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

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Environmental Effects Assessment for WideStrike™, MXB-13 Cotton Line Expressing *Bacillus thuringiensis* var. *aizawai* Cry1F (synpro) and *Bacillus thuringiensis* var. *kurstaki* Cry1Ac (synpro) Stacked Insecticidal Crystalline Proteins as part of Dow AgroSciences LLC Application for a FIFRA Section 3 Registration., EPA Reg. No.68467-G [1]

FROM: Zigfridas Vaituzis, Ph. D., Senior Scientist (signed 4-28-04)
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TO: Leonard Cole, Regulatory Action Leader
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Pesticide: Dow AgroSciences has submitted a request to register WideStrike™, the *Bacillus thuringiensis* insecticidal crystalline proteins (ICP) Cry1F (event 281-24-236) and Cry1Ac (event 3006-210-23) expressed in MXB-13 cotton (*Gossypium hirsutum* L.). MXB-13 cotton is a pyramided product produced from a backcross of genotype GC510 cotton expressing full-length synthetic protoxins (synpro) of Cry1F or Cry1Ac. The phosphinothricin acetyltransferase (PAT) herbicide-resistant selectable marker gene that provides glufosinate-ammonium resistance is also expressed in MXB-13 cotton. WideStrike™ is labeled to control the cotton bollworm (*Helicoverpa zea* B.), tobacco budworm (*Heliothis virescens* F.), pink bollworm (*Pectinophora gossypiella* S.), beet armyworm (*Spodoptera exigua* H.), fall armyworm (*Spodoptera frugiperda* S.), southern

[1] Parts of this document were prepared at the Oak Ridge National Laboratory (MRID No. 458084-20) managed and operated by UT-Battelle, LLC, for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

armyworm (*Spodoptera eridania* S.), soybean looper (*Pseudoplusia includens* W.) and cabbage looper (*Trichoplusia ni* H.).

Registrant: Dow AgroSciences LLC, 9930 Zionsville Road, Indianapolis, Indiana 46268

ENVIRONMENTAL HAZARD ASSESSMENT

I. Introduction

The Agency has conducted an environmental hazard assessment of the MXB-13 transgenic cotton line containing stacked ICPs (Cry1F/Cry1Ac). The assessment includes effects on wildlife, gene flow to related wild plants, development of weediness, fate of Cry1F/Cry1Ac proteins in the environment and effects on endangered species. The assessment is based on data submitted to the Agency during the developmental stages of the transgenic cotton lines, additional data submitted for registration, FIFRA Scientific Advisory Panel (SAP) recommendations for non-target testing of Plant Incorporated Protectants.(PIP), consultations with scientific experts, and public comments received on the PIP regulatory process.

II. Assessment Summary

Based on the evaluation of the submitted limit dose testing data and information on the general biology of Bt Cry proteins, no unreasonable adverse effects on the flora and fauna of the cotton agroecosystems are expected from the cultivation of MXB-13 transgenic cotton. Specific data are cited relating to aquatic and terrestrial wildlife, Cry protein fate in soils, potential effects on soil biota and field census data examining the effects on non-target foliar insects, and endangered or threatened species hazard assessment, particularly Lepidoptera listed by the USFWS. The submitted studies examined the effects of the Cry1F and Cry1Ac proteins separately and in combination to detect any possible synergistic effects. No synergistic effects or increase in non-target host range as a result of stacking were seen.

Summaries of these studies are presented here in both tabular (Table 1) and more detailed descriptive format. The complete review record of the submitted data can be found in the individual Data Evaluation Reports (DER) and the submitted studies, each designated by a separate MRID number. In order to assess long term environmental effects from the cultivation of MXB-13 transgenic cotton, EPA concludes that it is necessary to perform appropriately designed field monitoring during the initial years of the MXB-13 transgenic cotton registration. EPA believes that the development and review of such information will also address one of the major concerns of the general public regarding the cultivation of transgenic crops.

III. Non Target Wildlife Hazard Assessment

A. The Hazard Assessment Process

The Agency assesses the toxicity of a Cry protein (*B.t.* endotoxin) to representatives of potentially exposed non-target organisms by a tiered testing system starting with Tier I single species high dose laboratory data using mortality as the end point. This single high dose tiered testing approach was developed for EPA by the American Institute of Biological Sciences (AIBS) and approved in 1996 as an acceptable basis for ecological hazard assessment method by a FIFRA Scientific Advisory Panel for naturally occurring and altered microbial pesticides and microbial toxins, and by the December 9, 1999 SAP for protein Plant Incorporated Protectants (PIP). The tiered testing methods were last published as the Harmonized OPPTS Testing Guidelines (EPA 712-C-96-280, February 1996). The guidelines include (but are not limited to) bacteria and their toxins as defined in 40 CFR 152.20. The guidelines apply to microbes and microbial toxins when used as pesticides, including both those that are naturally occurring, and those that are strain-improved either by natural selection or by deliberate genetic manipulation. [The Cry proteins in MXB-13 transgenic cotton, being bacterial toxins, also fall under these testing guidelines.]

Tier I guideline testing reflects a maximum hazard approach to testing. Negative results from tests using this approach provide a high degree of confidence that no unreasonable adverse effects are likely to occur. The OPPTS Harmonized Testing Guidelines utilize the tier testing scheme to ensure, to the greatest extent possible, that only the minimum data sufficient to make scientifically sound regulatory decisions will be required. Moreover, the Agency believes that the Tier I maximum hazard dose testing requirement represents a reasonable approach to evaluating hazard related to the use of biological pesticides, and is one in which negative results allow a high degree of confidence in the safety of the test agents. The Agency expects that most of the plant incorporated Bt Cry proteins require testing only in the first tier for short term hazard assessment. Long range adverse effects have to be ascertained by higher Tier long term field testing. A SAP convened in October 2000 and the National Academy of Sciences (NAS 2000) also recommended testing non-target organisms directly in the field. This approach, together with an emphasis on direct testing of invertebrates found in the agricultural fields, was also recommended by the August, 2002 SAP, and was supported by several public comments.

The OPPTS Harmonized Guidelines call for testing of a single group or groups of test animals at the maximum hazard dose. In the initial Tier I testing, when the active ingredient is a toxin, the appropriate endpoint is death of the test organism. Each treatment and control group contains at least 10 test animals. When there is only one treatment group, at least 30 animals are tested at that treatment level. The guidelines provide that the duration of all Tier I tests be about 30 days long. Some test species, notably non-target insects, may be difficult to culture and the test duration has been adjusted accordingly. In cases where an insect species cannot be cultured for 30 days, the testing is continued until the negative control mortality rises above 20 percent.

