing definitions of "suspected" and "confirmed" resistance and the follow-up procedures for suspected resistance.

Further revisions to the remedial action plan were made for the steps to be implemented in the event of confirmed resistance. The previous (2000) plan included requirements to be undertaken within 30 days, 90 days, and 1 year from the event in an effort to mitigate the spread of resistance. ABSTC's 2008 revised plan removed the time dependent responses in favor of a

simplified list of procedures that include the following elements (taken from the terms and conditions for *Bt* corn registrations as amended in 2008 and 2009):

- EPA will receive notification within 30 days of resistance confirmation;
- Affected customers and extension agents will be notified about confirmed resistance within 30 days;
- Monitoring will be increased in the affected area and local target pest populations will be sampled annually to determine the extent and impact of resistance;
- If appropriate (depending on the resistant pest species, the extent of resistance, the timing of resistance, and the nature of resistance, and the availability of suitable alternate control measures), alternative control measures will be employed to reduce or control target pest populations in the affected area. Alternative control measures may include advising customers and extension agents in the affected area to incorporate crops residues into the soil following harvest to minimize the possibility of over-wintering insects, and/or applications of chemical insecticides;
- Unless otherwise agreed with EPA, sale and distribution of the relevant lepidopteran-active *Bt* corn hybrids will stop in the affected area immediately until an effective local mitigation plan approved by EPA has been implemented;
- [The registrant] will develop a case-specific resistance management action plan within 90 days according to the characteristics of the resistance event and local agronomic needs. The registrant will consult with appropriate stakeholders in the development of the action plan, and the details of such a plan shall be approved by EPA prior to implementation;
- Notification of affected parties (e.g., growers, consultants, extension agents, seed distributors, university cooperators and state/federal authorities as appropriate) in the region of the resistance situation and approved action plan; and
- In subsequent growing seasons, sales suspension and alternative resistance management strategies will be maintained in the affected region(s) for the *Bt* corn hybrids that are affected by the resistant population until an EPA-approved local resistance management plan is in place to mitigate the resistance.

6) Cross-Resistance

Cross-resistance is an area of major concern for resistance management and poses risks to both transgenic *Bt* crops and microbial *Bt* insecticides. Cross-resistance occurs when a pest becomes resistant to one *Bt* protein, which then allows the pest to resist other, separate *Bt* proteins. The threat of cross-resistance is particularly acute with *Bt* corn, since there are multiple *Bt* proteins and hybrids currently registered and commercially available (Cry1Ab and Cry1F are assessed in this document, though other Bt toxins have been registered for use in corn). In addition, some pests of corn are also pests of other crops for which *Bt* transgenic varieties are or may soon be available or of crops on which microbial *Bt* insecticides may be used (e.g., CEW on cotton, FAW on tomato). Cross-resistance also poses a risk to pyramid strategies, in which multiple proteins targeting the same pest complex are deployed simultaneously in the same hybrid. However, it should be noted that, to date, the development of cross-resistance has not been shown in insect pests exposed in the field to *Bt* crops producing different *Bt* proteins.

In general, it is possible for resistance to *Bt* proteins to occur through a number of different mechanisms, some of which may result in cross-resistance to other proteins. The most well documented mechanism of resistance is reduced (midgut) binding affinity to *Bt* proteins. Different Cry proteins may bind to distinct receptors in an insect gut. Modifications to these insect crystalline protein receptors have been implicated in resistance to Cry proteins. Other mechanisms that may lead to resistance (and ultimately cross-resistance) include protease inhibition, metabolic adaptations, gut recovery, and behavioral adaptations (Heckel 1994, Tabashnik 1994).

Regarding binding sites, cross-resistance may result if two proteins share the same binding site (receptor) in the insect midgut. Therefore, if exposure to one *Bt* protein results in a modification of the receptor, other proteins sharing this site will be affected as well. An example of a possible shared binding site resulting in cross-resistance was observed with tobacco budworm (TBW). In this case, TBW selected for resistance to Cry1Ac were also found to be resistant to the Cry1Aa, Cry1Ab, and Cry1F proteins (Gould et al. 1995).

Cross-resistance patterns in ECB, the major pest of corn, have proven to be complicated. The binding of three *Bt* insecticidal crystal proteins to the midgut epithelium of ECB larvae was characterized by performing binding experiments with both isolated brush border membrane vesicles and gut tissue sections (Denolf et al. 1993). Results demonstrated that two independent insecticidal crystal protein receptors were present in the brush border of ECB gut epithelium. From competition binding experiments, it was concluded that Cry1Ab and Cry1Ac are recognized by the same receptor. Also, the Cry1B protein did not compete for the binding site of Cry1Ab and Cry1Ac and was determined to have a different receptor. Cry1D and Cry1E, two proteins that are not toxic to ECB, were not bound to the gut epithelial cells. Other experiments using laboratory-selected resistant strains to predict survival and cross-resistance in the field on *Bt* corn with ECB have provided different results. A Cry1Ac-resistant ECB strain (produced by Dr. Hutchinson, University of Minnesota) and a Cry1Ab-resistant ECB strain (produced by Dr. Keil, University of Delaware) had a moderate level of resistance, about 30 to 60X. None of the resistant larvae survived on *Bt* corn beyond the second instar. It is interesting to note that the Cry1Ac-resistant ECB were not cross-resistant to Cry1Ab and that Cry1Ab-resistant ECB are not cross-resistant to Cry1Ac (Hutchison, personal communication, reviewed in U.S. EPA 1998). Based on receptor binding studies, one would have expected both resistant strains to survive on *Bt* corn. It can be concluded that although two proteins are closely related, there may be different binding mechanisms or binding affinity in ECB relative to other pests, such as DBM or TBW.

Based upon the binding properties of Cry1A and Cry2A proteins in CEW, TBW, and ECB larvae, there appears to be a much lower probability of cross-resistance developing to Cry2A delta endotoxins from resistance to Cry1Ab or Cry1Ac. Because the Cry1A and Cry2A proteins exhibit different binding characteristics and very low amino acid homology, they likely possess different modes of action. However, there is some evidence for the development of broad cross-resistance to Cry1 and Cry2A in at least two laboratory-selected strains: beet armyworm (BAW) (Moar et al. 1995) and TBW (Gould et al. 1992).

Binding studies have also been conducted with Cry1F (expressed in TC 1507 field corn) to determine cross-resistance potential with other *Bt* toxins including Cry1Ab, Cry1Ac, and Cry9C in ECB. The results showed that Cry1Ab likely recognizes multiple binding sites in ECB brush border membrane vessicles (BBMV), one of which may be shared by Cry1F (MRID# 450201-15; reviewed in R. Rose memo to M. Mendelsohn, 1/24/01). However, a second published study to assess midgut receptor binding patterns for Cry1Ab and Cry1F in ECB (Hua et al. 2001) suggested that Cry1F and Cry1Ab only weakly compete for BBMV binding sites. As in the previous study, ligand blotting analysis showed that Cry1Ab and Cry1F recognize multiple binding proteins in ECB. Both toxins recognized binding proteins of 154 and 220 kDas and Cry1Ab also recognized 145 and 167 kDa proteins. Despite these shared binding sites, pre-incubation with Cry1F did not inhibit Cry1Ab binding to BBMV in surface plasmon resonance studies, though Cry1Ab-incubated BBMV did reduce Cry1F binding. Cry1F reduced Cry1Ab BBMV binding in radioligand assays, but only at the highest tested concentrations of Cry1F. The study authors concluded that the "results are explained if Cry1F has low affinity for the Cry1Ab binding site." Another set of studies using resistant colonies of ECB showed little cross resistance potential between Cry1F and Cry1Ab. In the first study, four ECB colonies selected for resistance to Cry1Ab were shown to have low levels (< 5 fold) of cross resistance with Cry1F, but not Cry9C (Siqueira et al. 2004). Subsequently, a Cry1F resistant ECB strain was used to assess cross resistance to Cry1Ab, Cry1Ac, and Cry9C (Pereira et al. 2008). This strain possessed 3,000 fold resistance to Cry1F but was still highly susceptible to Cry1Ab and Cry9C. Cry1F was only slightly (7 fold) cross resistant with Cry1Ac.

Collectively, laboratory-selected strains and isolated field populations indicate that there is a genetic potential for *Bt* cross-resistance to develop to multiple or single Cry delta endotoxins in a number of corn pests from exposure to Cry1Ab. However, cross-resistance patterns and physiological mechanisms are complex and unpredictable, even within related groups of proteins and susceptible pests. Research has suggested that Cry1Ab and Cry1Ac may share binding sites in several tested insect species, although this may not necessarily result in cross-resistance in the field. Still, areas in which *Bt* corn (expressing Cry1Ab) and *Bt* cotton (Cry1Ac) are grown may pose additional selection pressure for resistance in CEW, a pest of both corn and cotton.

Given the unpredictability of cross-resistance among pest species, it would be useful to generate cross-resistance data for SWCB, SCSB, CSB, BCW, and other secondary pests, to gain a more complete understanding of the implications for *Bt* corn.

7) Compliance

a) ABSTC Compliance Program

As a term of the amended *Bt* corn registrations, registrants were required to develop and submit to EPA a compliance assurance program (CAP) to ensure grower adherence to IRM requirements (EPA letters to *Bt* corn registrants, 10/15/01). The terms of registration mandated a number of components for the compliance program including:

- Grower Agreements: Contractual arrangement between the registrant and grower to obligate adherence to IRM requirements.
- Annual IRM survey: The survey (conducted anonymously by an independent research firm) is intended to provide a statistically representative sample of growers from various corn-growing regions in the U.S. Results from the survey should assess levels of grower compliance with refuges as well as grower motivations, attitudes, and reasons for non-compliance.
- On-farm assessments: Registrants are required to develop an on-site assessment program in which trained personnel from each company make visits to farms growing *Bt* corn. During these visits, compliance with refuge requirements is assessed and growers out of compliance are identified for corrective action under the Phased Compliance Approach.
- Tips and complaints: Registrants must establish a means for the reporting and investigation of incidences of refuge non-compliance.
- Phased Compliance Approach (PCA): A consistent set of procedures (for all *Bt* corn registrants) to be employed to address non-compliance among growers and seed dealers.

The *Bt* corn registrants (under ABSTC) submitted a proposed CAP to the EPA in 2002. After review, EPA approved the cap (letter to *Bt* corn registrants dated November 1, 2002) to be fully implemented for the 2003 growing season. Subsequently, ABSTC submitted revised versions of the CAP in 2004 and 2005 in response to EPA reviews of annual growing season reports. Issues identified in these reviews are discussed in the sections below specific to each element of the CAP. EPA reviews of CAP reports include the following documents:

- 2002 and 2003 growing season reports: T. Milofsky memo to M. Mendelsohn, 7/15/04;
- 2004 growing season report and CAP amendments: S. Matten memo to M. Mendelsohn, 3/23/05);
- 2005 growing season report: S. Mattten memo to M. Mendelsohn, 11/2/06;
- 2006 growing season report: T. Milofsky memo to M. Mendelsohn, 8/30/07
- 2007 and 2008 growing season report: J. Martinez memo to M. Mendelsohn, 4/15/09

b) Grower Surveys (1996-2001)

Several surveys and estimates of the level of grower compliance for *Bt* corn IRM were conducted prior to the development and implementation of the 2002 ABSTC CAP. Dr. Marlin Rice (Iowa State University) conducted regular grower surveys to measure grower attitudes towards various aspects of *Bt* corn, including compliance with IRM guidelines. These surveys showed that the great majority of growers understood and were receptive to the need for refuge and resistance management. However, they also demonstrated that some level of non-compliance must be expected. The results from the 1996 grower survey showed that 23.5% of sampled growers would follow a prescribed IRM strategy, 57.1% would if compatible with their growing practices, 7.2% would not follow IRM, and 12.2% "didn't know" (Pilcher & Rice 1997). Results from the 1998 grower survey showed that 25.5% of growers would implement

recommended IRM, 58.9% would if compatible with their growing practices, 2.6% would not follow IRM recommendations, and 12.9% "didn't know" (Rice & Pilcher 1999).

In terms of compliance information submitted by industry, ABSTC (representing *Bt* corn registrants) conducted a compliance survey for the 2000 growing season (MRID# 453205-03). The ABSTC compliance plan consisted of grower contracts, intensified education for regions showing low compliance, and restrictions on future use of *Bt* corn for individual growers repeatedly out of compliance. The compliance survey was conducted by a marketing research firm and included telephone surveys of 501 total growers, each farming at least 200 acres. This survey did not involve visits to individual farms (i.e., grower audits). Compliance was assessed for two *Bt* corn IRM requirements: percent refuge (required to be 20% or greater) and refuge proximity (required to be within ½ mile of the *Bt* field). Survey respondents indicated that 87% planted an appropriate amount of refuge (at least 20%), while 13% had less than the required amount or no refuge. In terms of proximity, 82% of growers reported refuges planted within ½ mile of the *Bt* field (18% reported refuges planted greater than ½ mile from the *Bt* field). When both refuge percentage and proximity are considered together, 71% of growers were in total compliance. It should be noted that growers were sampled in southern cotton growing regions, where a 50% refuge is required. It is unclear from the survey whether these growers were counted as compliant for planting a refuge of less than 50%, but greater than 20%.

Collectively, these surveys indicate that 100% compliance is not likely and that some level of non-compliance must be expected. However, the 2000 SAP indicated that while surveys such as these are useful for tracking grower attitudes, they are not reliable for determining actual grower compliance (SAP 2001). The format of the surveys (mail or phone interviews) may encourage non-compliant growers to misrepresent their actions or "cheat" in their responses. Without confirmatory visits to individual farms (i.e., audits), it may be impossible to verify the accuracy of grower responses. The end result could be increased "false-positives," which may artificially inflate estimates of grower compliance. As such, actual non-compliance may be significantly higher than the survey results would suggest. To resolve this problem, the 2000 SAP suggested utilizing surveys created and conducted by independent parties to assess grower practices (SAP 2001). In addition to this recommendation, it may be useful to conduct some on-farm visits for firsthand verification of compliance. Such visits could be performed as part of a survey process, to evaluate the accuracy of grower survey responses.

c) Grower Surveys (2001- 2008)

As a term and condition of the Bt Corn product registrations in 2001, registrants were required to perform an "annual survey of a statistically representative sample of *Bt* corn growers conducted by an independent third party" (EPA letters to *Bt* corn registrants, 10/15/01). The grower survey functions to measure compliance adherence to refuge size and distance requirements at a regional level and to identify educational opportunities in these four regions to increase grower compliance with IRM requirements.

Beginning with the 2002 growing season, >500 growers from four separate regions have been anonymously surveyed. The methodology for conducting the grower survey has remained

largely unchanged since it was first conducted by Market Horizons, Inc in 2000 for ABSTC (MRID# 453205-03). However, in 2007 Marketing Horizons started to utilize an internet-based survey approach due to an increasing complexity of growers' *Bt* corn planting practices and a need to standardize the grower survey across insect-protected traits. Consistency in methodology is important because it allows for year-to-year comparisons of the results. There are four sampling regions that differ by pest and level of adoption: a) regions with high rates of *Bt* corn adoption (150 samples from the Eastern Iowa, Northern Illinois region and 200 samples from the South Dakota, Minnesota, Nebraska, Western Iowa region); b) areas where insecticides have historically been sprayed for control of Lepidopteran pests (100 samples from the Kansas, Oklahoma, Texas Panhandle region); and c) areas where *Bt* corn and *Bt* cotton may be grown simultaneously (100 samples from the North Carolina, South Carolina, Southeast Missouri, Tennessee, Mississippi region). Results are weighted to reflect the actual distribution of corn acres in each region.

Data from the 2002 through 2008 compliance surveys are summarized in Table D7 below. In 2007 and 2008, the online survey results were not broken down for the four regions, as was done in previous years, and therefore, it is impossible to determine which region had greater non-compliance with refuge requirements. It has been recommended that this information be provided in future reports so that it can be determined in which farming regions the registrants should focus their educational outreach (see EPA review in J. Martinez memo to M. Mendelsohn, 4/15/09).

Overall, the survey results through 2008 show that grower compliance with refuge size requirements for *Bt* corn has declined and is now at the lowest level since initiation of the surveys in 2000. A report issued by the Center for Science in the Public Interest (CSPI) that analyzed the same data set also highlighted this declining trend in refuge compliance (Jaffe 2009). Most growers continue to adhere to the refuge requirements and a portion of the non-compliant growers likely have planted some refuge (though not large enough in size). However, decrease in refuge compliance is a concern and can increase the risk of resistance by reducing the availability of susceptible insects to mate with any resistant survivors of *Bt* corn. It is noted that the observed decrease has occurred despite increased efforts by registrants and the National Corn Grower Association (NCGA) to provide more outreach tools, improved educational efforts, and more avenues of communication with growers. In addition, grower awareness of IRM requirement has consistently increased since 2002 through 2008, suggesting that there is a degree of willful non-compliance by some growers. The reasons for the increase in non-compliance are not clear but could include financial incentives (i.e., increased yield on *Bt* acres), logistical difficulties with planting refuges, lack of availability of refuge seed, or ignorance of refuge requirements and IRM obligations.

Table D7. Summary of Telephone/Online Survey Results for Corn Borer-Protected *Bt* Corn Growers from 2002 to 2008 (Data from ABSTC annual reports 2002 through 2008)[1]

Survey Question	Survey Year						
	2002	2003	2004	2005	2006	2007	2008
	----------% respondents----------						
Adherence to Refuge Size							
Weighted average across all four regions surveyed	86	92	91	92	89	80	78
NC/SC/SE MO/TN/MS (50% refuge in cotton-growing areas)	77	82	78	82	80	N/A	N/A
E IA/N IL (20% refuge)	88	96	95	94	89-99		
SD/MN/NE/W IA (20% refuge)	85	93	90	91			
KS/OK/TX (20% refuge) [High Plains]	81	82	93	95	82		
Adherence to Distance Requirements							
Weighted average across all four regions surveyed	89	93	96	96	96	88[5]	88[5]
NC/SC/SE MO/TN/MS [cotton-growing region]	78	83	84	80	80	N/A	N/A
E IA/N IL	95	96	96	97	89-99		
SD/MN/NE/W IA	89	94	98	97			
KS/OK/TX [High Plains]	79	92	94	94			
Awareness of IRM Requirements							
All growers	88	93	92	92	92	96	98
Unaided Recall of IRM Requirements							
Refuge size (weighted average across all four regions surveyed)	42	52	53	59	N/A	63[4]	79[4]
Refuge distance (weighted average across all four regions surveyed)	37	49	55	53	49	79[4]	89[4]
Refuge size (Corn Belt and High Plains) [2]					61	N/A	N/A
Refuge size (cotton-growing region) [3]					31	N/A	N/A

[1] The data in this table include only growers planting single trait Bt corn registrations expressing Cry1Ab and Cry1F. Growers with stacked products containing Cry1Ab or Cry1F (and other toxins for rootworm control) are not included.
[2] Corn Belt and high plains = Iowa, Illinois, South Dakota, Minnesota, Nebraska, Kansas, Oklahoma, Texas, i.e., three of four regions surveyed
[3] Cotton-growing region = North Carolina, South Carolina, south-eastern Missouri, Tennessee, and Mississippi
[4] Cotton Belt numbers are not separated from Corn Belt survey results
[5] Percent reflects only those growers that could remember the layout of all their Bt fields; 447 out of 467 (95.7%) in 2007; 298 out of 317 (94%) in 2008.

ABSTC has focused the survey on growers who plant more than 200 corn acres in the Corn Belt

and 100 cotton-growing areas. Annually, these larger growers plant more than 85% of the *Bt* corn acreage. EPA's initial review of the survey (see T. Milofsky memo to M. Mendelsohn, 7/15/04 and EPA letter to ABSTC, 8/19/04) noted concerns associated with the use of farm size as a component of the grower survey participant selection criteria. First, the terms and conditions of the registration state that the annual survey shall consist of a "statistically representative sample of *Bt* corn growers." Since corn borer-protected corn is grown on farms of all sizes, a survey that excludes responses from smaller growers would fail to provide a "statistically representative" picture of all growers and their compliance. It would exclude a body of growers who plant approximately 15% of the *Bt* corn acres. A 2003 CSPI report (Jaffe 2003) using USDA/National Agricultural Statistics Survey (NASS) data from 10 corn-growing states found substantially lower IRM compliance on small farms (<200 acres). This finding may have been indicative of inadequate grower education among smaller farmers.

In response to EPA's concerns, ABSTC proposed to amend the terms of the registration to limit the surveys to growers planting more than 200 acres (100 total acres in the cotton-growing region) (amendment requests from *Bt* corn registrants, October and November, 2005). ABSTC provided four reasons as to why the third-party survey should continue to exclude growers with low acreage:

1. The farm size restriction ensures that the survey covers the largest number of corn acres. 2002 USDA/NASS statistics indicated that nearly 88% of all *Bt* corn in the 10 states sampled in 2002 was planted on farms with greater than 200 total acres of corn.
2. The farm size restriction has been in place since 2000 and ensures consistency in methodology for year-to-year statistical comparisons.
3. Inclusion of smaller farms would dilute the number of acres surveyed if current sample sizes remained constant.
4. The cost of the survey would increase significantly if sample size was increased and/or extra efforts were made to survey smaller farms. The extra cost would not be justified and inclusion of small farmers would have limited impact on the potential for resistance development.

After review (see S. Matten memo to M. Mendelsohn, 2/15/06), EPA accepted ABSTC's rationale for focusing the third-party survey on growers with larger acreages. It was recognized that the grower survey is not a direct measurement of individual compliance and that the on-farm assessment program (intended to detect non-compliant individuals) includes all growers regardless of size. An analysis of the 2003 NASS data indicated that there would have been only a difference of 2.3% in compliance (range of 1.7% to 5.7% for individual states) if both larger and smaller farms were included in the NASS assessment. According to this same data set, less than 5% of farms in the 10 states would have been classified as potentially contributing to a localized cluster of small farmers growing *Bt* corn that could have led to higher local selection pressure for resistance. It is unlikely that such farms could form clusters that were not interspersed amongst the other 95% of larger farms. Further, even if refuge adherence on smaller farms was found to be lower than on larger farms, the overall acreage of *Bt* corn not protected by the required refuge would be extremely small. Therefore, the lower overall amount

of *Bt* corn on smaller farms would not likely increase the risk of resistance evolution to *Bt* corn.

d) On-Farm Assessments

The on-farm assessment program is the portion of the CAP that identifies individual non-compliant growers (regardless of farm size) for remedial IRM education, follow-up reassessments, and other activities as part of the phased compliance approach (PCA). It can also serve as a tool to enhance the registrant's understanding of the obstacles growers face in implementing IRM requirements. The mandatory on-farm assessment program was fully implemented for the first time in 2003 and has typically encompassed approximately 2,000 - 2,200 growers. Company representatives are trained to conduct on-farm assessments. Topics covered in these trainings include IRM requirements, messages that should be communicated to growers, follow-up actions if growers are found to be out of compliance and how to use the IRM assessment form. All assessment forms contain the same questions, but the introductory paragraphs and company representative sections are customized to suit the needs of each registrant. Registrants respond to all compliance deviations identified in the assessments according to the common set of standards outlined in the PCA.

Data from the on-farm assessments (2003 through 2008) of corn borer-protected corn are summarized in Table D8 below. The assessments do not have the statistical power associated with the consistently stratified and randomized telephone/on-line surveys and are not used to measure representative rates of non-compliance. Fewer on farm assessments were made in 2008 relative to previous years, though this coincided with an increase in the on-farm assessments for growers of stacked *Bt* corn PIPs for lepidopteran and corn rootworm control. In 2007 and 2008, no information was provided regarding specific non-compliance with refuge size and distance. This information should be provided in future reports to be consistent with previously collected data and to illustrate how growers are out of compliance (see EPA review in J. Martinez memo to M. Mendelsohn, 4/15/09).

Table D8. Cumulative Results (2003-2008) for the First-Time On-Farm Assessments of Corn Borer-Protected *Bt* Corn Growers (Data from ABSTC annual reports 2003 through 2008)[1]

	Number of growers					
	2003	**2004**	**2005**	**2006**	**2007**	**2008**
Growers assessed	1961	2130	2215	2020	2083	1312
Compliant growers	1,789 (91%)	2032 (95.4%)	2089 (94.3%)	1930 (95.5%)	1895 (91.0%)	1132 (86.3%)
Noncompliant growers [2]	172 (9%)	98 (4.6%)	126 (5.7%)	90 (4.5%)	188 (9.0%)	180 (13.7%)
Refuge size deviations	64 (3%)	27 (1.3%)	33 (1.5%)	33 (1.6%)	N/A	N/A
Refuge distance deviations	127 (7%)	69 (3.2%)	68 (3.1%)	64 (3.2%)	N/A	N/A
Insignificant deviations	68 (4%)	59 (2.8%)	51 (2.3%)	45 (2.2%)	59 (2.8%)	46 (3.5%)
Significant deviations [3]	104 (5%)	39 (1.8%)	75 (3.4%)	45 (2.2%)	129 (6.2%)	134 (10.2%)

[1] The data in this table include only growers planting single trait *Bt* corn registrations expressing Cry1Ab and Cry1F. Growers with stacked products containing Cry1Ab or Cry1F (and other toxins for rootworm control) are not included.
[2] Some growers had compliance deviations other than refuge size or distance or some growers may have had both a refuge size and a refuge distance deviation; thus, the total of refuge distance and size deviations does not equal the number of non-compliant growers.
[3] Significant deviations are defined as: A *Bt* corn grower who planted less than 15% non- *Bt* corn refuge (except in certain cotton growing areas in which case it would be less than 40% non- *Bt* corn refuge); or planted fewer than 2/3 of the *Bt* corn fields are planted within 2 mile of a non- *Bt* corn refuge.

e) Tips and Complaints

As required by the terms of registration, *Bt* corn registrants must have a "tips and complaints" system as a mechanism for individuals (e.g., growers, sales representatives, etc.) to report alleged instances of IRM noncompliance. A system was developed by ABSTC and implemented for the first full-season in 2003. It allows each registrant to develop a "tips and complaints" system that is "compatible with their business operations..." The registrants have developed mechanisms (e.g., customer service numbers) to receive alleged instances of non-compliance with the IRM requirements. The tips and complaints number is publicized through a variety of IRM communications mechanisms (e.g., technical bulletins, grower guides, letters, seed catalogs, newsletters, etc.).

The number of tips and complaints (for all *Bt* corn registrations) received through 2008 is summarized in Table D9 below. Each of these growers identified through the tips and complaints mechanism were visited as part of the on-farm assessment program. However, it is not possible to determine whether any of the non-compliant growers identified via the tips and complaints route were subject to the Phased Compliance Approach.

EPA's initial review of the tips and complaints system identified a number of flaws that could make it difficult to report instances of non-compliance (see T. Milofsky memo to M. Mendelsohn, 7/15/04). This was highlighted by the fact that there were no legitimate tips and complaints in 2003 or 2004. In response, ABSTC stated that "the companies are planning to modify language in *Bt* corn product use guides and other grower communications to clearly state how growers can register a tip or complaint about an allegedly non-compliant grower by calling a toll free number or by contacting a company representative" (October 4, 2004 letter to EPA). Since the 2005 growing season anonymous tips have been reported, though they have been relatively rare.

Table D9. Anonymous Tips and Complaints about Non-Compliance with IRM requirements (Data from ABSTC annual reports 2003 through 2008)

Year	Number of Tips and Complaints
2003	0
2004	0
2005	5
2006	3
2007	14

2008	5

f) Phased Compliance Approach

ABSTC's 2002 CAP included a standard set of procedures (shown in Table D10) known as the Phased Compliance Approach (PCA), which is to be used by registrants when responding to instances of grower noncompliance with the IRM requirements. The PCA also established a tiered approach for non-compliance with "significant" deviations and "other" deviations. For refuge requirements of 20% (Corn Belt) and 50% (cotton-growing regions) significant deviations were defined as: a *Bt* grower planted less than 15% non-*Bt* corn refuge (in cotton growing areas less than 40% non-*Bt* corn refuge); or fewer than 2/3 of the *Bt* corn fields were planted within ½ mile of a non-*Bt* corn refuge. Under the PCA, sales are to be suspended to individual growers for one year after two years of significant deviations. Following the one-year suspension, growers will need to requalify to purchase seeds.

Table D10. Phased Compliance Approach (PCA) – Standards for *Bt* Corn Refuge Non-Compliance (submitted with the ABSTC 2002 CAP)

	Mandatory Responses	Additional Responses
Significant Deviations	• IRM education. • Warning letter. • Compliance assistance contact prior to planting. • Compliance assessment contact for the following growing season. • Deny access to the *Bt* corn product for any significant deviation two years in a row.	• Invoice monitoring. • Technical assistance. • Grower IRM training. • Reaffirmation of IRM obligations. • Deny access to the *Bt* corn product for other deviations that are repeated over a period of years.
Other Deviations	• IRM education. • Letter and/or compliance assistance contact prior to planting. • Compliance assessment contact in the following growing season.	