The maximum hazard dose approach is based on a safety factor times the maximum amount of active ingredient expected to be available to terrestrial and aquatic plants and animals in the environment (the expected environmental concentration, or EEC). Therefore, data that establishes an LC_{50}, ED_{50}, or LD_{50} that is greater than the maximum hazard dosage level (e.g. LD_{50} >10 X EEC) is sufficient to evaluate adverse effects and lower dose testing is not necessary. If the LD_{50} is lower than the maximum hazard test dose used in the Tier I test, additional testing with sequentially lower doses to establish a definitive LD_{50} with confidence limits is required. The OPPTS Harmonized Guidelines call for testing of multiple groups at lower, incremental doses in order to quantify the hazard. Sufficient doses and test organisms are required to determine an LD_{50} value and, on a case-by-case basis, the No Observed Effect Level (NOEL), or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. In the final analysis, the hazard assessment is made by comparing the lowest observed effect concentration (LOEC) to the expected environmental concentration (EEC), and when the EEC is lower than the LOEC, a no hazard assessment is made. Appropriate statistical methods are used to express trends, and to evaluate the significance of differences in data obtained from different test groups. The statistical methods used must reflect the current state-of-the-art with appropriate statistical power.

On December 9, 1999, the Agency presented the maximum hazard dosing approach to testing of protein PIP and for possible new data requirements to a FIFRA Scientific Advisory Panel for their recommendations. The December 1999 SAP report was generally supportive of the Agency's testing and hazard evaluation. The Panel also recommended more testing of non-target invertebrates more closely related to the target species and species more likely to be present in the field of the GM crops. In addition, the October 2000 SAP recommended appropriate field testing be conducted for non-target organisms. The August, 2002 SAP and certain public comments also agreed with this approach with some additions. It was recommended that the choice of appropriate indicator organisms for testing be based on the potential field exposure as deduced from data on Cry protein activity and expression in the plant. The SAP thought that appropriately chosen single species Tier I laboratory tests showing no detrimental effects are sufficient to make a short term hazard assessment and that field studies be conducted when these tests show toxicity (as higher Tier testing described in the OPPTS Microbial Testing Guidelines) but that proper multi year commercial field studies with appropriate statistical power are needed to determine long term ecological effects. This comment is in agreement with the Agency's OPPTS Testing Guideline discussed above.

Bt Cry endotoxins are proteins and, unlike inorganic chemicals, do not have the potential to bioaccumulate and thereby result in delayed adverse 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 Cry protein binding by certain soils under certain circumstances, the bound Cry proteins are also reported to be rapidly degraded by microbes upon elution. The same sources also report that Bt proteins in the soil in Bt crop fields have no detectable effect on soil invertebrates or culturable microbial flora. In addition, 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.

B. Hazard Assessment of Cotton Expressing Cry1F and Cry1Ac Insecticidal Crystalline Proteins to Non-target, Beneficial and Endangered Wildlife

1. Summary of Non-Target Wildlife Toxicity Testing

The following environmental hazard assessment summarizes data from numerous studies to analyze the effects of MXB-13 cotton to non-target organisms which inhabit areas in and adjacent to cotton agroecosystems with special emphasis on beneficial and endangered lepidopteran insects.

Two separate SAP reports (October, 2000 and August, 2002) recommended that non-target testing be focused primarily on species exposed to the crop being registered. However, in addition to testing species directly exposed to the Cry1F/Cry1Ac proteins in the field, the full battery of non-target wildlife species testing was conducted to comply with the published Agency non-target data requirements for microbial toxins (in the absence of PIP-specific data requirements, EPA requires applicants for PIP registrations to meet the 40 CFR. Part 158 data requirements for microbial toxins). The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic cotton fields were beneficial insects feeding on cotton pollen and nectar, and soil invertebrates. Direct field census data on the abundance of invertebrates in the field were also requested, received and evaluated. The August, 2002 SAP, however, found small plot field census data unsatisfactory because of low statistical power. While protocols for valid field testing are being developed, the field census data are used as supplemental information to confirm the findings of the maximum hazard dose single species laboratory toxicity testing on representative beneficial invertebrates.

The toxicity of the Cry1F/Cry1Ac proteins has been evaluated following challenge of several species of invertebrates, including: adult and larval honey bees, a parasitic hymenopteran (*Nasonia*), green lacewings, lady beetles, Collembola (springtail), monarch butterfly and earthworms. Reproductive and developmental observations were also made on Collembola, honey bee and lady beetle larva maturation studies. The August, 2002 SAP however, found the green lacewing and parasitic wasp studies lacking and recommended testing of alternative species. The August, 2002 SAP also suggested that additional soil degradation testing is desirable in a larger variety of soils and climactic conditions.

The non-target organisms tested are chosen as representative indicators of the major groups of wildlife and on the potential for field exposure as deduced from data on Cry1F/Cry1Ac protein expression in the plant. Although Bt Cry proteins are very specific in their activity to only certain lepidopteran insect species and even though a recent SAP (March, 2001) recommended against testing of non-targets species not related to those susceptible to the specific activity of Bt Cry proteins, the EPA has

examined the toxicity of Cry1F/Cry1Ac proteins in cotton to birds, fish, honey bees and certain other beneficial insects. In order to comply with the published Agency data requirements (40CFR Part 158) for registration of microbial toxins, the Agency asked for avian and aquatic invertebrate toxicity data, as well as Collembola and earthworm species to ascertain effects on beneficial soil decomposers because prolonged exposure to Cry1F/Cry1Ac proteins in soil was a possibility. Effects on honey bee brood as well as adults were required as some exposure to the Cry1F/Cry1Ac protein in pollen is a possibility.

The form of the test substances used in the studies for this assessment are plant material such as leaves, pollen and purified bacterially-produced Cry1F/Cry1Ac proteins, separately and in combination, incorporated into the test species diet. The October 2000 SAP provided guidance to the Agency that while actual plant material is the preferred test material, bacterially-derived protein is also a valid test substance, especially in testing where the test animals do not consume cotton plant tissue and where large amounts of Cry protein are needed for maximum hazard dose testing. As per the OPPTS Harmonized Testing Guidelines, the adult insect studies were generally of 30 days duration or until the negative control mortality reached 20%. Larval studies were through pupation and adult emergence.

Table 1. Tabular results of non-target wildlife and soil fate studies

Guideline No	Study	Results	MRID No.
USEPA OPPTS 885.4150	Wild Mammal Testing, Tier I	Mammalian wildlife exposure to Cry1F/Cry1Ac proteins is considered likely; however, the Cry1F/Cry1Ac protein toxicity data for Human Health Assessment indicate that there is no significant toxicity to rodents from testing at the maximum hazard dose. Therefore no hazard to mammalian wildlife is anticipated.	Not Applicable
885.4050	A Dietary Toxicity Study with the Northern Bobwhite Quail	The acute dietary LC_{50} value for northern bobwhite exposed to cotton meal prepared from seeds expressing Cry1F and Cry1Ac proteins for 8 days. was determined to be greater than the 0.021µg Cry1F/g cotton meal and 0.012 µg Cry1Ac/g cotton meal (> 100,000 ppm diet). No adverse effects on avian wildlife is expected from incidental field exposure to WideStrike™ cotton. A higher concentration and longer duration broiler study is recommended. **Acceptable**.	458084-14
885.4100	Avian Pulmonary/Inhalation Testing, Tier I,	Data not required for non-infectious active ingredients	Not Applicable
885.4200	Freshwater Fish Testing	The Fish Acute Toxicity Test, Freshwater and Marine (USEPA OPPTS 850.1075) MRID NO: 458084-13 in the table below is **Acceptable** to fulfill this data requirement.	458084-13