Grower identified as non-compliant (significant or other deviations) are required to receive a "compliance assessment contact" the following year under the PCA. Non-compliant growers are typically identified through the on-farm assessment program (see discussion in the on-farm assessment section below). Table D11 summarizes the numbers of non-compliant growers

reassessed under the PCA and the growers still found to be out of compliance. As of the 2008 growing season, two growers have been denied access to *Bt* corn technology due to consecutive years of significant non-compliance.

Table D11. Reassessment of Non-Compliant Corn Borer-Protected Corn Growers Under the Phased Compliance Approach (taken from ABSTC annual CAP reports) [1]

Year	Reassessments [2]	Significant Deviations [3]	Loss of Access to Technology
2004	172	0	0
2005	98	0	0
2006	126	2	2
2007	90	0	0
2008	188	0	0

[1] The data in this table include only growers planting single trait Bt corn registrations expressing Cry1Ab and Cry1F. Growers with stacked products containing Cry1Ab or Cry1F (and other toxins for rootworm control) are not included.
[2] Reassessments of growers identified with deviations (significant and other) to refuge requirements the previous growing season.
[3] Significant deviations recorded the following season. Two successive years of significant deviations results in loss of access to Bt corn technology.

EPA's reviews of the PCA and annual reports identified several concerns with the overall approach (see T. Milofsky memo to M. Mendelsohn, 7/15/04). First, one year of seed suspension may be insufficient to reform noncompliant growers. Second, it is unclear how the PCA will address repeat violators. However, the PCA results indicate that the reassessment process and the penalty for repeated non-compliance in the second year have been effective in bringing non-compliant growers back into compliance. Through 2008, only two growers (out of 674 assessed) have lost access to Bt corn due to repeated years of significant non-compliance.

8) Grower Education

Growers are perhaps the most essential element for the implementation and success of any IRM plan as they will ultimately be responsible for ensuring that refuges are planted according to guidelines and that *Bt* fields are monitored for unexpected pest damage. Therefore, a program that educates growers as to the necessity of IRM and provides guidance as to how to deploy IRM should be an integral part of any resistance management strategy. The 2000 SAP also suggested that a comprehensive education program may help increase IRM compliance (SAP 2001). Ideally, the educational messages presented to growers should be consistent (among different registrants) and reflect the most current resistance management guidelines. Specific examples of education tools for growers can include grower guides, technical bulletins, sales materials, training sessions, Internet sites, toll-free numbers for questions or further information, and educational publications.

9) Annual Reports

Written reports on various aspects of IRM, submitted on an annual basis to EPA, are of great aid in the evaluation of the success of resistance management for *Bt* corn. The Agency has received annual reports from *Bt* corn registrants (as a requirement of registration) on *Bt* corn sales/market penetration, IRM-related research, grower education, grower compliance and resistance monitoring. It is particularly useful to receive reports from *Bt* corn registrants on grower compliance and resistance monitoring.

10) *Bt* Sweet Corn IRM

Attribute *Bt* sweet corn is a BT11 hybrid and expresses the Cry1Ab protein. It is thought that Attribute, like BT11 field corn, contains a high dose for ECB. The other targeted pests, for which there is not a high dose, are CEW and FAW.

Refuge for *Bt* sweet corn was not recommended for the following reasons: 1) sweet corn is typically harvested earlier than field corn (18-21) days after silking (before most lepidopteran larvae complete development); and 2) all *Bt* sweet corn residues were to be destroyed within one month of harvest (a practice that presumably would destroy any live larvae left in corn stalks). The 2000 SAP agreed that this approach should be sufficient to mitigate pest resistance to *Bt* sweet corn. Several panelists, however, suggested a shorter crop destruction period (i.e., 14 days instead of one month) (SAP 2001).

The terms and conditions of the *Bt* sweet corn registration stipulate that, based on IRM concerns, the product is for commercial use only and is not available to growers planting less than 40 acres. However, should smaller growers (i.e., those planting less than 40 acres) adhere to the crop destruct requirements for *Bt* sweet corn (to destroy any overwintering insects), it is unlikely these growers will pose a threat to pest resistance given the limited acreage involved. As such, from an IRM perspective, it should be possible to lift the acreage restrictions on smaller growers for *Bt* sweet corn.

Regarding crop destruction, it is possible that the crop destruct requirement may not be adequate in itself to mitigate the threat of resistance for ECB. Specifically, there are data (Mason et al. 1983) that show variance among different crop destruct techniques in terms of the number of surviving ECB. The variation in the efficacy of crop destruct techniques may increase the risk for ECB resistance in *Bt* sweet corn. This risk may be mitigated by either: 1) Prescribing a specific and effective crop destruct technique; or 2) Utilizing structured refuge. Regarding option #1, it should be noted that corn cultivation practices vary (i.e., plow vs. no-till) and certain crop destruct techniques may not be compatible with all practices. Furthermore, additional research could help to verify the most appropriate crop destruct technique.

The threat of resistance for CEW and FAW in sweet corn should be lower than ECB due to the fact that CEW and FAW typically complete development in corn ears (unlike stalk-boring ECB), which are mostly harvested and removed from the field prior to crop destruction (Lynch et al. 1999). Also, FAW is known to overwinter only in south Florida, south Texas, and the

Caribbean.

As part of the registration, a FAW monitoring program has been developed to determine susceptibility to Cry1Ab (other *Bt* sweet corn target pests, ECB and CEW, are part of the monitoring program for *Bt* field corn, described in the Monitoring section - D.2.b.4). Susceptibility was determined with diet assays utilizing toxin overlays for FAW populations collected from four geographic locations in 1998 and 1999. For 1998, the LD_{50} range was 0.90 - 1.50 µg Cry1Ab/cm^2 and for 1999, the LD_{50} range was 2.14 - 10.22 µg Cry1Ab/cm^2 (Lynch et al. 2000). Susceptibility data for FAW and Cry1Ab are summarized in Table D12 below. The decreased susceptibility observed in 1999 was not likely an increase in tolerance to the toxin (the lab colony used as a control showed similar trends) and presumably was the result of population variability or experimental effects.

It should be noted that for FAW, resistance monitoring is less of a concern due to the fact that resistance is not likely. However, should there be significant *Bt* sweet corn acreage in areas where FAW overwinters (south Florida and south Texas), it would be beneficial for FAW to be monitored for resistance. As a term of registration, FAW monitoring was required for Attribute Bt sweet corn in counties where acreage exceeds 5,000 acres and FAW is capable of overwintering. However, FAW data have not been submitted since the 2000 growing season because this 5,000 acre/county requirement has not been met. Given the low acreage, there has likely been low pest pressure for resistance with *Bt* sweet corn and it is unlikely FAW tolerance to Cry1Ab has increased due to the planting of *Bt* sweet corn.

Table D12. FAW Susceptibility (LC_{50}) to Cry1Ab (Lynch et al. 2000, Hamm et al. 2001)

Year	Susceptibility (LC_{50} - µg Cry1Ab/cm^2)
1998	0.90 - 1.50
1999	2.14 - 10.22
2000	0.27 - 0.94

c. Stacked and Pyramided PIPs Containing Cry1Ab and/or Cry1F

Since the initial registrations of Cry1Ab and Cry1F, these traits have been "stacked" (i.e., combined with one or more other toxins for control of multiple pest complexes) or "pyramided" (i.e., combined with one or more other toxins for control of the same target pest) into additional PIP products.

Both Cry1Ab and/or Cry1F have been combined with other *Bt* toxins for control of corn rootworm (*Diabrotica* sp.) in stacked PIPs. For these products, IRM must be addressed for lepidopteran and corn rootworm by deploying either separate refuges (for each pest complex) or a combined refuge that addresses both pest complexes. Complete discussions of corn rootworm IRM (as well as combination refuges for lepidoptera and corn rootworm) can be found in the

regulatory documents (i.e., BRADs) for the relevant corn rootworm PIP traits. The following products with Cry1Ab or Cry1F have been stacked with corn rootworm traits (some have also been pyramided with multiple lepidopteran toxins):

- MON 863 x MON 810 (YieldGard Plus) – EPA Reg. No. 524-545 (Cry1Ab, Cry3Bb1)
- MON 88017 x MON 810 (YieldGard VT Plus) – EPA Reg. No. 524-552 (Cry1Ab, Cry3Bb1)
- DAS-59122-7 x TC1507 (Herculex Xtra) – EPA Reg. No. 68467-6 (Cry1F, Cry34/35Ab1)
- MIR 604 x Bt11 (Agrisure CB/RW) – EPA Reg. No. 67979-8 (Cry1Ab, Cry3A)
- Bt11 x MIR 162 x MIR 604 – EPA Reg. No. 67979-13 (Cry1Ab, Vip3Aa20, mCry3A)
- MON 89034 x TC1507 x MON 88017 x DAS-59122-7 (SmartStax) – EPA Reg. No. 524-581 and 68467-7 (Cry1F, Cry1A.105, Cry2Ab2, Cry3Bb1, Cry34/35Ab1)
- DAS-59122-7 x TC1507 (Herculex Xtra) + TC1507 (Herculex) (Optimum AcreMax 1 Seed Blend) – EPA Reg. No. 29964-6 (Cry1F, Cry34/35Ab1)
- TC1507 x DAS-59122-7 x MON 810 – EPA Reg. No. 29964-8 (Cry1F, Cry1Ab, Cry34/35Ab1)
- DAS-59122-7 x MON 810 – EPA Reg. No. 29964-9 (Cry1Ab, Cry34/35Ab1)

Pyramided products for enhanced lepidopteran control have also been registered. Such products (with no stacked toxins for rootworm control) include the following:

- Bt11 x MIR 162 – EPA Reg. No. 67979-12 (Cry1Ab, Vip3Aa20) – discussed in the BRAD for Vip3Aa20
- TC1507 x MON 810 – EPA Reg. No. 29964-7 (Cry1F, Cry1Ab) – discussed below

TC1507 x MON 810 (EPA Reg. No. 29964-7)

TC1507 x MON 810 was registered by Pioneer and expresses both Cry1Ab and Cry1F for lepidopteran control. The product was registered with the same refuge requirements and IRM plan as the single toxin Cry1Ab (MON 810) and Cry1Ab (TC1507) registrations (see details on the IRM plan in section D.1.a of this document).

For stacked and pyramided products of previously-registered toxins developed by conventional breeding, IRM data typically consists of efficacy and protein expression data. These data are used to confirm that the stacked/pyramided products will have comparable dose profiles as the single toxin constituent products. Along these lines, Pioneer submitted protein expression data and field efficacy data for the major target pests.

Protein expression data (MRID# 475109-04) were generated for the pyramided product (1507 x MON 810) and the single toxin constituent PIPs. Studies were conducted in greenhouses and plant tissues were analyzed by ELISA for protein expression. Pioneer sampled leaf, stalk, root, whole plant, and grain tissues for the analyses (pollen tissue was also collected but was often below the limit of detection). Corn tissues were sampled during the R1 corn growth stage, except for grain which was taken from the R6 stage. The results from the ELISA assays showed

that Cry1F and Cry1Ab, protein were expressed at comparable levels in the pyramided product as the single toxin constituent products. Expression was consistent throughout all tissues -- only small differences in protein levels were noted between the pyramid and single toxin products which were not statistically significant.

Field efficacy data (MRID# 476778-03) were generated for ECB, FAW, and SWCB with TC1507 x 59122 x MON 810 (a PIP that also contains a corn rootworm toxin). Efficacy for ECB was tested for both first and second generation using artificial infestation in five states (Illinois, Indiana, Iowa, Minnesota, and Nebraska). First generation ECB were assessed with a "reversed" Guthrie 1-9 damage scale (i.e., 1 = heavy damage, 9 = no damage). Second generation ECB damage was assessed by sampling stalks from infested plots and measuring tunnel (feeding) damage (in cm). The results for first generation ECB were consistent across test locations. Untreated controls (isoline corn) experienced moderate ECB feeding, with a damage ratings range of 4.8 to 7.9. The stacked product (1507 x 59122 x MON 810) suffered no damage (rating 9 for all locations). A similar pattern was also observed for second generation ECB. Stalk tunneling in the untreated control groups was moderate to high (average tunnel length 7.5 to 26.2 cm) while damage was very low in *Bt* corn, with average tunnel lengths generally less than 0.2 cm (two locations had 0.6 and 0.8 cm avg. tunneling). SWCB testing was conducted similarly to ECB but using natural infestation at two sites in Tennessee and Kansas. Damage at the R4-R5 reproductive stage was assessed through measurements of stalk tunneling. Results showed that non-*Bt* corn (isoline) experienced moderate damage (average 10.9 cm tunnel length) while the *Bt* treatments had little or no tunneling (0 to 1.33 cm avg. length). FAW tests were conducted using artificial infestation on whorl stage corn (V6). FAW feeding damage was scored after three weeks of infestation with a 1 to 9 scale (1 = heavy damage, 9 = no damage) much like the one used for ECB testing. Data from the field trials showed that FAW inflicted significant damage (average damage ratings of 2.22 to 3.10) on isoline corn but the Bt corn treatments experienced very slight to no damage from FAW (avg. ratings of 8.93 to 9.0).

Both MON 810 (Cry1Ab) and Herculex (Cry1F) have been considered "high dose" for ECB and likely SWCB, but not for CEW. Given the confirmatory protein expression and efficacy studies, the stacked products can also be considered high dose for ECB and SWCB and non-high dose for CEW. With similar dose profiles, the IRM assessments and refuge plans developed for the single toxin PIPs (i.e., TC1507 and MON 810 can be applied to the pyramided PIP (1507 x MON 810). Therefore, the submitted data supported the use of the existing lepidopteran IRM plan including a 20% refuge (50% in cotton growing regions) for TC1507 x MON 810 corn (see EPA review in A. Reynolds memo to A. Sibold, 1/7/10).

d. Information to Improve the Risk Assessment

Although the Agency has considered the most up-to-date scientific information in this risk assessment, resistance management is a developing field. Therefore, the IRM strategies may be improved with the collection of additional information, the results of which can be submitted in annual research reports. As part of the 2001 *Bt* crops reassessment, a number of areas were identified that would benefit from additional research. These data goals are summarized in Table D13 below with a 2010 status update.

Table D13. Summary of Data That Could Improve Insect Resistance Management Strategies for *Bt* Corn Products

Data Need (As Identified in 2001)	Pests	2010 Status
Pest Biology: e.g., larval movement, adult movement, mating behavior, pre- and post-mating dispersal, ovipositional behavior, fitness, and overwintering habitat and survival	ECB, CEW, SWCB	No additional data were required but research has been conducted and reported in the public literature. No information has been submitted to EPA that would change this risk assessment for Cry1Ab or Cry1F corn.
North to South Movement	CEW	Data were required as a condition of registration in 2001. A summary of the data and conclusions can be found in section D.2.b.3 of this document.
High Dose Verification (using 1998 SAP techniques)	ECB and SWCB	No additional data have been received, although it is likely that both Cry1Ab (MON 810, BT11) and Cry1F (TC 1507) corn express a high dose for both of these insects.
Resistance Allele Frequency	ECB, CEW, SWCB, FAW (Bt sweet corn)	Some additional information has been received for ECB and FAW populations of interest as part of the resistance monitoring data (see section D.2.b.4)
Cross-Resistance - Cry1F, Cry2A, Cry1A proteins	ECB, CEW, SWCB	Additional data have been developed for Cry1F and Cry1Ab (see section D.2.b.6). Data for Cry2A, Cry1F, and Cry1A proteins are further discussed in the BRAD for MON 89034 *Bt* corn.
Evaluation (field studies and models) of Refuge Options (20% external refuge (sprayable) v. 20% in-field) - [Issues to consider: production of susceptible insects (500:1 ratio) in insecticide treated and non-insecticide treated refuges, adequacy of size, structure, and deployment of the refuge, rotation of refuge.]	ECB, CEW, SWCB	Data on the impacts of insecticide use on *Bt* corn refuges were required as a condition of registration. These data are discussed in section D.2.b.3 of this document.
Models: development, validation, refinement of existing and new models	ECB, SWCB, CEW	New models have been conducted for stacked and pyramided products containing Cry1Ab and Cry1F and are discussed in the regulatory documents for those products. New modeling has been conducted for single trait Cry1Ab and Cry1F products to evaluate insecticide use in refuges (see section D.2.b.3).
Collection of Baseline Susceptibility	ECB, SWCB, CEW, FAW (Bt sweet	These data have been required as

Data Need (As Identified in 2001)	Pests	2010 Status
Data and Validation of Discriminating/Diagnostic Dose	corn)	conditions of registration. Submitted information is discussed in section D.2.b.4 of this document.
Evaluation of Resistance Monitoring Techniques, e.g., discriminating v. diagnostic dose, F_2 screen, sentinel plots, gene mapping	ECB, CEW, SWCB	No additional data were required or submitted to EPA.
Grower Compliance - more detailed information on refuge (%, deployment, and management), impact of non-compliance	ECB, CEW, SWCB	Development of a compliance program was required as a condition of registration. A full discussion of this program can be found in section D.2.b.7 of this document.

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E. Benefits Assessment (2001 Bt Corn Assessment)

Bt Corn Plant-Incorporated Protectants

a. Usage Estimates

There have been two grower benefit studies of *Bt* corn (field corn) (Marra, Carlson & Hubbell, 1998 and Carpenter & Gianessi, 1999) and both used essentially the same information about yield advantages due to reduced insect damage, technology fee and reductions in conventional pesticide use. As discussed above, only a very small portion of field corn acres are treated with foliar insecticides.

Field corn is the most widely grown crop in the 48 contiguous states and USDA/NASS reports acres planted for all of these states. Table E.1. contains the states which average in excess of 1 million acres planted to field corn. These 16 states account for about 90 percent of field corn acres and 93 percent of the value of field corn grown for grain. EPA estimates of planted *Bt* corn are based on registrant submissions of annual sales. Since the first registrations in 1995, planting of *Bt* corn increased to a maximum of almost 20 million acres in 1999 and decreased slightly in 2000 to 19.5 million acres. USDA estimates of acres planted to *Bt* field corn for those states covered by the corn estimating program represents about 15 million acres versus the 19.5 million acres estimated by this Agency. The states in the corn estimating program were not readily available and USDA has estimates published for 1999 and 2000. We have compared state level usage estimates from the registrants (which is claimed to be Confidential Business Information) with the USDA estimates for those states where USDA has published adoption estimates and they do not agree. The USDA estimate is based on a survey while the EPA estimate is based on sales data. Each method has its potential problems and we have no basis to prefer one over the other except that the EPA estimate covers all corn producing states. If the USDA estimates covered more states (those states listed in Table E.1 below), we would have reason to prefer the USDA estimates. The EPA estimate is used as the basis for the benefit calculation. However, we recognize that this could present an overestimate of benefits.

The Agency has recently registered an additional plant-incorporated protectant known as Cry1F which has efficacy against those pests currently controlled plus some control against the black cutworm and armyworms. This product does not provide any control of the corn rootworm which is the primary soil borne insect pest of field corn. There would have been little, if any, commercial planting of Cry1F in 2001 so we do not know actual farmer experience with this material.

Table E. 1. Acres Planted to Field Corn and Crop Value for Selected States

State	Acres planted	Crop value	Percent *Bt* corn
	1,000	1,000 dollars	
Colorado	1,253	306,309	NA
Illinois	10,867	3,007,964	14
Indiana	5,767	1,506,910	7
Iowa	12,300	3,119,481	25
Kansas	3,200	811,489	26
Kentucky	1,317	291,899	NA
Michigan	2,183	449,236	8
Minnesota	7,167	1,674,917	30
Missouri	2,667	568,464	22
Nebraska	8,633	2,109,197	26
New York	1,087	127,698	NA
Ohio	3,517	896,085	6
Pennsylvania	1,533	237,273	NA
South Dakota	3,933	649,020	37
Texas	2,150	465,761	NA
Wisconsin	3,600	718,233	14
U.S. total	79,032	18,215,745	
Percent of U.S. total in these States	90	93	89

Source: Crop Production 2000 Annual Summary, Crop Values 2000 Annual Summary, and Acreage. NA indicates USDA did not estimate *Bt* corn usage.

b. Insect Pests

The *Bt* protein expressed by the field corn plant targets Lepidopteran insects. This protein is

effective in controlling the European corn borer, the Southwestern corn borer and provides some control of the corn earworm. The European corn borer and the Southwestern corn borer are difficult to control since they bore into the corn stalk where most conventional pesticides do not provide any control. Some control can be achieved with foliar applications prior to entry into the stalk but control is not adequate since the pests usually arrive on the plant over a period two to three weeks. *Bt* corn presents a method to control virtually all of these pests. Corn borers consume plant energy and weaken the corn stalks so they are more likely to lodge (blow down) under windy conditions. Lodged stalks are difficult, if not impossible, to mechanically harvest.

The corn earworm enters the ear via the silk and feeds on the ears. Again once the insect larvae is in the ear under cover of the husk, it is difficult to control with conventional insecticides. These pests populations are not constant from year to year nationwide nor within regions. Varying population levels results in varying pest pressure, varying need for pest control and variation in the benefit a farmer will gain from controlling the pest.

The populations of these pests vary geographically and from year to year depending on environmental conditions including farming practices. This includes the past years planting of *Bt* corn. The planting of *Bt* corn on a significant portion of the corn acreage in a region probably would reduce the populations of corn borers available to survive the winter and reproduce the next year. This means that a farmer is not guaranteed to be making a wise investment with a decision to plant *Bt* corn. Some areas have historically high levels of European corn borer or the Southwestern corn borer. These include southwestern Kansas plus parts of Colorado, New Mexico, Texas and Oklahoma for the Southwestern corn borer and Iowa, Minnesota, Nebraska and South Dakota for the European Corn Borer. There is some probability that some farmers have adopted *Bt* corn when their actual damage did not warrant the additional expense. We have not been able to estimate the magnitude of this. It is reasonable to expect farmers to be examining losses in their mandated refuge acres to determine whether they should continue to plant *Bt* corn and that if damage does not reach economic thresholds, they would not plant *Bt* corn the next year.

Major states where these pests are regularly considered economic pests recommend farmers examine yields for previous years and projections for the current year (Nebraska 2001). We understand *Bt* corn is less expensive to plant than the cost of non *Bt* seed plus spraying a conventional pesticide. If a farmer were to apply two sprays, the cost would be significantly lower. It needs to be noted that something less than about 20 percent of *Bt* field corn acres were likely to have been treated with an insecticide which could target corn borers (this is discussed below).

Bt corn has been on the market for a number of years and the levels of reported damage have been down from the historical average. It is not clear if there is just an unusually long period of reduced pest pressure or if the use of *Bt* corn has resulted in a lowering of insect pressure. Either way, it is logical to expect some farmers will make a decision to reduce the portion of their corn acres planted with *Bt* corn unless the monitoring of damage in refuge acres implies continued planting of *Bt* corn is financially justified.

The addition of Cry1F *Bt* corn provides some degree of control against the black cutworm and armyworms in addition to the European corn borer and the Southwestern corn borer.

c. Potential to Replace Conventional Pesticides

Insecticide use on field corn is largely to control the soil pest complex, rather than the *Bt* corn target pests. As discussed above, those pests targeted by *Bt* corn, are difficult to control with conventional pesticides. Therefore, little conventional pesticide has been used on field corn. Five to eight percent of field corn acres may have been treated with conventional pesticides to control these pests implying that 6.3 million of those 79 million acres planted to field corn could have been treated with conventional insecticides. A look at changes from 1995 and 2000 indicates a reduction of around 3.9 million acre treatments due to all causes.

Cry1F *Bt* corn has the potential to replace conventional pesticides in areas where corn growers rotate corn with a crop such as soybeans which is not a host to the corn rootworm and their crop land is river bottomland. These farmers are unlikely to also have corn rootworm and it may be a wise economic decision to plant Cry1F *Bt* corn and would be part of the 6.3 million acres planted to field corn discussed above. Some of these farmers probably adopted other *Bt* corn products but if they have, they may have used conventional pesticides to control the black cutworm. Where there are cutworms, farmers may make the decision to plant Cry1F *Bt* corn and be able to reduce or eliminate use of conventional insecticides. While we can predict there will be some reduction, we do not have data to accurately estimate the magnitude of the potential reduction.

Assuming there was a 3.9 million acre treatment reduction due to the corn borer pests, this equates to 0.2 acre treatments per *Bt* corn acre (all 19.5 million acres). The majority of field corn growers who adopted *Bt* corn did not consider reductions in cost of conventional pesticides applied when they made the decision to plant or not plant *Bt* corn because they had not been applying conventional pesticides to control these pests.

d. Benefits for Field Corn

Field corn is grown on a average (1998-2000) of about 79 million acres with production close to 10 billion bushels having a market value of about $18 billion (Table E.2). The 1994-1996 average for acres planted was 76.6 million acres with production of 8.9 billion bushels and a market value of $24.6 billion, reflecting the fact that higher yields have been more than offset by lower prices. Other market factors may have been contributing to the change in market value.

Table E.2. 1998-2000 National Field Corn Data

Item	Unit of measure	1998	1999	2000	Average
Acres planted	1,000 acres	80,165	77,386	79,545	79,032

Acres of *Bt* corn	1,000 acres	14,500	19,800	19,500	17,933
Corn production	1,000 bushels	9,758,685	9,430,612	9,968,358	9,719,218
Yield	Bushels/acre	134.4	133.8	137.1	135.1
Value	1,000 dollars	18,922,084	17,103,991	18,621,160	18,215,745
Price per bushel	dollars	1.94	1.82	1.85	1.87
Source: Crop Production, 2000 Annual Summary, USDA/NASS Crop Value, 2000 Annual Summary, USDA/NASS Acres of *Bt* corn are EPA estimates obtained from industry sales reports.					

Two studies estimated farmer level benefits from the adoption of *Bt* field corn. Marra, Carlson and Hubbell (1998) utilized a 4 to 8 percent increase in yield, nominal reduction in pesticide use and a technology fee of about $11 per acre. They discussed economic thresholds for adoption of *Bt* corn but did not estimate overall benefits. Gianessi and Carpenter (1999) estimated benefits for 1997 through 1999. They estimated farmers had significant gains in 1997 and a net loss in 1998 and 1999 because yield gains went from 12 bushels in 1998 to 4.2 in 1998 and 3.3 in 1999 and the price of field corn went down. The decrease in prices was due to a combination of market forces largely unrelated to the introduction of *Bt* corn.

We conducted a partial budgeting analysis for grower benefits of field corn using three most recent year averages and obtained estimates of benefits from $38 million (about $2 per acre to $219 million (about $12 per acre) per year (table 3). Thirty-eight million dollars per year across 19.5 million acres of *Bt* corn implies benefits of less than $2 per acre which may be too close to the margin to make economic sense for many farmers. It is likely that benefits would be about $38 million or less in years of low insect pressure and benefits would be in the area of $219 million in years of high insect pressure. It may be clearer to state that benefits of *Bt* corn are likely to be less than a maximum of $219 million per year. They would be around $38 million or less in years of low infestations. There is considerable uncertainty regarding the magnitude of yield changes and the amount of the technology fee. The technology fee is charged by the seed dealer and can be subject to discounts due to market factors. It is likely that farmers who find planting of *Bt* corn to be a profitable move will lose money planting *Bt* corn in years of low insect pressure but that the expected gain (the returns from averaging out impacts over the years of high to low pest pressure) warrant planting *Bt* corn or that *Bt* corn is viewed as insurance against the high infestation years.

This partial budget estimate does not include the cost of the insect refuge. Acres planted to refuge would not obtain the gains of $12 per acre. That is, the grower would plan for the gain on up to 80 percent of field corn acreage.

The introduction of Cry1F *Bt* corn may result in additional acres being planted to *Bt* corn and/or could result in a shift in acres from another *Bt* corn products to Cry1F. It is reasonable to expect some corn growers have not planted *Bt* corn because the economic gain from control of European corn borers or Southwestern corn borers did not warrant *the cost, but* Cry1F *Bt* corn controls additional pests and its use may be warranted. We are not projecting the numbers of corn acres that may be impacted.

The partial budgeting estimates assume there are no price effects which would affect the distribution of benefits. Typically, if growers increase production, value per unit goes down implying some of the benefit is being passed to processors or the consumer resulting in less benefit to the growers. The other implication is non-adopters of the technology also will receive less money per acre (or per bushel) for their crop. These effects apply to any new technology which results in increased efficiency.