Guideline No	Study	Results	MRID No.
850.1075	Fish Acute Toxicity Test, Freshwater and Marine	The 8-day LC50 for rainbow trout is greater than 100 mg a.i./kg-diet. No mortality or sublethal effects were observed. In view of the lack of toxicity and minimal aquatic exposure, no fresh water fish hazard is expected from cultivation of WideStrike™ cotton crops. **Acceptable**	458084-13
850.1010	Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids,	In a 48-hour static test with *Daphnia magna*, there were no observed adverse effects with Cry1F and Cry1Ac in combination at respective concentrations of 510 and 2,500 µg/L. Therefore, no hazard to aquatic invertebrates is expected from incidental exposure to WideStrike™ cotton pollen. **Acceptable** to fulfill the OPPTS 885.4240 data requirement	458084-12
885.4280	Estuarine and Marine Animal testing, Tier I	The Fish Acute Toxicity Test, Freshwater and Marine (USEPA OPPTS 850.1075) MRID NO: 458084-13 in the table above is **Acceptable** to fulfill this data requirement.	458084-13
885.4300	Nontarget Plant Studies, Tier I	Since the active ingredient in this product is an insect toxin (Bt endotoxin) that has never shown any toxicity to aquatic or terrestrial plants, these studies have been waived for this product. Outcrossing issues are addressed below.	Not Applicable
885.4380	Honey Bee Larva Testing Tier I	At 1.98 µg Cry1F + 11.94 µg Cry1Ac per mL sugar water no effect on survival of larvae to adult emergence was seen.. The LC_{50} is >4X pollen expression. Therefore no hazard to honey bee larvae and adult bee emergence is anticipated. **Acceptable**	455423-16
885.4340	Parasitic Hymenoptera Larva Testing Tier I	At 5.2 µg Cry1F + 46.8 µg Cry1Ac per mL sugar water at 10 d no effect of limit dose with LC_{50} > 13X pollen expression was seen. Minimal exposure and no hazard to parasitic Hymenoptera from Cry1F/Cry1Ac protein is expected. Testing of a species more common to cotton fields is recommended. **Acceptable**.	458084-11
885.4340	A Dietary Toxicity Study with Green Lacewing Larvae Tier I	No effect is noted at Cry protein levels expressed in pollen that would be encountered by green lacewings in the field. Because of questionable ingestion of the test material another species (e.g, minute pirate bug) which is more likely to be exposed should be tested. **Supplemental** to testing *Orius insidiosus*	458084-10
885.4340	Adult Lady Beetle Testing. Tier I *(H. convergens)*	300 µg Cry1F + 22.5 µg Cry1Ac per mL sugar water no effect of limit dose with LC_{50} > 780X Cry1F pollen expression and > 8X Cry1Ac pollen expression Based on these results , no hazard to *H. convergens* is expected when feeding on WideStrike™ cotton pollen in the field. **Acceptable**	455423-15

Guideline No	Study	Results	MRID No.
885.4340	Collembola Chronic Dietary Toxicity Study Tier I	The combination of 709 µg Cry1F + 22.6 µg Cry1Ac per g diet and cotton leaf tissue showed no effect on adult survival and reproduction at up to 10X the anticipated field level of expression. Therefore, no hazard to decomposers represented by collembola is expected from exposure to WideStrike™ cotton in the field. **Acceptable**	458084-09
OECD Guideline 207	Earthworm Toxicity Study	A 14-day study for earthworms exposed to soils treated with microbial-produced Cry1Ac and Cry 1F, individually and in combination was performed. There were no overt signs of toxicity to earthworms exposed to soils containing nominal concentrations of Cry1F and Cry1Ac at 50x the expected worst case EEC. **Supplemental**	455807-01
885.4340	Monarch Butterfly Larval Pollen Exposure Calculation	The calculated EC_{50} $>10^5$ the dietary pollen exposure for Cry1F and >10X the dietary pollen exposure for Cry1Ac. The calculations indicate that young monarch larvae (at the most sensitive stage) will not be adversely affected by exposure to WideStrike™ cotton. This is not a Guideline data requirement. **Supplemental**.	458084-20
Not Guideline Data	Insecticidal Activity Spectrum studies	The activity spectrum of of Cry1F and Cry1Ac ICP was determined for nine insect species representing three orders and four families. Both Cry1F and Cry1Ac activity was restricted to lepidopteran insects. **Supplemental**.	458084-20
154-3500	Field evaluation of WideStrike™ cotton exposure on non-target organisms Tier IV	The preliminary results from Tier IV field census studies. are supplemental to Tier I maximum hazard dose testing. The data do not show any WideStrike™ cotton related adverse effect on non-target and beneficial invertebrate abundance in the field. **Supplemental**	458084-19
885.5200	Expression in a terrestrial Environment Tier II	The soil half-life of the plant expressed Cry1F and Cry1Ac was estimated as 1.3 days in a laboratory study with a representative soil from a cotton growing region. The Cry proteins were not detectable after 14 days. These results verify that the Cry1F and Cry1Ac proteins degrade rapidly. Additional multi year field testing is requested. **Acceptable**.	455568-01

2. Non-target Wildlife Testing and Hazard Assessment

Exposure Estimates

Exposure estimates for organisms directly feeding on cotton plants or plant parts containing Cry1F and Cry1Ac ICPs are based on the high-end expression for the relevant plant tissue to which a non-target organism may be exposed. High-end exposure estimates (HEEE) represent the 90% upper bound of

the reported expression. Indirect exposures represent inadvertent exposures to Cry1F and Cry1Ac protein through soil, water, pollen on host plant tissue or multitrophic interactions. These exposures are expressed as Estimated Environmental Concentrations (EEC) and are conservatively calculated using high-end estimates for input parameters. Risk is characterized by comparing the exposure estimates (HEEE or EEC) to toxicity levels.

Direct feeding on plants or plant parts constitutes the primary route of exposure of organisms to Cry1F and Cry1Ac expressed in MXB-13. Plant parts subject to feeding are leaves, roots, stems, pollen and nectar. Plant pests which directly feed on cotton as their primary food source are not germane to this assessment. Organisms incidentally exposed to cotton plants or plant residues as an occasional or supplementary food source are considered non-target organisms of concern in this exposure assessment. Secondary exposure to ICP residues by tritrophic interactions may occur for predators or plant-feeding organisms.

Evaluation of protein expression concentrations and routes of exposure provide estimated levels of exposure conservatively projected to occur in the environment. Levels of Cry1F and Cry1Ac measured in tissues collected from transgenic cotton line MXB-13 are presented in Table 2. Also, the HEEE are presented for tissues relevant to estimating exposure concentrations.

Table 2. Cry1F and Cry1Ac expression levels in cotton tissue.