Table E. 3. Grower Benefits of *Bt* Field Corn (Partial Budgeting Approach)

Item	Unit of measure	Low Insect Pest Pressure**	High Insect Pest Pressure
Acres planted 1/	1,000	79,032	79,032
Yield 1/	bushels/acre	135.1	135.1
Acres *Bt* corn 1/	1,000	17,933	17,933
Yield increase 2/	bushels/acre	5.4	10.81
Value of yield change 3/	dollars/acre	10.11	20.21
Technology fee 4/	dollars/acre	8.00	8.00
Per acre benefit 5/	dollars	2.11	12.21
Total grower benefit 6/	1,000 dollars	37,758	218,979

1/ Three Year Average from Table E. 2.
2/ based on 4 to 8 percent yield increase from Marra et al. (1998) and from Carpenter and Gianessi (1999).
3/ change in yield times average grower price received of $1.87/bushel (Table E. 2.).
4/ The 1999 technology fee as estimated by Carpenter & Gianessi (1999)
5/ value of yield increase less technology fee.
6/ per acre benefit times acres *Bt* corn.
**This is intended to be representative of the lower bound benefits. Various conditions could result in actual benefits below this lower bound estimate for some years.

e. Mycotoxin Reduction

Mycotoxins are chemicals produced by fungi, that are toxic or carcinogenic to animals and humans. The most commonly occurring mycotoxins on corn are produced by the fungal genus *Fusarium*, and are known as fumonisins (Munkvold, 2000). There are several different kinds of fumonisins: FB_1, FB_2, FB_3, FB_4, FA_1, and FA_2 (Marasas, 1996; Ross et al., 1992). Another class of corn mycotoxins are those produced by the genus *Aspergillus*, including the notorious aflatoxins. The economic impact of aflatoxins is greater than that of other mycotoxins because they can be passed into milk if dairy cows eat contaminated grain (Munkvold et al., 1999).

Damage by insect pests such as the European corn borer can be an important factor for mycotoxin development in corn. Insect pests promote the growth of mycotoxin producing fungi in two ways: 1) They carry fungal spores from the plant surface to the surfaces of damaged kernels, and 2) They create entry wounds on the kernels for the fungi. Even when the insect pests do not directly carry fungal spores to the corn wounds, ambient spores deposited later on tissue wounded by pest feeding are more likely to infect the plant (Munkvold, 1999). Field studies have shown that damage due to southwestern corn borer (SWCB) can increase aflatoxin levels (Windham, et.al. 1999).

When mycotoxin contamination occurs in corn, the potential damages can be both economic costs to growers and health risks to humans and livestock. Corn grain that contains mycotoxins above a certain level is more likely to be rejected in the market, forcing growers to accept the lower price for non-food uses. In particular, the FDA's new proposed guidelines about recommended levels of fumonisins in grain may have a significant impact on amount of corn sold at the better food/feed prices. While these FDA guidelines for fumonisin levels are not yet set as action levels, they have been proposed to industry as safety thresholds (Randall A. Lovell, Center for Veterinary Medicine/ FDA, personal communication). The guidelines in human food and animal feed are shown in Tables E1 and E2.

Table E1. FDA guidelines for total fumonisins in human foods (FDA, 2001a).

Product	**Total Fumonisins (FB_1+FB_2+FB_3) parts per million (ppm)**
Degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, corn flour with fat content of < 2.25 %, dry weight basis)	2 ppm
Whole or partially degermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour with	4 ppm
Dry milled corn bran	4 ppm

Cleaned corn intended for masa production	4 ppm
Cleaned corn intended for popcorn	3 ppm

Table E2. FDA guidelines for total fumonisins in animal feed (FDA, 2001b).

Animal or Class	Recommended Maximum Level of Total Fumonisins in Corn and Corn By-Products (ppm[1])	Feed Factor[2]	Recommended Maximum Level of Total Fumonisins in the Total Ration (ppm[1])
Horse[3]	5	0.2	1
Rabbit	5	0.2	1
Catfish	20	0.5	10
Swine	20	0.5	10
Ruminants[4]	60	0.5	30
Mink[5]	60	0.5	30
Poultry[6]	100	0.5	50
Ruminant, Poultry & Mink Breeding Stock[7]	30	0.5	15
All Others[8]	10	0.5	5

[1] total fumonisins = $FB_1 + FB_2 + FB_3$.
[2] fraction of corn or corn by-product mixed into the total ration.
[3] includes asses, zebras and onagers.
[4] cattle, sheep, goats and other ruminants that are $\geq$ 3 months old and fed for slaughter.
[5] fed for pelt production.
[6] turkeys, chickens, ducklings and other poultry fed for slaughter.
[7] includes laying hens, roosters, lactating dairy cows and bulls.
[8] includes dogs and cats.

Fumonisins are toxic to livestock, especially horses, swine, and cattle; and are carcinogenic in laboratory animals. The 1989 US corn crop had particularly high levels of fumonisins, resulting in dramatic increases in the horse disease equine leukoencephalomalacia (ELEM) and the swine disease porcine pulmonary edema (PPE) (Marasas, 1996; Ross et al., 1992). At the time of the 1989 mycotoxicosis outbreaks, FB1 concentrations in suspect swine feeds were 20-360 ppm, and in equine feeds were 8-117 ppm. Non-problem feeds contained concentrations below 8 ppm (Ross et al., 1992). Epidemiological studies have linked consumption of fumonisin-contaminated grain with elevated esophageal cancer incidence in humans (Marasas, 1996). A definitive link between fumonisin levels and human cancer is not possible from these studies due to the presence of possibly confounding effects in the study. Other documented toxicological effects of fumonisins in laboratory studies include toxicity and carcinogenicity in rats, cytotoxicity to mammalian cell cultures, and phytotoxicity to weeds and other plants including tomatoes (inhibiting growth and chlorophyll synthesis) (Marasas, 1996).

One of the benefits of Bt corn (a genetically modified, pest-protected corn) is that it has demonstrated drastically reduced occurrences of contamination by the mycotoxin fumonisin. This is because Bt corn is far less prone to insect injury, which in turn prevents the growth of fumonisin producing fungi. Certain events of Bt corn, such as MON810 and BT11, can reduce fumonisin levels by as much as 90% (Munkvold, 2000). This implies both private and social benefits: economic returns on corn sales would increase, and there would be potential reductions in mortality and morbidity among livestock and, presumably, humans.

Munkvold (2000) estimated that, if the current FDA guidelines for fumonisins in food were to become action levels, about 160 million bushels of corn in just the states of Iowa, Illinois, Missouri, and Nebraska would be at risk of rejection – an annual loss of value in the tens of millions of dollars in just these states. Depending on the amount of Bt corn planted in these states in lieu of conventional corn, the *savings* that Bt corn would afford might also range in the tens of millions. Vardon (2000) made similar predictions on potential economic losses due to fumonisins in corn, estimating an annual loss of $11 million. Hence, the economic benefits from Bt corn by this estimation is in the several millions. One of the reasons this value is lower than Munkvold's estimates (in the tens of millions of benefits) is that Vardon's model assumes that the corn that is rejected for food is still acceptable for animal feed. This in fact may no longer be the case, as FDA's most recent proposed guidelines in animal feed are at about the same level as for human food. Neither study calculates the costs of the fumonisin mycotoxin to human health, acknowledging the difficulty of extrapolating from available epidemiological studies directly to

human health benefits.

Aflatoxins are known carcinogens to laboratory animals and presumably man; hence, the presence of aflatoxins in foods is restricted to the minimum levels practically attainable by modern processing techniques. Historically, aflatoxin levels in corn have been highest in the Southeastern states. Corn from anywhere in the US may be affected, however, depending on the growth, harvesting and storage conditions involved, as was the case with aflatoxin contamination in the Midwest in 1988 and Texas in 1987 (FDA, 1999).

Currently, the action level for aflatoxins in corn grain for human food is 20 ppb (FDA, 1994; Munkvold, 1999). When dairy animals consume feed containing high levels of aflatoxin, one of the metabolized aflatoxins (B_1) may be secreted into the animals' milk as aflatoxin M_1. Dairy cattle consuming corn feed that contains less than the FDA action level of 20 ppb total aflatoxins, however, should produce milk under the 0.5 ppb action level for aflatoxin M_1 in milk (FDA, 1999). In 1969, the FDA had established the action level of 20 ppb aflatoxins in all foods, including animal feed; however, subsequent tests showed that aflatoxin levels above 20 ppb could be fed to certain food-producing animals without endangering either these animals or consumers of food derived from the animals (FDA, 1994). The action levels for aflatoxins in corn are summarized in Table E3:

Table E3. FDA's action levels for corn aflatoxins in human and animal foods (FDA, 1994).

Product or animal	***Aflatoxin action level (ppb)***
Human food	20
Milk	0.5
Beef cattle	300
Swine over 100 lbs	200
Breeding beef cattle, swine, or mature poultry	100
Immature animals	20
Dairy animals	20

FDA compliance monitoring program from 1990 to 1996 indicate that 6.6 percent of corn samples exceeded the aflatoxin action level for food (Vardon, 2000). The potential value of crops lost because of aflatoxin contamination has been estimated to be $47 million per year in food crops (corn and peanuts) and $225 million per year in feed corn. The cost of livestock morbidity was estimated at $4 million per year (Vardon, 2000).

Studies comparing Bt with non Bt hybrids have usually show no significant difference in

aflatoxin levels. The variability in aflatoxin levels due to environmental factors overwhelms the beneficial effects related to insect control seen in the current Bt products (Odvody 2001, personal communication). Even though insect damage ratings are lower for Bt hybrids, apparently the amount of insect feeding is sufficient for *A. flavus* establishment and subsequent aflatoxin contamination (Windham, et.al 1999). Studies across 10 states in 2000 found little or no aflatoxin to begin with, and in cases of substantial aflatoxin contamination, no significant differences were seen between Bt and non Bt hybrids (Headrick, 2001). Two studies in Alabama in 1999 also showed no difference in aflatoxin levels while yields were significantly higher for the Bt hybrids (Delamar, et. al, 1999).

A study in 1999 in Corpus Christi, Texas actually showed that under conditions of extreme drought and artificial inoculation with *A. flavus*, Bt corn hybrids had higher aflatoxin levels compared with non-Bt isolines (Odvody 2000). The study was expanded in 2000 to include more locations with mixed results in terms of aflatoxin contamination levels between Bt and non-Bt isolines. However, Bt corn hybrids had less aflatoxin contamination than the non-Bt hybrids, on average in 2000 when the comparison is done excluding one of the 9 Bt/non-Bt hybrids (Pioneer 3394). The author concludes that differences in individual hybrid susceptibility to infection by *A. flavus* was the primary factor influencing aflatoxin accumulation (Odvody 2001, personal communication). The reasons for the negative performance of the one particular commercial line are not known. Factors that were forwarded as hypothesis by the researcher are the particular adaptability of a hybrid in a region, differences in the test material (i.e., not true isolines), or unintended effects from the insertion of the Bt gene to the plant's natural defense system against infection. Better pest control is viewed as only one of many defenses in the attempt to develop hybrids with improved performance against aflatoxin contamination (Odvody 2001, personal communication).

e. Future Benefits

This analysis has used the benefits which have occurred from the adoption of *Bt* corn as a basis to project future benefits. It is expected that benefits will continue at about the magnitude of those for the period of the analysis. Individual growers will have more experience with *Bt* corn and should have the experience to determine whether the reduced damage warrants the additional cost for the technology. The European corn borer and the Southwestern corn borer cause significant damage in certain regions of this country most years. Those growers in these areas with regular infestations, will continue to utilize this technology and others with significant damage will adopt the technology. Those whose infestations is not serious, will not continue to utilize the technology. It will be interesting to see whether forecasting of insect problems can become sufficiently sophisticated to enable growers throughout major corn growing areas to know enough about the probability of an economic infestation to plant *Bt* corn only in those years when the problem will warrant it.

4. *Bt* Sweet Corn Plant-Incorporated Protectant

In 1998, EPA approved the registration of Syngenta's (formerly Novartis) Cry1Ab (*Bt*11) sweet corn. Major pests controlled are European corn borer (ECB), corn earworm (CEW), and fall

armyworm (FAW). Approximately 742,000 acres of sweet corn is grown in the United States, including processed and fresh corn. EPA recently registered Cry1F *Bt* sweet corn which has control of the Black cutworm as well as the ECB, CEW and FAW. This material has not been available for a sweet corn growing season. Therefore, while the addition of Cry1F has the potential to increase acres planted to *Bt* sweet corn, we have not factored this new registration into the analysis.

Table E. 4 . Top States Growing Sweet Corn (Acres Planted in 1999)

State	Processed	Fresh	Total Sweet Corn
Minnesota	127,400	0	127,400
Wisconsin	107,100	8,900	116,000
Washington	99,400	2,100	101,500
New York	33,100	35,900	69,000
Oregon	44,200	6,900	51,100
Florida	0	38,900	38,900
California	0	31,000	31,000
Illinois	16,600	7,600	24, 200
Pennsylvania	2,800	20,800	23,600
Georgia	0	22,000	22,000
Ohio	0	17,200	17,200
Idaho	15,900	0	15,800
Michigan	0	11,500	11,500

State	Processed	Fresh	Total Sweet Corn
New Jersey	0	10,500	10,500
Selected States	446,400	213,300	659,700

Source: NASS, USDA, 2000

a. Potential to Replace Chemical Insecticides

Commercial field data studies for *Bt* sweet corn submitted by Syngenta suggest the potential to achieve equivalent yields to traditional varieties while reducing the quantity of insecticides used to control these pests. According to NASS data, about 3.3 million acre treatments of insecticide are applied annually to sweet corn. Based on the pest complex being targeted, the potential market for *Bt* sweet corn is 2.0 million acres, or 60% of total acre treatments (Doane, 1998). The major chemical insecticide alternatives are cyhalothrin-lambda, permethrin, and methomyl with esfenvalerate, carbaryl, chlorpyrifos, cyfluthrin, and methyl parathion. *Bt* microbial sprays are used to a lesser extent. (Doane, 1998).

b. Benefits for Sweet Corn

The majority of sweet corn acres are planted to processed corn while the value per acre of fresh corn is over 3 times the market value of processed corn.

Table E. 5. Value of Processed and Fresh Sweet Corn

Year	1997	1998	1999
Processed			
Acres Planted	478,900	486,400	473,400
Value ($000's)	250,329	238,748	234,448
Value/acre	522.72	490.85	495.24
Fresh			

Acres Planted	254,900	255,700	268,300
Value ($000's)	418,617	452,410	458,632
Value/acre	1,642.28	1,769.30	1,709.40
Total			
Acres Planted	733,800	742,100	741,700
Value ($000's)	668,946	691,158	693,080
Value/acre	911.62	931.35	934.45

Source: NASS, USDA, 2000

On average, sweet corn is treated for all insect pests 5.5 times per year: 4.3 times for processed corn and 8.6 times for sweet, although the variability is quite significant among states.

Table E. 6. Fresh Sweet Corn Insecticide Treatments, 1998 (thousands of acres)

State	Acres Planted	Acre Treatments	No. of Applications/Yr
California	31.0	389.4	12.56
Florida	38.9	657.6	16.9
Georgia	22.0	115.8	5.26
Illinois	7.6	30.3	3.99
Michigan	11.5	50.4	4.39
New Jersey	10.5	82.2	7.83

State	Acres Planted	Acre Treatments	No. of Applications/Yr
New York	35.9	136.2	3.79
Oregon	6.9	5.8	0.84
Washington	2.1	11.3	5.4
Wisconsin	8.9	36.7	4.12
Total for Top States	175.3	1,479.0	8.65

Source: NASS, USDA, 2000

A simulation model based on a demand curve for *Bt* sweet corn shows an average net benefit/acre of $3.55 for processed corn and $5.75 for fresh corn. Upper limits benefits for *Bt* sweet corn are based on savings from reduced insecticide applications. An upper limit application savings of $45/acre is based on 9 applications per year, 60% (5.4) of which target *Bt* pests, and each application costs an average of $8.25 per acre (Doane, 1998). The source for market share estimates for *Bt* sweet corn is USDA's Pest Management Practices 1999 summary. The USDA estimated 4% of vegetables in 1999 were planted with genetically modified seed to resist insects and sweet corn is the only crop with a registered plant-incorporated protectant. However, Syngenta Seeds considers their market share information for *Bt* sweet corn information to be confidential business information. Information available from USDA indicates the quantity of *Bt* plant-incorporated protectant on all vegetables for 2000 was too small to quantify (*Bt* plant-incorporated protectants for vegetables are only registered for use on potatoes and sweet corn). If we assume less than 5% of sweet corn is *Bt* sweet corn, seed premium cost $30/acre (personal communication: Warnick, Debra, Novartis Seeds, Inc [year]), upper limit benefits $45/acre, and upper limit *Bt* specific costs are $58/acre (which is 6.2% of the average value per acre grown in 1999). Net benefits are $5.38/acre.

Table E. 7 Estimated Benefits for *Bt* Sweet Corn

Item	Unit of Measure	Value
Acres planted	acres	739,200
Average benefits to *Bt* adopters	dollars per acre	40

Average other costs to *Bt* adopters	dollars per acre	5
Technology fee	dollars per acre	30
Net benefit	dollars per acre	5
Insecticide treatments saved	per *Bt* acre	4.8

Source: Acres planted-average from Table E.5

Average benefit to *Bt* adopters was estimated by the simulation model using the subset of observations for which benefits exceeded all costs.

Other costs (insect resistant management, discounts for marketability, and underlying hybrid yield) equals the average cost to the adopters using the subset of observations for which benefits exceeded all costs. Other costs have been estimated indirectly by the model.

Technology fee is the seed premium.

Net benefit equals average benefit less other costs less technology fee.

Insecticide treatments saved equals average benefit divided by average treatment cost ($8.25 per treatment) based on the assumption that the principal benefits are reduction in treatment costs.

The average *Bt* sweet corn user must cover all costs (the seed cost premium & other costs), and if benefits are mainly to reduce cost, then use reduction can be deduced from the average benefits plus seed cost premium divided by the chemical cost per acre. At a cost per treatment of $8.25 and average benefit of $40.00/acre, the use reduction of 4.8 treatments per year. Applied to the 29,600 acres assumed treated with *Bt* plant pesticides, total pesticide use reduction is estimated to be 142,000 acre treatments for 1999.

c. Environmental Benefits of *Bt* Sweet Corn

A number of comments addressed the potential environmental benefits of *Bt* sweet corn. Because of the low adoption rates, potential benefits have not been realized. Biorationals with novel modes of action have not significantly replaced the more acutely toxic organophosphate and pyrethroid insecticides. *Bt* sweet corn allows a transition to more selective toxins and increase the beneficial arthropod community (Fleisher, 2000). The benefits of reducing toxic insecticide use are as follows:

1. Sweet corn in Florida is still mostly hand picked and packed in the field. Detasseling operations also bring workers into direct contact with sweet corn (Nuessley, 2000).

2. Maryland growers of sweet corn and potatoes are concerned about worker exposure risks and believe that the *Bt* technology offers an alternative to toxic insecticides (Dively, 2000).

3. Studies in Maryland conducted in 1999 clearly showed that BT11 corn had significantly less fumonism contamination of up to 96% compared to its non-transgenic isoline (Dively, 2000).

4. Adoption of *Bt* corn (field and sweet corn) may help an areawide suppression of the corn earworm since corn serves as the primary nursery for recruitment of CEW populations, which later in the season infest soybeans, lima bean and tomatoes. Further insecticide use reductions could therefore occur in these other crops as well as corn (Dively, 2000).

d. Future Benefits

Information available suggests adoption of *Bt* sweet corn has not grown as expected which implies fear of consumer rejection or that the technology is not working as expected. We expect the seed companies marketing this technology will resolve any technology problems and consumers will accept the product in time.

5. Summary of Results

a. General Findings

EPA believes that significant benefits accrue to growers, the public, and the environment from the availability and use of certain *Bt* plant-incorporated protectants. Direct benefits to growers for all *Bt* products is estimated to be less than $350 million in 2000. Indirect or environmental benefits occur as improved pest resistance decreases corn diseases that result from insect interactions. Insect pests that damage ears, kernels or stalks are causative agents for mycotoxin development by carrying fungal spores to the surfaces of damaged kernels and by creating entry wounds on the plant. Common mycotoxins on corn are fumonisins and aflatoxins. Fumonisins are toxic to livestock, especially horses, swine, and cattle; and are carcinogenic to humans and animals. Aflatoxins are known carcinogens to laboratory animals and presumably man. Growers must accept lower prices if mycotoxin levels exceed FDA food standards or total loss if the lower feed standards are not met. The public costs of mycotoxins to human health has not been quantified due to the difficulty of extrapolating from available epidemiological studies.

There are several *Bt* corn plant-incorporated protectant products registered by three basic registrants with more than 19 million acres planted to *Bt* corn. The per acre benefits are modest but there are a large number of acres where the only control before *Bt* corn was a hybrid with corn stalks with some resistance to corn borers. *Bt* corn provides season long control and became a viable control. Annual benefits are estimated to be up to $220 million.

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F. Benefits Assessment for MoCry1F Corn (2005 Conclusion)

Benefit Claims Made in Maize-optimized Cry1F-protected Corn Event TC6275 Public Interest Document (Zabik et al., 2003; MRID# 460193-12)

Dow AgroSciences (Dow) believes that the maize-optimized Cry1F-protected corn is clearly in the public interest and provides data to support the following claims:

1. Maize-optimized Cry1F-protected corn provides highly efficacious control of key Lepidopteran pests of field corn

2. Maize-optimized Cry1F-protected corn has the potential to provide comparable or superior pest control to existing commercial Bt corn products for key corn pests and affords a broader spectrum of pest control than do corn products expressing the Cry1A(b) insect resistance trait.

3. The use of moCry1F-protected corn is expected to reduce the use of chemical insecticides.

4. Yields of moCry1F-protected corn varieties will be significantly greater than the yields of the non-Bt varieties.

5. Economic models show that moCry1F-protected corn maximizes economic benefits as compared to the application of conventional chemical insecticides to control Lepidopteran pests that are susceptible to Cry1F.

6. Maize-optimized Cry1F-protected corn represents a competitive choice for insect control.

7. Maize-optimized Cry1F-protected corn will solidify and extend the benefits of insecticide use reduction that have been established for plant-optimized Cry1F-protected corn.

8. Cry1F expressed in corn poses no foreseeable risks to human health or the environment.

Field Efficacy

Field efficacy trials were conducted over multiple locations and years. EPA reviewed the moCry1F-protected corn field efficacy data submitted by Dow AgroSciences (Babcock and Bing, 2003) and found these data to be Aacceptable@ (see EPA review, Hill, 2004). Dow has submitted sufficient field efficacy data to demonstrate that event TC6275 (maize-optimized Cry1F-protect corn) offers excellent control of European corn borer (*Ostrinia nubilalis* (Huebner), ECB), southwestern corn borer (*Diatraea grandiosella* (Dyar), SWCB), fall armyworm (*Spodoptera frugiperda* (J.E. Smith), FAW), black cutworm (*Agrostis ipsilon* (Hufnagel), BCW), Western bean cutworm (*Richia albicosta* (Smith), WBCW), and suppression of the corn earworm(*Helicoverpa zea* (Boddie), CEW). TC6275 performed comparably to TC1507 (plant-optimized Cry1F-protected corn, Herculex I7) and showed significantly less infestation than the non-Bt isogenic hybrid. No additional data are required at this time.

Comparative Product Performance

Dow submitted an analysis of the comparative efficacy of event TC6275 (moCry1F) corn against several commercial standards (conventional and transgenic) for insect control in corn. A no treatment (no Cry1F) control was also included. Based on this analysis, TC6275 provides comparable performance relative to TC1507. TC6275 performed better or comparably with the commercial standards against ECB, SWCB, BCW, FAW, CEW, and WBCW.

Yield and Economic Benefits

The primary economic benefit of corn hybrids containing Cry1F insect resistance trait (mo and po Cry1F) is the protection of yield. At the individual farm level, there will be cost savings from reduced field scouting and applying fewer chemical insecticides. The two factors that drive a farmer to choose a Bt corn hybrid versus conventional hybrids treated with chemical insecticides are : 1) the higher level of field efficacy that Bt corn hybrids, such as Cry1F corn hybrids, offer in comparison to current chemical insecticides and 2) the Ainsurance@ factor of a Bt corn hybrid as a prophylactic control measure. Both of these factors result in potential yield benefits to the farmer.

Yield and Agronomic Characteristics

Previously, EPA concluded that Event TC1507 corn hybrids expressing plant-optimized Cry1F were competitive with other *Bt* hybrids as well as with non-*Bt* corn varieties (EPA, 2001b). Event TC6275 corn hybrids expressing maize-optimized Cry1F was also competitive with plant-optimized Cry1F corn hybrids (Event TC1507) as well as non-*Bt* corn varieties.

Agronomic performance traits of TC6275 hybrids were compared to isogenic hybrids containing TC1507 and isogenic hybrids containing no transgenic trait in field trials for early (11 locations) and late (15 locations) season hybrids in 2002. The comparison of both late and early maturity hybrids found no statistically significant differences within either maturity group among the TC6275 hybrid, the TC1507 hybrid, and non-transgenic isogenic hybrid for grain density, plant stature, emergence vigor, root lodging, and dropped ears (see Tables 5 and 6 in Zabik et al., 2003). There were no statistically significant yield differences between either early or late maturity comparisons of TC6275 and TC1507 hybrids. Both the early maturity TC6275 and TC1507 hybrids were significantly higher yielding than the early maturity non-transgenic isogenic hybrid. This is most likely due to the protection offered by Cry1F against European corn borer-induced yield loss. Both early and late maturity TC6275 hybrids had significantly better top integrity scores than the non-transgenic hybrids. Although there were some statistically significant differences found in other parameters, these differences were not considered to biologically or commercially significant. Based on this analysis, there would be no expectation of increased likelihood of weediness due to the presence of the mo-Cry1F transgene.

Economic Benefits

EPA has previously analyzed the economic benefits resulting from Cry1F-protected corn (Event TC1507) (EPA, 2001b). At product maturity, grower benefits of Cry1F-protected corn are estimated to be between $28 and $81 million per year on 7.3 to 12.5 million acres of field corn. The range depends upon the technology fee, from $7.50 to $13.13/acre. Bt-related costs are assumed to be $10/acre. These costs cover refuge requirements and marketability concerns and apply to situations where Cry1F replaces chemical control or no control. Acres at risk are estimated to be 25 million acres, based on the states affected and the extent of area infested. Grower benefits could vary by an average of $3.90/acre to $6.51/acre. The very wide range is due to the wide range of the proposed technology fee. It should be noted that these annual benefits would occur at product maturity, or 3 to 5 years after commercialization. The analysis does not consider possible stacked products which offer multiple protections and efficiencies, the effect of new competitor products, or the impact of increased competition on overall market equilibrium conditions. Increased competition should offer growers more choice and lower the cost of pest control. The benefits are the incremental improvement to grower profits compared to current practice. All costs are eventually passed along to consumers in the long run, but this analysis did not deal with when that would occur.

The economic benefit to the grower who plants corn varieties containing Event TC6275 (moCry1F) was evaluated on the basis of data on yield and efficacy as reported in Zabik et al. (2003) using the Herculex Value Calculator. The Herculex Value Calculator, http://www.dowagro.com/herculex/calc/index.htm, is a tool that allows growers to evaluate the advantages/disadvantages of using corn varieties using the Cry1F insect resistance trait. Three scenarios using both early and late maturity hybrids were used to illustrate the economic benefit

associated with Event TC6275 (see Table 7, p. 33, in Zabik et al. 2003). As summarized in Table 7 of Zabik et al. (2003), the value of the Event TC6275 corn ranged from $40 to $98 per acre. The economic benefit of ECB protection accounts from from $24 to $81 per acre of this total with an added benefit of from $16 to $17 per acre for BCW protection.

Marketing Issues

TC6275 (moCry1F) and TC1507 (poCry1F) are positioned to compete against the following chemical alternatives: bifenthrin, carbofuran, chlorpyrifos, cyhalothrin-lambda, permethrin, fipronil, tebuprimiphos/cyluthrin, tefluthrin, terbufos, and zeta-cypermethrin and against the Cry1Ab-protected corn hybrids. The registered chemical alternatives commonly used to treat the target pest complex protected by Cry1F are restricted use for the most part. They have precautionary label statements such as extremely toxic to fish and aquatic organisms, wildlife and require protective clothing for workers. The specific organophosphate and pyrethroid pesticides likely to be replaced are ranked in the top 15 of all pesticides with respect to reported incidents of mortality to non-target wildlife. Many of these products also control corn rootworm, which is the most significant pest of corn and is frequently treated along with the lepidopteran target pest complex of Cry1F. Cry1F performance data have shown performance equal to or better than any of the conventional chemical alternatives or other Bt corn hybrids against ECB, BCW, SWCB, FAW, WBCW, and CEW. Growers may be more likely to choose Cry1F protected corn due to better product performance and broader spectrum of control. Cry1F protected corn is also expected to be economical on some unprotected fields and provide insurance against the risk of crop loss and the need to replant. But without rootworm protection, the use of Cry1F to reduce conventional pesticide use is somewhat limited.