Matrix	**Mean**	**Standard Deviation**	**Min/Max Range**	**HEEE[a]**
	*(ng **Cry1F**/mg tissue[b])*			
Young leaves (3-6 wk)	6.81	3.58	2.8-19.2	
Terminal leaves	8.19	3.5	3.0-19.5	15.05
Squares	4.88	1.8	0.97-9.9	
Flower	5.44	1.84	1.9-11.4	
Whole Plant (seedling)	14.1	5.6	8.0-28.4	
Whole Plant (pollination)	25.3	11	0.05-48.0	
Whole Plant (defoliated)	22.0	11	7.6-40.2	43.56
Root (seedling)	0.88	0.73	0.18-0.27	
Root (pollination)	0.54	0.4	0.13-1.8	

Matrix	Mean	Standard Deviation	Min/Max Range	HEEE[a]
Root (defoliated)	0.51	0.2	0.26-0.87	0.90
Boll (early)	3.52	1.7	0.91-8.8	
Seed	4.13	1.11	1.4-6.6	6.31
Pollen	0.06	0.15	ND[c]-0.51	0.35
Nectar	ND	NA[d]	ND-ND	
	*(ng **Cry1Ac**/mg tissue[b])*			
Young leaves (3-6 wk)	1.82	0.6	0.50-3.7	
Terminal leaves	1.31	0.4	0.43-2.1	2.09
Squares	1.82	0.5	0.83-3.0	
Flower	1.83	0.4	1.1-2.8	
Whole Plant (seedling)	1.37	0.4	0.94-2.4	
Whole Plant (pollination)	1.05	0.2	0.79-1.3	
Whole Plant (defoliated)	0.6	0.2	0.31-0.92	0.99
Root (seedling)	0.17	0.06	0.06-0.27	
Root (pollination)	0.07	0.06	ND-0.15	0.19
Root (defoliated)	ND	NA	ND-0.09	
Boll (early)	0.64	0.2	0.21-1.0	
Seed	0.55	0.07	0.44-0.70	
Pollen	1.45	0.5	1.0-2.5	2.43
Nectar	ND	NA	ND-ND	

[a]High end exposure estimate (HEEE) = 90% upper bound = [mean + 1.96 x (standard deviation)]

[b]Seed, pollen and nectar are reported in a fresh weight basis; all other results are reported on a dry weight basis for lyophilized samples.

[c]ND-not detected, limit of quantitation (LOQ) = 0.15 ng/mg (pollen, root), ~ 0.05ng/mg (nectar)
[d]NA-not applicable

Estimated Environmental Concentrations [MRID No. 458084-20]

EEC's in soil and water matrices were calculated to conservatively represent exposure by indirect routes for comparison against tier I non-target species testing endpoints. The basis for EEC computations is expression data for MXB-13 cotton which describe relevant HEEE for Cry1F and Cry1Ac proteins in plant tissues at harvest and conservatively based models that predict concentrations in soil and water. The basis for calculation of EEC reported here is predicted biomass production and partitioning as determined for average cotton yield. From this and literature estimates of biomass production and dry matter partitioning in cotton, the HEEE for expression are converted into EEC in soil and water. The EEC in soil for Cry1F is 0.317 mg a.i./kg soil and that for Cry1Ac is 0.0196 mg a.i./kg soil.

The EEC for Cry1F and Cry1Ac occurrence in surface water was estimated using the GENEEC farm pond scenario. Conservative model inputs for Koc (100 L/kg) and solubility (1000 g L^{-1}) result in EEC estimates in water of 1,710 and 107 ng/L for Cry1F and Cry1Ac, respectively.

Cotton is predominately a self-pollinated crop with some amount of cross-pollination facilitated by bees. Lepidopteran insects are not pollinators of cultivated cotton and indirect exposure to cotton pollen is negligible.

Both target and non-target insect herbivores serve as food sources for beneficial insect predators and prey which constitute a relevant exposure route within a multitrophic context. The concentrations of Cry1Ab protein found in aphids were a minimum of 100-fold lower upwards to several thousand-fold lower than in food sources containing the Cry1Ab protein. Similarly, Lepidoptera showed reduction in Cry1Ab protein concentration in comparison to their food source but the level of reduction was less dramatic than for aphids.

a. Mammalian Wildlife Hazard Assessment

Wild Mammal Testing Tier I, USEPA OPPTS 885.4150

Mammalian wildlife exposure to Cry1F and Cry1Ac protein is considered likely; however, the mammalian toxicology information gathered to date on Cry1F and Cry1Ac proteins does not show a hazard to wild or domesticated mammals. Microbial protein preparations of Cry1F and Cry1Ac were administered to CD1 mice by oral gavage for Human Health Assessment indicate that there is no significant toxicity to rodents from acute oral testing at the maximum hazard dose (Cry1F >600 mg a.i./kg and Cry1Ac >700 mg a.i./kg.). Therefore no hazard to mammalian wildlife is anticipated.

b. Avian Hazard Assessment

Avian Oral, Tier I, USEPA OPPTS 885.4050. [MRID No. 458084-14]

The acute dietary toxicity of a basal avian diet fortified with cotton seed expressing the Cry1F and Cry1Ac insecticidal proteins was evaluated in young northern bobwhite (*Colinus virginianus*) for 8 days. Thirty bobwhite chicks, 10-days old, were fed a diet fortified with 10% cotton meal from cotton seeds expressing the Cry1F and Cry1Ac protein for five days. The bird feed was amended with 10% cotton seed meal from cotton seed containing nominal 0.021 µg Cry1F/g cotton seed meal and 0.012 µg Cry1Ac/g cotton seed meal. Although no mortality was observed from the two control groups, nor in birds exposed to the insecticidal proteins, clinical symptoms of wing droop, lethargy and ruffled appearances were evident in the majority of birds from both groups receiving diets amended with cotton seed meal. These clinical symptoms were attributed to markedly high levels of gossypol–8-10X the maximum allowable in animal feeds. The dietary LC_{50} value for northern bobwhite exposed to cotton meal prepared from seeds expressing Cry1F and Cry1Ac proteins was determined to be greater than the 0.021 µg Cry1F/g cotton meal and 0.012 µg Cry1Ac/g cotton meal (> 100,000 ppm diet prepared with cotton seed from transformed cotton with both Cry proteins). The no mortality concentration was >0.021 µg Cry1F/g cotton seed meal and >0.012 µg Cry1Ac/g cotton seed meal. The study is insufficient to assess hazards to avian species which may be exposed continuously to high levels of Cry1F and Cry1Ac in domestic poultry feed which normally contain 60 to 70% corn, and/or 10% cottonseed meal. However, in consideration of gossypol toxicity noted in the transgenic cottonseed and the reference control cottonseed, a six-week broiler chicken study in which 60-70% corn meal from Cry1F/Cry1Ac-corn is used for the diet will be an acceptable protocol to make a dietary assessment for the two combined proteins.