Human Health and Environmental Risks and Benefits

The Agency=s human health and environmental safety assessments attest to the safety of the Cry1F protein (both as poCry1F and moCry1F) that it is expected to pose no unreasonable adverse effects to human health or the environment (Matten, 2005a and b; Hill, 2005; EPA 2001 a and b). Based on the Agency=s previous evaluation of the benefits of Event TC1507 (EPA, 2001a and b), Event TC6275 can also substantially reduce the health and environmental risks associated with the use of traditional chemical insecticides. Cry1F-protected corn varieties will potentially decrease the reliance on conventional pesticides when used as part of an integrated pest management program. Reductions in the use of conventional pesticides would eliminate the need to transport, mix, apply, and dispose of these pesticides, reduce spray-drift and run-off associated with some of the registered alternatives, and reduce potential adverse effects to non-target organisms. Increased use of Cry1F-protected corn would also improve worker protection as compared to chemical insecticides.

The data submitted show that poCry1F and moCry1F corn produce the same Cry1F protein and therefore have the same expected toxicity to target pests. Data also demonstrate that Cry1F is not heat labile (MRID 452748-01), is rapidly digested in simulated gastric fluid (MRID 447149-03),

and does not share any amino acid sequence similarity to known allergens (MRID 449717-01). These data indicate there is no likely potential for the Cry1F protein to be a food allergen.

Expression data reveal that moCry1F plants express lower concentrations of Cry1F protein in pollen and grain than poCry1F plants (1/6 and1/2, respectively) while moCry1F plants express the Cry1F protein three to eight times greater in the leaf than the poCry1F plants (Zabik et al. 2003, see p. 36, Table 8). Increased expression in the stalks and leaves should decrease the risk of resistance development by borers (a higher, high dose) whilst the decreased expression in pollen and grain should decrease the non-target exposure to Cry1F expressed in corn (Zabik *et. al* 2003).

Similarly, testing with bacterially prepared Cry1F protein at levels greatly exceeding those found in maize optimized plants resulted in no effect with several beneficial species including the monarch butterfly. Additionally, field monitoring for effects of poCry1F corn on non-target insects confirmed the absence of adverse effects to non-target organisms (MRID 450201-13, see Table 1). Data were also provided regarding the rapid soil degradation of poCry1F protein, approximately 3.13 days (MRID 450201-05), although the Agency required additional data to study long-term soil degradation (EPA, 2001b).

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III. Regulatory Position on Bt Corn

A. Overview (2001 Conclusions)

Currently registered *Bt* corn products were conditionally approved for commercial use in August 1996 (Bt11 Cry1Ab field corn amendment), December 1996 (MON810 Cry1Ab field corn registration), February 1998 (Bt11 Cry1Ab sweet corn registration), May 2001 (Cry1F field corn

registration) under FIFRA Section 3(c)(7)(B). The data reviewed for the initial commercial approvals as well as new data and reports received, results of public meeting, hearings, workshops, forums, and Scientific Advisory Panel meetings, and public comments received regarding the *Bt* crops reassessment have been taken into consideration. The scientific assessment has included product characterization, human health effects, gene flow, effects on non-target organisms, ecological exposure, insect resistance management, and benefits. Over the last five years, new data and information have been provided to the Agency in each of these areas and these data have been incorporated into the science assessment and has been taken into account in making regulatory decisions.

Tests have shown no toxicity to mammals from the Cry1Ab and Cry1F proteins; the proteins are readily digestible in gastric fluids and are non-glycosylated, the proteins are inactivated by typical food processing, and anticipated exposure of farm workers to the proteins is negligible. The Cry1Ab protein acute oral toxicity data submitted demonstrated no effects at the relatively high dose level of 4,000 mg/kg. The Cry1F protein acute oral toxicity data submitted demonstrated no effects at the relatively high dose level of 5,050 mg/kg. The Cry1Ab and Cry1F proteins were readily degraded in gastric fluid *in vitro*. Exposure via the skin or inhalation is not likely since the Cry1Ab and Cry1F proteins are contained within corn plant cells which essentially eliminates or reduces exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed products and drinking water. Worker exposure to the Cry protein via seed dust is also expected to be negligible because of the low amount of protein expressed in seeds of the transformed plants. Taken in total, these data allow the Agency to make a determination that for human health, there is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1Ab and Cry1F proteins and the genetic material necessary for their production. Thus, EPA concludes that there are no adverse effects on human health from the use of Cry1Ab or Cry1F proteins expressed in corn.

EPA has also reviewed the original data base and the new data, information, and comments regarding ecological effects. EPA has reviewed the potential for gene capture and expression of the Cry1Ab/Cry1F endotoxin in corn by wild or weedy relatives of corn in the United States, its possessions or territories. The Agency has determined that there is no significant risk of gene capture and expression of any *B.t.* endotoxin by wild or weedy relatives of corn product registrations in the U.S., its possessions or territories. In addition, the USDA/APHIS has made this same determination under its statutory authority under the Plant Pest Act.
The Agency has concluded that based on the weight of evidence there are no unreasonable adverse effects of Cry1Ab or Cry1F protein expressed in corn to non-target wildlife or beneficial invertebrates. However, EPA is requiring insect census estimates from representative fields to determine if there are long-term adverse impacts from the use of *Bt* corn, field tests of Cry1Ab and Cry1F protein accumulation and/or persistence in soil under a range of conditions typical of *Bt* crop cultivation as confirmatory data, and chronic avian data.

In the Cry1Ab ecological effects testing done, no treatment related effects were observed in Bobwhite quail or catfish fed Cry1Ab corn as part of their diet. No measurable deleterious effects from the Cry1Ab protein on honey bee larvae, honey bee adults, parasitic wasps,

Ladybird beetles, green lacewings, Collembola (springtails), and Daphnia were observed in submitted studies.

In the Cry1F ecological effects testing done, no treatment related effects were observed in Bobwhite quail fed Cry1Ab corn as part of their diet. No measurable deleterious effects from the Cry1F protein on honey bees, parasitic wasps, Ladybird beetles, green lacewings, Collembola (springtails), earthworms, Daphnia, and Monarch butterflies were observed in submitted studies.

MON 810 and Bt11 show relatively low toxicity to monarch larvae and the Cry1F protein has no detectable impact on monarch larvae. Overall, the available information indicates a very low probability of risk to monarchs in areas beyond the near edge of corn fields. Inside corn fields and at the near edge of corn fields there is low probability of monarch larvae encountering a toxic level of pollen for the Bt corn products covered by this risk assessment.

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. Cry1Ab protein bioactivity from Cry1Ab corn tissue added to the soil decreased with an estimated DT_{50} (Degradation Time) of 1.6 days and an estimated DT_{90} of 15 days. The bioactivity of purified Cry1Ab protein in soil decreased with an estimated DT_{50} of 8.3 days and a an estimated DT_{90} of 32.5 days. The bioactivity of purified Cry1F protein in soil decreased with an estimated DT_{50} of 3.13 days.

The issue of insect resistance management has generated more data, meetings, and public comments than all of the other sections covered in this BRAD. Insect resistance management (IRM) is the set of practices aimed at reducing the potential for insect pests to become resistant to a pesticide. *Bt* IRM is of great importance because of the threat insect resistance poses to the future use of *Bt* plant-pesticides and *Bt* technology as a whole. EPA considers protection of insect (pest) susceptibility of *Bt* to be in the "public good." EPA has determined that development of resistant insects would constitute an adverse environmental effect. In order to delay the development of insect resistance to *Bt* field corn by maintaining insect susceptibility, growers must choose at least one of structured refuge (a portion of the total acreage using non-*Bt* seed) options listed in Section V.B.4.a. above.

For *Bt* sweet corn, no specific refuge requirements are necessary because sweet corn is typically harvested much earlier than field corn, 18-21 days after silking, and before most lepidopteran larvae complete development. However, to mitigate the development of resistance, EPA has determined that crop residue destruction is necessary within 30 days. This practice will likely destroy any live larvae left in *Bt* sweet corn stalks and prevent overwintering of any resistant insects.

The IRM program for *Bt* field and sweet corn also require: 1) anyone purchasing *Bt* corn to sign a grower agreement which contractually binds the grower to comply with the IRM program and that there will be a mechanism by the year 2003 by which every grower affirms, annually, their contractual obligations to comply with the IRM program, 2) an IRM education program, 3) an IRM compliance monitoring program including a third party compliance survey and mechanisms

to address non-compliance, 4) an insect resistance monitoring program for each target insect pest, 5) remedial action plans to be implemented if resistance does develop, and 6) annual reporting of the IRM (and other) activities. No other pesticide products than the *Bt* crop products have such extensive IRM requirements.

In addition to assessing the risks from the use of Cry1Ab and Cry1F expressed in corn, EPA has evaluated the benefits from the use of these products. Direct grower benefits include improved yield and profitability, improved crop management effectiveness, reduction in farming risk, and improved opportunity to grow field corn in case of severe pest infestation. Total annual monetary grower benefits from the use of Bt field corn are less than $219 million annually. The magnitude of benefits for any year is largely a function of the level of lepidopteran insect pressure in that year. That is, other things being equal, the higher the insect pressure, the higher the benefits. The major environmental benefit is potential reduction in mycotoxins. EPA believes that use of Bt sweet corn would result in significant reductions in the use of chemical pesticides. However, the current use of Bt sweet corn is very low.

Pursuant to FIFRA Section 3(c)(7)(A), EPA may conditionally amend the registration of a pesticide if the Agency determines (i) that the pesticide and proposed used are identical or substantially similar to a currently registered pesticide and use thereof, or differs only in ways that would not significantly increase the risk of unreasonable adverse effects on the environment, and (ii) approving the amendment in the manner proposed by the applicant would not significantly increase the risk of unreasonable adverse effect on the environment. FIFRA defines "unreasonable adverse effects on the environment" in pertinent part as: "any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide . . . ." Thus, the FIFRA unreasonable adverse effects standard requires EPA to balance the risks and benefits of using the pesticide in reaching its regulatory decision.

EPA finds that the use of Cry1Ab or Cry1F expressed in corn will not significantly increase the risk of unreasonable adverse effects on the environment. This finding, however, applies only to the use of Cry 1Ab or Cry1F protein expressed corn under the terms and conditions of registration specified below, and only for the limited time period of 7 additional years (to October 15, 2008). The following sections set forth the basis for EPA's finding in general, and the basis for the decision to approve the registration subject to the specific terms and conditions identified below.

B. General Finding (2001 Conclusions)

EPA's finding that Cry1Ab or Cry1F protein as expressed in corn will not significantly increase the risk of unreasonable adverse effects on the environment is based on the analysis contained in the preceding sections of this BRAD and the specific terms and conditions that are imposed upon this registration, as set forth in Section V. In general terms, EPA concludes that use of Cry1Ab or Cry1F expressed in corn is effective at controlling significant lepidopteran pests of corn including European corn borer, corn earworm, and southwestern corn borer. Therefore, these

products have clear benefits for users. Beyond these economic benefits, EPA determines that Cry1Ab and Cry1F corn hybrids, to the extent they are an alternative to the use of other corn insecticides, will provide benefits in that use of Cry1Ab or Cry1F protein expressed in corn results in less human and environmental risk than chemical alternatives. In addition, EPA finds that the use of these products, subject to the specific terms and conditions set forth below, would not pose risks to human health or to non-target species. EPA also concludes that the use of Cry1Ab or Cry1F corn hybrids expressed in corn raises concerns with respect to insect resistance management. As discussed below, the registrations for Cry1Ab and Cry1F proteins expressed in corn is subject to specific terms and conditions that effectively restrict the use of the product in ways that EPA determines will adequately mitigate these concerns. Therefore, EPA determines that the allowed use will not significantly increase the risk of unreasonable adverse effects on the environment. Finally, EPA has identified the need for certain confirmatory data on potential accumulation of Cry1Ab and Cry1F proteins in soil and field impacts of Cry1Ab and Cry1F proteins on non-target species. The registration of these products is specifically conditioned on submission of these data.

By this reassessment, EPA has completed its tolerance reassessment for Cry1Ab (180.1173) under 408(q) of the FFDCA. The tolerance exemption for Cry1F (180.1217) does not require reassessment at this time.

C. Insect Resistance Management (IRM) Program (2001 Conclusions)

Rationale for IRM Requirements:

In deciding on the size, proximity, configuration, and management of the non-*Bt* corn refuge, EPA has taken into account empirical data on the pest biology and ecology of the three primary target pests, European corn borer (*Ostrinia nubilalis* (Huebner)), corn earworm (*Helicoverpa zea* (Boddie)), and southwestern corn borer (*Diatreae grandiosella* (Dyar)), models that predict the estimated time that resistance would develop to compare the effectiveness of various IRM strategies, economics, sustainability, and grower feasibility.

Beginning with the first *Bt* plant-incorporated pesticide registration, the Agency has taken steps to manage insect resistance to *Bt* with IRM plans being an important part of the regulatory decision. The Agency identified (later confirmed by the 1995 SAP) seven elements that should be addressed in a *Bt* plant-incorporated protectant resistance management plan: 1) knowledge of pest biology and ecology; 2) appropriate dose expression strategy; 3) appropriate refuge; 4) resistance monitoring and a remedial action plan should resistance occur; 5) employment of integrated pest management (IPM); 6) communication and education strategies on use of the product; and 7) development of alternative modes of action. IRM plans also include grower education and measurement of the level of compliance.

The Agency has determined that the 20% non-*Bt* field corn refuge requirements for *Bt* corn grown in the Corn-Belt and the 50% non-*Bt* corn refuge requirements for *Bt* corn grown in cotton-growing areas are scientifically-sound, protective, feasible, sustainable, and practical to

growers. Models have been developed by scientists in academia to predict the estimated time that insect resistance would develop to compare IRM strategies for *Bt* field corn. For example, if a high dose is achieved to control ECB (as it is for the currently registered *Bt* corn products), then these models predict that ECB will not evolve resistance for at least 99 years if a 20% refuge is implemented in the Corn Belt. Models are also used to predict the evolution of CEW resistance have also been used. These models indicate that 50% non-*Bt* field corn refuge in cotton-growing areas is sufficient to delay CEW resistance for at least the time frame of the registrations. A 20% non-*Bt* field corn refuge in the Corn Belt is sufficient to delay CEW resistance because CEW do not overwinter in the Corn Belt. EPA believes that the use of these models provides confidence that resistance will not evolve under the time frame of the registrations.