A summary of an acute oral study (referenced in MRID #45808420) found the acute oral LD_{50} for northern bobwhite quail exposed to a single oral dose of Cry1F/Cry1Ac (7.9:1 ratio) to be >128 mg a.i./kg, the limit test dosage. No mortality, clinical signs of toxicity or treatment related effects were observed from exposure to the limit dose.

c. Aquatic Species Hazard Assessment

There is no evidence for sensitivity of aquatic (including endangered) species to anti-lepidopteran Cry proteins. Toxicity studies with lepidopteran-active Cry proteins on aquatic organisms show no hazard for fish or invertebrates exposed to either pollen or to bacterially expressed Cry protein. In addition, aquatic exposure from Bt cotton is extremely small. [The October, 2000 and August, 2002 SAP reports recommended that non-target testing be focused on species exposed to the crop being registered. Therefore, testing of aquatic species was performed primarily to satisfy the testing requirements for microbial toxins published in 40 CFR Part 158.]

i. Freshwater Fish Hazard Assessment

Fish Acute Toxicity Test, Freshwater and Marine USEPA OPPTS 850.1075. [MRID NO.

458084-13]

In an eight day study, the acute dietary toxicity to the rainbow trout (*Onchorynchus mykiss*) was determined by providing a basal fish diet fortified with 100-mg a.i./kg diet mixture of Cry1F/Cry1Ac in a ratio of approximately 7.9:1. No mortality or sublethal effects were observed. The 8-day LC50 for rainbow trout is greater than 100 mg a.i./kg-diet. In view of the lack of demonstrated toxicity and minimal aquatic exposure, no fresh water fish hazard is expected from the proposed uses of WideStrike™ cotton crops.

ii. Aquatic Invertebrate Hazard Assessment

Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids USEPA OPPTS 850.1010 [MRID NO. 458084-12]

The acute toxicity of microbial-produced 510 µg Cry1F/L + 2500 µg Cry1Ac/L of test solution to instars of *Daphnia magna* was assessed in a 48-hour static test. No immobility or other adverse effects were seen during the study. Based on biological interpretation of the data, the 24-hour and 48-hour EC_{50}s for daphnia exposed to the Cry1F + Cry1Ac mixture were >510 µg Cry1F/L and >2500 µg Cry1Ac/L (represents a worst-case exposure of one kg of transgenic cotton pollen per liter of pond water). This rate of fortification represents 298X and >23,000X the anticipated EEC for Cry1F and Cry1Ac protein in surface water. Therefore, no hazard to aquatic invertebrates is expected from incidental exposure to WideStrike™ cotton pollen.

iii. Estuarine and Marine Animal Hazard Assessment

Fish Acute Toxicity Test, Freshwater and Marine USEPA OPPTS 850.1075. [MRID NO. 458084-13]
In an eight day study, the acute dietary toxicity to the rainbow trout (*Onchorynchus mykiss*) was determined by providing a basal fish diet fortified with 100-mg a.i./kg diet mixture of Cry1F/Cry1Ac in a ratio of approximately 7.9:1. No mortality or sublethal effects were observed. The 8-day LC50 for rainbow trout is greater than 100 mg a.i./kg-diet. In view of the lack of demonstrated toxicity and minimal aquatic exposure, no estuarine or marine animal hazard is expected from the proposed uses of WideStrike™ cotton crops.

iv. Terrestrial and Aquatic Plant Hazard Assessment

Nontarget Plant Studies, Tier I USEPA OPPTS 885.4300

Since the active ingredient in this product is an insect toxin (Bt endotoxin) that has never shown any toxicity to plants, these studies have been waived for this product. Outcrossing issues are addressed below.

3. Terrestrial Invertebrate Testing and Hazard Assessment

Background:

The October 2000 SAP concluded that invertebrates such as earthworms and springtails (Collembola) are appropriate indicator species for Cry protein testing despite the specific nature of the Cry protein toxicity to select target species. When EPA initially reviewed the applications for PIP products that were registered in 1995, EPA considered requiring studies evaluating effects upon the representative beneficial soil invertebrates Collembola and earthworms. The Agency 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 growing season add to this concern. However, the Agency understands that routine agronomic practices have included the long term use of chemical pesticides, which have adverse effects on soil organisms, and this practice has not resulted in an accumulation of significant amounts of plant detritus in soils. Thus, Cry protein expressed in crops is expected to have less impact on these species than chemical pesticides and should not result in any increased build up of plant detritus or Cry proteins at toxic levels. Supporting this conclusion are data received by EPA that indicate that such proteins are known to degrade rapidly in field soils. Cry proteins that become bound to soil particles have been shown to be rapidly degraded by soil microbes upon elution from the soil particles. Therefore the potential for significant soil buildup and adverse effects to non-target soil organisms are not anticipated. It has been confirmed in published literature that Bt Cry protein released from root exudates and biomass of Bt plants 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 some Bt crops, or from the degradation of the Bt crop biomass, is not toxic to a variety of organisms in the soil environment. It has also been reported that the same degree of Bt Cry protein persistence takes place in soils that have been exposed to repeated Bt microbial spray applications. In addition, new plants grown in Bt containing soil do not take up the Bt protein. Nevertheless, data on insects closely related to the target pest, as well as other studies to address the published data requirements for registration of microbial toxins (40 CFR §158) have been received and reviewed.

a. Single Species Laboratory Testing

The test material fed to the invertebrate species in several of the studies is purified microbial Cry1F and Cry1Ac protein in a ratio equivalent to the Cry1F and Cry1Ac proteins in whole WideStrike™ cotton plants. The toxicity tests conducted with non-target arthropods indicate that no adverse effects are expected when exposed to Cry1F and Cry1Ac protein concentrations exceeding the EECs.

i. Effects on Honey Bee Larvae

Honey Bee Testing, Tier I USEPA OPPTS 885.4380 [MRID No 455423-16]

Honey bee larvae were exposed to sucrose solutions containing the following 3 test substances; genetically modified pollen (Cry1F), genetically modified pollen (Cry1Ac) and *Bacillus thuringiensis* with both Cry1F and Cry1Ac expression. Honey bee larvae control groups were exposed to non-genetically modified pollen, a positive control of potassium arsenate and a negative control of sucrose only. Honey bee larva survival to capping and to emergence as adults, was 90.00% in the genetically modified pollen (Cry1F) group, 76.25% in the genetically modified pollen (Cry1Ac) group and 90.00% in the group exposed to *Bacillus thuringiensis* with both Cry1F and Cry1Ac expression. All three treatment groups were not statistically different from the negative control (sucrose only) which had a survival of 95% to capping and 93.75% to adult emergence. The positive control exhibited only 45% survival to capping and adult emergence. Based on these data, the LD_{50} for honey bees is > 2 mg cotton pollen expressing the Cry1F or Cry1Ac proteins and >1.3 mg Cry1F delta-endotoxin plus >8.5 mg ± 5% of Cry1Ac delta-endotoxin from bacterial sources per 100 mL 30% sucrose. These results indicate honey bee mortality as evaluated by capping and adult emergence was not affected by exposure to any of the test substances, therefore no honey bee hazard is expected from the proposed uses of WideStrike™ cotton crops.

ii. Parasitic Hymenoptera hazard assessment

Parasitic Hymenoptera Testing Tier I USEPA OPPTS 885.4340 [MRID No 458084-11)

In a limit test, the study authors concluded that prepared diets containing 5.2 µg Cry1F/mL, 46.8 µg Cry1Ac/mL, or 5.2 µg Cry1F + 46.8 µg Cry1Ac/mL did not affect the mean mortality of the parasitic hymenopteran *Nasonia vitripennis* after ten days of exposure. Surviving larvae in all groups were generally normal in appearance and behavior. Based on this study, the dietary LC_{50}s were: > 5.2 µg Cry1F/gram of diet, >46.8 µg Cry1Ac/gram of diet, >0.52 µg Cry1F + 4.68 µg Cry1Ac/gram of diet, >5.2 µg Cry1F + 46.8 µg Cry1Ac/gram of diet, and > 5.2 µg heated Cry1F Ac + 46.8 µg heated Cry1Ac per gram of diet. A 40% mortality observed in the Cry1F + Cry1Ac group at 32X the EEC is less than a LC_{50} at 32X the possible field exposure (EEC). The EPA level of concern for terrestrial wildlife is a LC_{50} at less than 5X the field exposure ($EEC/LC_{50} = RQ > 0.2$). Therefore since the LC_{50} in this study is greater than 32X the EEC, no hazard to parasitic Hymenoptera is expected at field exposures which are minimal to nonexistent. [The August 27, 2002 SAP concluded that the parasitic Hymenoptera testing was not appropriate. Testing another beneficial organism rather than a parasitoid was recommended as more suitable.]

iii. Green Lacewing Larva Hazard Assessment

Green Lacewing testing Tier I. USEPA OPPTS 885.4340 [MRID No 458084-10)

In tests of dietary toxicity (mean survival to pupation) of Cry1F, Cry1Ac, and Cry1F + Cry1Ac

mixtures to green lacewing larvae (*Chrysoperla carnea*), the dietary LC_{50}s were: > 5.2 µg Cry1F/gram of diet, >46.8 µg Cry1Ac/gram of diet, >0.52 µg Cry1F + 4.68 µg Cry1Ac/gram of diet, >5.2 µg Cry1F + 46.8 µg Cry1Ac/gram of diet, and > 5.2 µg heated Cry1F Ac + 46.8 µg heated Cry1Ac per gram of diet. Mortality was increased and pupation was affected in the Cry1F/Cry1Ac at 32X the concentration found in pollen. (LC_{50} > 14X pollen expression). No effect is noted at Cry protein levels expressed in pollen that would be encountered by green lacewings in the field.

However, the appropriateness of the methodology for the green lacewing acute toxicity study is questionable. In addition, green lacewing are difficult to test in the laboratory because of a high rate of mortality. The August 2002 FIFRA Scientific Advisory Panel (SAP) also noted concerns regarding the green lacewing methodology. The SAP questioned whether the green lacewings are ingesting the Cry protein that is coated around moth eggs in a diet. Since green lacewing have piercing-sucking mouthparts, they may not be exposed to the protein on the external surface of the egg diet. Finally, the SAP questioned the appropriateness of testing green lacewing and recommended testing an alternate natural enemy such as the minute pirate bug (*Orius insidiosus*). Therefore, an additional Tier 1 non-target insect test with the minute pirate bug should be conducted with the Cry1F and Cry1Ac proteins. *Orius* typically occur in fields as egg predators and they typically feed on pollen. Therefore, a laboratory study should be conducted feeding *O. insidiosus* both pollen and purified protein in diet. Feeding *O. insidiosus* Cry proteins in diet will allow for a test at the maximum hazard dose; whereas, feeding *O. insidiosus* pollen expressing the Cry proteins will provide an evaluation of potential effects from actual exposure scenarios.

iv. Lady Beetle Hazard Assessment

Lady Beetle Testing. Tier I. USEPA OPPTS 885.4340 [MRID No. 455423-15]

Adult Lady beetles (*Hippodamia convergens*) were exposed to either a single dietary dose of 300 µg a.i./mL of Cry1F, a single dose of 22.5 µg a.i./mL of Cry1Ac or a combined dose of 300 µg a.i./mL of Cry1F plus 22.5 µg a.i./mL of Cry1Ac as a mixture with sugar water. Four replicates of 25 beetles each were used for treatment and control groups which were observed for mortality and clinical changes until the negative control mortality exceeded 20% on day 15 of the test. Cumulative mortality and signs of toxicity observed in the treatment groups were used to calculate the dietary LC_{50}. The dietary LC_{50} was greater than 300 µg a.i./mL for Cry1F, greater than 22.5 µg a.i./mL for Cry1Ac and greater than the combined dose of 300 µg a.i./mL for Cry1F plus 22.5 µg a.i./mL for Cry1Ac. This study demonstrates that lady beetles will not be adversely affected by the proposed uses of WideStrike™ cotton.

v. Collembola Hazard Assessment

Collembola Testing. Tier I. USEPA OPPTS 885.4340 [MRID No. 458084-09]

The chronic effects of Cry1F and Cry1Ac were assessed on Collembola (*Folsomia candida*) using microbially-derived Cry1F and Cry1Ac added to brewers yeast. In 28-day dietary toxicity tests, 709 mg Cry1F/kg of diet or 702 mg Cry1F + 22.6 mg Cry1Ac per kg of diet did not adversely affect mortality or reproduction of Collembola. The dietary concentration of approximately 709 mg a.i. of Cry1F /kg diet and 22.6 mg a.i. of Cry1Ac /kg diet represents >1,100-fold higher levels than those anticipated in the field. Diets containing 22.6 mg Cry1Ac/kg alone did not affect mortality but decreased reproduction by up to 45%; however, the toxicity was attributed to impurities in the Cry1Ac test material. Lyophilized Cry1Ac cotton leaf at 5% or 50% of the diet had no adverse effect on mortality or reproduction. The combination of Cry1F and Cry1Ac showed no effect at up to 10X the anticipated field level of expression. Therefore, no hazard to decomposers represented by Collembola is expected from exposure to WideStrike™ cotton in the field.

This study adequately addresses potential concerns for Cry1F and Cry1Ac protein expressed in transgenic cotton to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry1F and Cry1Ac proteins found in transgenic cotton pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial non-lepidopteran soil insects. It is notable that recent recommendations by the SAP (March, 2001) are that invertebrates of different orders than those known to be affected by the Cry protein in question need not be tested.

vi. Earthworm hazard assessment

Acute Toxicity to Earthworms. OECD Guideline 207 [MRID NO. 455807-01]

A 14-day limit dose study was conducted on earthworms exposed to soils treated with microbial-produced Cry1Ac and Cry 1F, individually and in combination. There were no overt signs of toxicity to earthworms exposed to soils containing nominal concentrations of Cry1F and Cry1Ac at 50x the expected worst case EEC [this represents concentrations which are 792X and 5479X higher than the expected EEC for incorporation of defoliated cotton plants into the top 15 cm of soil.] The 14-day LC50s were >247 mg a.i./kg for Cry1F; >107 mg a.i./kg for Cry1Ac, and > 247 mg a.i./kg Cry1F + >107 mg a.i./kg Cry1Ac in the test with the two proteins combined. These data show that no adverse effects to earthworms are expected in fields growing WideStrike™ cotton. [This study was rated as Supplemental. The study was conducted at nominal test material concentrations.]