However, it should be noted that these predictive models cannot be validated without actual field resistance. They have limitations and the information gained from the use of such models can only be used as a part of the weight of evidence determination conducted EPA to assess the risks of resistance developing in target pest populations. EPA agrees with the October 2000 SAP that models are an important tool in determining appropriate *Bt* crop IRM strategies and that model design should be peer reviewed and parameters validated. In the absence of field resistance, EPA agrees with the October 2000 SAP that models are "the only scientifically rigorous way to integrate all of the biological information available, and that without these models, the Agency would have little scientific basis for choosing among alternative resistance management options." While the absolute number of years to resistance is not precisely determined from the models, the relative difference in effectiveness between refuge options can be determined. Thus, the utility of the models is not that they make accurate quantitative predictions, rather, it is that they enable the Agency to make informed judgments of the potential effects of using various refuge options.

In addition to assessing the likelihood of resistance, EPA has mandated specific requirements for annual resistance monitoring to determine, in a pro-active fashion, whether insect susceptibility has changed or whether resistance is likely to occur (or is occurring). After five years of analyzing resistance monitoring data (1996-2000), there is no evidence of European corn borer, corn earworm, or southwestern corn borer resistance developing in the field to the Cry1Ab delta-endotoxins produced by current registered *Bt* corn products. There are no resistance monitoring reports yet available for Cry1F field corn products because they were just registered in 2001. Therefore, EPA believes that resistance is not occurring in the field based on the available data. The Agency is mandating enhancements to the resistance management programs that will improve the certainty of detection of resistance. If insect resistance occurs, EPA has also mandated a specific remedial action plan that will contain (and perhaps eradicate) resistance prior to the occurrence of any widespread, endemic resistance.

In addition, to the use of biological data, predictive models, and resistance monitoring information, EPA also weighed practical considerations in deciding which refuge options to allow. Grower education and compliance with refuge options are essential to the success of any IRM strategy. Growers must be able to implement the refuge options within the constraints of their farming operations. Based upon the currently available scientific data and information and

understanding of farming operations, EPA believes that the 20% non-*Bt* field corn refuge options in the Corn-Belt and the 50% non-*Bt* field corn refuge options in the cotton-growing areas provide an adequate time-to-resistance for *Bt* field corn and are practical, sustainable, and feasible to growers. If the 20% non-*Bt* field corn refuge options in the Corn-Belt and the 50% non-*Bt* field corn refuge options are deployed correctly then, there is a very limited chance of insect resistance evolving over the next seven years of the registration of these products.

EPA has determined that a mandatory refuge strategy was not necessary for *Bt* sweet corn products to reduce the likelihood of resistance for the following reasons: 1) sweet corn is typically harvested earlier than field corn (18-21) days after silking (before most lepidopteran larvae complete development); and 2) all *Bt* sweet corn residues are mandated by the terms and conditions of the registration to be destroyed within one month of harvest (a practice that presumably would destroy any live larvae left in corn stalks). The 2000 SAP agreed that destruction of *Bt* sweet corn residues would be sufficient to mitigate pest resistance to *Bt* sweet corn.

To strengthen the IRM strategies for *Bt* field and sweet corn, EPA has mandated that the registrants have grower agreements that contractually bind the grower to the IRM requirements, ongoing grower education programs, ongoing research programs, ongoing resistance monitoring programs, and a multi-faceted compliance monitoring program (including an annual third-party compliance survey) to ensure that IRM strategies are deployed correctly. EPA will be obtaining annual reports on grower agreements, grower education programs, resistance monitoring programs, research programs, and compliance monitoring programs. EPA has asked for additional data on the effect of insecticides on refuge effectiveness for *Bt* field corn and north-south movement of CEW to further enhance the IRM strategies. Part of the compliance monitoring program (to be developed as part of the terms and conditions of registration) includes specific actions for growers or growers in a region who are found to be non-compliant with IRM requirements. As noted above, if resistance were to occur, EPA has mandated refinements to specific remedial action plans for the *Bt* corn products to contain (and perhaps eradicate) resistance.

D. 2010 Update

Conditional Amendment for Bt11 Sweet Corn

Section 3(c)(7)(A) of FIFRA provides for the registration or amendment of a pesticide when the pesticide and proposed use "…are identical or substantially similar to any currently registered pesticide and use thereof, or differ only in ways that would not significantly increase the risk of unreasonable adverse effects on the environment, and (ii) approving the registration or amendment in the manner proposed by the applicant would not significantly increase the risk of any unreasonable adverse effect on the environment." Unreasonable adverse effects on the environment are defined under section 2(bb) of FIFRA as "… any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide…" Thus, pursuant to section 3(c)(7)(A), EPA may conditionally register a pesticide if (1) the pesticide and its proposed use are identical or substantially similar

to a currently registered pesticide; or (2) the pesticide and its proposed use differ only in ways that would not significantly increase the risk of unreasonable adverse effects; and (3) approving the registration would not significantly increase the risk of any unreasonable adverse effect.

The Agency concludes that the Attribute Bt Sweet Corn, Reg. # 65268-1 Bt Corn Event Bt11 with Cry 1Ab (Sweet Corn), registration, set to expire in September 2010 and described in this BRAD, meets both criteria (1) and (2):

This Bt corn product is identical in both composition and use (corn) to plant-incorporated protectants that are currently registered. Thus, criterion (1) has been fulfilled.

With regard to criterion (2), the Agency maintains, as was previously determined for the original registration of this product, that cultivation of Cry1Ab -containing corn will not cause unreasonable adverse effects on the environment. The conditional environmental effects data, submitted in response to terms and conditions of registration and summarized in section II(C) of this BRAD, strengthen the Agency's initial position and also confirm that long-term effects on non-target organisms are not anticipated. Lastly, the continued use of this product will likely still provide many of the benefits as were evaluated in section II(E) of this BRAD to support the 2001 Bt crops reassessment of Cry1Ab Bt corn (e.g., reduction in use of conventional insecticides that are highly toxic to both humans and the environment).

In conclusion, as the expiring Cry1Ab sweet corn product has met the required criteria under section 3(c)(7)(A) of FIFRA, the Agency is amending this registration to extend the expiration date until September 30, 2015[a].

Although data provided were satisfactory to make the determinations required by section 3(c)(7)(A) of FIFRA, they were not sufficient to support an unconditional registration under FIFRA section 3(c)(5). Additional data, specifically in relation to Bt11 sweet corn expression data on a dry weight basis are necessary for a finding of registrability under FIFRA section 3(c)(5) and will remain as terms or conditions for the purposes of the proposed amendment.

Unconditional Registrations for Cry1Ab and Cry1F Field Corn

Pursuant to FIFRA section 3(c)(5), EPA may unconditionally register a pesticide if EPA determines that, when used in accordance with widespread and commonly recognized practice, it will not generally result in unreasonable adverse effects to the environment. Mycogen Seeds c/o Dow AgroSciences LLC, Monsanto Company, Pioneer Hi-Bred International, and Syngenta Seeds Inc. have submitted or cited data sufficient for EPA to determine that unconditional time-limited registration of 1) Field corn uses of *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material (via elements of vector pZO1502) necessary for its production in corn (SYN-BTØ11-1), 2) Bacillus thuringiensis Cry1Ab delta-endotoxin and the genetic material necessary for its production (Vestor PV-ZMCT01) in event MON 810 corn (OECD Unique

[a] See section III(E) of this BRAD for an explanation describing how the proposed expiration dates were determined.

Identifier: MON-ØØ81Ø-6), *3) Bacillus thuringiensis* Cry1F protein and the genetic material (plasmid insert PHI8999) necessary for its production in corn event DAS-Ø15Ø7-1, 4), and 4) *Bacillus thuringiensis* var. *aizawai* strain PS811 Cry1F protein and the genetic material necessary for its production (plasmid insert PHP12537) in corn event DAS-Ø6275-8 under FIFRA 3(c)(5) will not result in unreasonable adverse effects to the environment. The aforementioned companies have submitted and/or cited satisfactory data pertaining to the proposed uses. The human health effects data and nontarget organism effects data are considered sufficient for the period of the unconditional registrations. These data demonstrate that no foreseeable human health hazards or ecological effects are likely to arise from the use of the products and that the risk of resistance developing to Cry1Ab and Cry1F proteins, during the limited registration period is not expected to be significant.

The expiration date of these registration have been set to September 30, 2015, as reflected in the chart below.

Product (EPA Reg. No.)	Expiration Date
67979-1 Bt Corn Event Bt11 with Cry 1Ab (Field Corn)	**September 30, 2015**
524-489 Bt Corn Event MON 810 Cry1Ab	**September 30, 2015**
68467-2 Bt Corn Event TC1507 with PO Cry 1F	**September 30, 2015**
29964-3 Bt Corn Event TC1507 with PO Cry 1F	**September 30, 2015**
68467-4 Bt Corn Event DAS-06275-8 with MOCry1F	**September 30, 2015**

E. Period of Registration

In the 2001 *Bt* Corn reassessment, EPA determined that it was appropriate to amend the then-existing registrations to extend the period of registration of those products to an expiration date of October 15, 2008. All of the products being assessed at that time were efficacious against lepidopteran pests. EPA based this action on the finding that use of Cry1Ab or Cry1F expressed in corn will not significantly increase the risk of unreasonable adverse effects on the environment "for the limited time period of 7 additional years (to October 15, 2008)." These registrations were later amended to extend the period of registration to an expiration date of September 30, 2010. EPA subsequently granted time-limited registrations to products efficacious against coleopteran corn rootworm pests. For example, EPA registered Cry3Bb1 on February 24, 2003, to May 1, 2004, and extended that registration twice, to February 24, 2008, and September 30, 2010.

As set forth elsewhere in this document, EPA's primary concern for the *Bt* protected transgenic corn products is the possibility that target pests will develop resistance to one or more of the plant-incorporated protectant (PIP) toxins. Development of resistance to a *Bt* toxin would be a grave adverse effect, and, for over 15 years, EPA has imposed stringent requirements intended to countermand the potential development of resistance. Registrants similarly have been busily

developing various products, product mixes (i.e., so-called "pyramids" and "stacks"), and resistance strategies, to maximize agronomic benefits and address resistance management issues. The result has been a vast array of product combinations and, occurring over the past couple of years, a re-emergence of varying refuge requirements for different products.

As discussed in the 2001 *Bt* PIP BRAD (at IID13), the earliest *Bt* corn registrations did not include mandatory refuge requirements. There was a lack of scientific consensus as to what the appropriate refuge requirement should be, and, it was assumed that the limited market penetration of these early crops would be so low as to guarantee that adequate natural refuges would be available from neighboring non-*Bt* corn fields. From 1995 to 1997, *Bt* corn registrations included voluntary refuge requirements of 0% to 20% in the Corn Belt. In 1999, the Agricultural Biotechnology Stewardship Technical Committee (ABSTC), in conjunction with the National Corn Growers Association, proposed uniform insect resistance management (IRM) requirements for *Bt* corn registrations. With some modifications, this proposal, put in place for the 2000 growing season, formed the baseline IRM requirements for almost all *Bt* corn registrations for the better part of a decade: farmers were required to plant a 20% refuge that could be treated for insects, or a 50% treated refuge in cotton-growing areas; all refuges to be planted within one-half mile of the *Bt* corn field.

These uniform requirements brought certainty and consistency to the market after the initial period where many *Bt* corn products had different refuge requirements. Recently, however, as product developers have begun to conceive of products with different combinations of "pyramided" products (i.e., products containing two or more toxins efficacious against the same pest) and "stacked" products (i.e., products combining toxins efficacious against different pests), the refuge requirements have begun to vary. For example, certain products require a 20% external refuge; some products permit a 5% external refuge; one product incorporates a 10% seed blend refuge; we have applications in process for products that propose to incorporate a 5% seed blend refuge; and other permutations are possible.

Given the profusion of various toxin combinations and refuge options, we can no longer proceed on the basis that, as concerns insect resistance management, all products are equal. It was a relatively simple proposition when the default requirement of a 20% sprayed refuge applied to almost all of the *Bt* corn crops in the market. Under those circumstances, the relative durability of products against the development of resistance was functionally equivalent, and, as a consequence, imposing functionally equivalent registration periods was appropriate. That is now no longer the case.

As part of our continually evolving regulatory approach to the continually evolving product mix wrought by developers, we think it appropriate to revise our regulatory requirements in scientifically defensible ways to reflect the comparative level of risks posed by the products that we regulate. Here, for example, where we've determined that a particular product, or category of products, likely will pose less risk of insect resistance developing to a particular PIP protein, we think it appropriate to grant that particular product, or category of products, a registration for a period greater than that granted a corresponding product that poses a greater risk of insect resistance developing. This approach is reflective of complementary principles: first, to ensure

that we apply our limited resources to the products that pose greater risk of adverse effects to the environment; and, second, to conserve the resources that registrants and applicants must expend in amending the registrations of products that pose less risk of adverse effects to the environment.

The scheme that we are following includes registration periods of five, eight, and twelve years; a fifteen-year registration period will also be available, if adequately supported by our science assessment. In this scheme, (i) a product with a single PIP toxin, and a 20% external refuge, qualifies for a five-year registration; (ii) a product with pyramided PIP toxins (i.e., two or more toxins with distinct, non-cross reacting modes of action), that are non-high dose (the definition for a high dose product remains unchanged), with either a seed blend or external refuge, qualifies for an eight-year registration; (iii) a product with pyramided PIP toxins (i.e., two or more toxins with distinct, non-cross reacting modes of action), that are high-dose, with either a seed blend or external refuge, qualifies for a twelve-year registration; (iv) a product with pyramided PIP toxins (i.e., two or more toxins with distinct non-cross reacting modes of actions), with either a seed blend or external refuge, that has been determined by EPA's science assessment to be 150% as durable as the baseline single toxin product with a 20% external refuge, would qualify for a fifteen-year registration. Products determined by EPA's science assessment to be less than 100% as durable as the baseline single toxin product with a 20% external refuge would not qualify for a five-year registration and the registration period for such products will be determined on a case-by-case basis consistent with the level of risk they pose. Similarly, instances where other risk issues may arise, or where novel resistance concerns may be present, would also be determined on a case-by-case basis, as will novel refuge configurations that may present unique durability profiles.