Earthworm feeding studies submitted to the Agency for all of the registered Cry proteins demonstrate that the Cry proteins are not toxic to earthworms at the worst case environmental concentration. Some public comments have voiced concerns as to whether the earthworms actually ingested the Bt Cry proteins when these are incorporated into the soil in the test systems used. Recently published data show that the earthworms do, however, ingest the Bt Cry proteins with the soil without harmful effects. These reports also show 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, in fallow fields, or after 45 days in soil amended with biomass of Bt or non-Bt corn or not amended. The Bt Cry protein was shown to be

present in both the casts and guts of the worms.

The reviewed data show that no adverse effects to earthworms are expected in fields growing WideStrike™ cotton.

vii. Monarch Butterfly Risk Assessemnt

Non-target insect testing. Tier I. USEPA OPPTS 885.4340 [MRID No. 458084-20]

Studies conducted by Hellmich, et al. (Proc. Nat. Acad, Sci. 98 [21]: 1925-11930) were used to show that the density of cotton pollen on milkweed leaves (11 grains of MXB-13 pollen per cm^2) is 10X less than the minimum pollen density required to elicit subchronic or developmental effects on monarch butterfly larvae. The EC_{50} $>10^5$ the dietary pollen exposure for Cry1F and > 10X the dietary pollen exposure for Cry1Ac. The calculations indicate that young monarch larvae (at the most sensitive stage) will not be adversely affected by exposure to WideStrike™ cotton pollen expressing Cry1F and Cry1Ac proteins in the field.

viii. Insecticidal Activity Spectrum Study

Susceptible insect spectrum of Cry1F and Cry1Ac ICPs. Non-Guideline studies. [MRID No. 458084-20]

The insecticidal activity spectrum of Cry1F and Cry1Ac was determined for nine insect species exposed to microbially-expressed Cry1F and Cry1Ac in artificial-diet studies. The insects represent taxonomically diverse cotton pests including three orders (Lepidoptera, Heteroptera and Coleoptera) and four families (Miridae, Curculionidae, Noctuidae and Gelchiidae). Insects evaluated were:

tobacco budworm (TBW) - *Heliothis virescens*
cotton bollworm (CBW) - *Helicoverpa zea*
beet armyworm (BAW) - *Spodoptera exigua*
western tarnished plant bug (WTPB) - *Lygus hesperus*
boll weevil (BW) - *Athonomus grandis*
soybean looper (SBL) - *Pseudoplusia includens*
fall armyworm (FAW) - *Spodoptera frugiperda*
cabbage looper (CL) - *Trichoplusia ni*
pink bollworm (PBW) - *Pectinophora gossypiella*
cotton aphid (CA) - *Aphis gossypii*

Both Cry1F and Cry1Ac activity was restricted to lepidopteran insects lending support to the contention that the combination of two Cry proteins did not expand the insect host range. These data also support to the observations that Bt Cry proteins have a very specific and narrow range of target

species.

b. Field Evaluation of WideStrike™ Cotton Effects on Invertebrates

Non-target Beneficial Arthropod Field Survey (MRID No. 458084-19). Supplemental to submitting 2003 field survey data and conducting additional field surveys on large plots that have been planted with Cry1F/Cry1Ac cotton for at least three consecutive years.

The submitted field monitoring studies substantiate the Tier I single species data showing a lack of adverse non-target invertebrate effects of MXB-13 cotton. The beneficial arthropods present in field plots of MXB-13 cotton were compared with those in field plots of non-transgenic cotton with comparable genetics as well as those with and without insecticide application at locations in Louisiana and Arizona. Preliminary results show no adverse effect of MXB-13 on the numbers of insects from over 50 taxa monitored using scouting, whole plant sampling and sweeps. Synthetic (chemical) insecticide treatment, however, reduced the population of some taxa of non-target arthropods at certain times of sampling.

Field surveys using sweep net and sticky trap sample methods were conducted to evaluate potential effects on non-target beneficial arthropods of MXB-13 stacked cotton line (Cry1F/Cry1Ac) in 2002. at two locations. Analyses of all data collected at Winnsboro, LA revealed that there were no adverse effects of MXB-13 on non-target beneficial arthropods. The MXB-13 with no chemical insecticide treatment for Lepidoptera showed significantly higher seasonal survey counts for beneficial Heteroptera in sweeps and for lady beetle adults in leaf sampling while demonstrating effective control of bollworm larvae. Likewise, field studies conducted at the Maricopa Agricultural Research Center in Arizona showed no apparent major negative effects to non-target organisms from the MXB-13 cotton line to the nearly 200 arthropods examined from sweep net collections and the 143 arthropods examined from aerial traps. Several insect groups were significantly more numerous in the MXB-13 plots than in the control plots sprayed with chemical insecticides for Lepidoptera.

This study was only conducted for one year using two sample methods in small plots. The study author also indicated that this study will be repeated which implies that it was replicated during the 2003 growing season. Additional field studies are needed on larger plots that have been planted with Cry1F/Cry1Ac cotton for at least three consecutive years. Since large plots are typically only available after registration, this study should be conducted three years after registration. In addition to sweep net and sticky trap sampling, the soil-dwelling arthropod community should be evaluated with a method such as pitfall trap sampling.

[These preliminary field and field census study design methodologies have been presented to a Scientific Advisory Panel (August, 2002). The SAP commented that the study designs lack appropriate statistical power, but that methodology for conducting statistically valid field census studies at the scale necessary to determine ecosystem effects is not available. Such methodology is yet to be developed. As a result, the Agency is reviewing the available field studies as data supplemental to the maximum

hazard dose single species laboratory testing but useful for short range assessment of non-target invertebrate abundance in Cry protein expressing crop plots. It is an accepted practice in the Office of Pesticide Programs to use the trends seen in several supplemental studies for hazard assessment when a perfect study is not available. The August, 2002 SAP concluded that field experiments must be appropriately designed to provide a measure of ecological impacts. In addition, the SAP opinion was that a two year field study would not be sufficient to determine if a PIP crop will have long term impact on non-target invertebrates. Several public comments also expressed this concern. Short-term field studies are not adequate to draw conclusions on the variations in non-target invertebrate populations. Large field-scale studies conducted for at least three to four years would be needed to draw a conclusion on non-target impacts. The Panel generally concluded that "the state-of-the science" needed for long-term studies must improve to provide meaningful results. The statistical power (avoiding Type II experimental error) needed to gain useful results from field studies would require very large fields, more replications and more samples per plot (e.g., 10 soil and pitfall samples) plus the addition of visual plant samples (e.g., >50/plot). Since the endpoint for field census studies has not been determined, it is difficult to determine how large the fields should be, how many replications are needed and how many samples per plot are needed to achieve appropriate statistical power. Therefore, additional field census studies should not be conducted until the endpoints and logistics of the study have been determined. If Tier I maximum hazard dose single species laboratory studies show a hazard, intermediate field or semi-field studies between laboratory and full-scale field studies should be conducted. Additional full scale field or semi-field studies with appropriate end points and statistical power should also be considered based on recommendations of the August 27, 2002 SAP.]

4. Soil Degradation Studies (Environmental Fate)

Expression in a terrestrial Environment, Tier II (Environmental Fate) [MRID No. 455568-01]

The soil half-life of the plant expressed Cry1F and Cry1Ac was estimated as 1.3 days in a laboratory study with a representative soil from a cotton growing region (Wayside, Mississippi). Soil fortification rates for the study were 0.072 mg a.i. Cry1Ac and 0.853 mg a.i. Cry1F per kg of oven dry soil. These levels represent approximately 3.2 X the EEC for incorporation of defoliated whole plants of MXB-13 into the top 15-cm of soil.

The soil degradation study was conducted with cotton leaf tissue expressing Cry1F(synpro) and Cry1Ac(synpro) insecticidal proteins (2.87 µg/g Cry1Ac and 34.1 µg/g Cry1F). Lyophilized cotton leaf tissue was mixed with soil, incubated under standard laboratory conditions and sampled for bioassay at various intervals. Insect bioassay was conducted to measure degradation via biological activity by applying aqueous-agar mixtures of soil samples to the top of artificial diet and allowing neonate tobacco budworms (*Heliothis virescens*) to feed on the treated media. Test concentrations of Cry1Ac/Cry1F in the surface diets were 0.0762, 0.229, 0.686, 2.06, 6.17, 18.5, 55.6 and 167 µg/cm^2. Mortality and insect weight data were collected from the insect bioassays. Growth inhibition (GI_{50}s-concentration estimated to reduce growth by 50%) were used to estimate the potency of each sample. Based on the increase seen in GI_{50}s over time, the half-life of the Cry1Ac/Cry1F proteins in a representative cotton soil was 1.3 days under laboratory conditions, indicating a rapid decay rate in soil. The Cry proteins were not detectable after 14 days.

Soil organisms may be exposed to Cry proteins by exposure to 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 the Cry protein into the soil. In addition, some evidence suggests that Cry proteins while bound to some soil components, e.g. clays and humic acids, are recalcitrant to degradation by soil microorganisms, but without eliminating their insect toxicity. Several factors influence either the affinity of binding or the rate of degradation. In particular, pH near neutrality generally substantially increases degradation. These issues are being evaluated on a case-by case basis by environmental fate studies designed to determine the rate of Cry protein degradation over sufficiently long periods to assure an accurate assessment of degradation in agricultural soils

The August 27, 2002 SAP Panel concluded that several different soils should be examined and monitored for a minimum of one growing season after harvest and continued until the Cry protein can no longer be detected. The Panel also recommended that an additional sample or two should be examined to verify that an analytical error was not the cause for the lack of detection. According to the Panel, at least two additional soil types should be evaluated for Cry protein persistence. Soils that are high in organic matter and clay should be concentrated on since there is the highest potential of persistence in these soil types. The Panel also recommended that the soil degradation studies be conducted under less than optimum conditions such as high or low temperatures or high or low moisture content. Since roots grow deep into the soil to areas with reduced microbial activity, degradation rates may be reduced. Therefore, degradation of Cry proteins from deep sites should also be examined. The Panel also addressed the protein source that is appropriate for the soil degradation studies. Future studies should utilize plant material that is representative of actual field conditions. For example, whole plant tissue should be incorporated. Plant tissue should not be ground prior to incorporation because it artificially increases the surface area exposed to microorganisms which may lead to an increase in the rate of degradation of the protein. Since more protein may be present than is detected by a single method, an ELISA and an insect bioassay using a sensitive species such as the Colorado potato beetle should be conducted. The SAP concluded that "[r]eal life or true persistence is likely to be equal to or less than that measured with ELISA." If an ELISA is conducted, the results should be compared to results from an insect bioassay.

Because the Agency believes that additional studies would be useful in completing the database for long term effects assessment, it is requesting additional supplementary studies regarding Cry1F/Cry1Ac cotton protein degradation in soil.

5. Effects on Soil Microorganisms

Published studies performed by the EPA Office of Research and Development on the impact of transgenic Cry cotton and other plants indicates that adverse effects on soil microorganisms are unlikely. No effects have been seen due to the protein itself, and only a minimal, transient increase observed in soil microbes attributed to the transgenic cotton plant tissue rather than the Cry protein expressed in that tissue. No adverse effects have been observed in a similar season long field study with Cry3A potato.

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

The Agency has evaluated the potential for horizontal gene transfer (hgt) from Bt crops and has considered possible risk implications if it occurred. Several experiments published in the scientific literature have been conducted to assess the likelihood of hgt, and have been unable to detect gene transfer under typical field conditions. Hgt has only been detected under conditions designed to favor transfer. In addition, the genes that have been engineered into the 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 or toxins to other microorganisms or plants is not known to occur. Therefore, the Agency concluded that hgt is at most an artificial event, and the traits engineered into the Bt crops are already present in soil bacteria or are unlikely to have selective value for soil microorganisms. In considering these data the Agency further concludes that there is no significant risk from hgt from the transgenes found in Cry1F/Cry1Ac cotton.

7. Gene Flow and Weediness Potential

EPA has reviewed the potential for gene capture and expression of the Cry proteins in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories. There is a possibility for gene transfer in locations where wild or feral cotton relatives exist. Therefore, EPA requires stringent sales and distribution restrictions on *Bt* cotton within these areas to preclude outcrossing or hybridization from the crop to sexually compatible relatives. There are only three areas in the United States and its territories wherein cultivated cotton has the opportunity to outcross to wild or feral species which are genetically compatible: (1) southern Arizona, (2) Hawaiian islands, and (3) southern Florida. *G. thurberi* (Arizona Wild Cotton), is present in the elevated regions of Arizona and does not grow in areas of commercial cotton production. *G. thurberi* is a diploid and produces sterile, triploid progeny when crossed with the tetraploids *G. hirsutum* or *G. barbadense*. In the very south of Florida, feral *G. hirsutum* exists in apparently self-sustaining populations. Since these would readily cross with cultivated cotton, sale of *Bt*-Cotton is restricted south of Interstate 60. There is currently no commercial cotton production in the southern part of Florida. Evidence from germplasm collections indicates that feral *G. barbadense* and possibly *G. hirsutum* exist in the Carribean, including Puerto Rico and the U.S. Virgin Islands. There is presently no production of commercial cotton in either of these places, hence, outcrossing is not an issue.

Under FIFRA, the Agency 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 territories. The detailed reviews may be found in the EPA Biopesticides Registtration Action Document (BRAD) for the *Bacillus thuringiensis* (Bt) Plant-Incorporated Protectants, dated October 15, 2001.

8. Endangered Species Considerations

Based on the Cry1F/Cry1Ac cotton protein toxicity and exposure data reviewed there will not be a "may effect" situation for endangered mammals, birds, plants and aquatic species. A comparison of the county-level distribution of endangered lepidopteran species relative to cotton producing counties in the

US indicate that only the Kern primrose sphinx moth (*Euproserpinus euterpe*) is know to occur in a cotton producing county. However, cotton is not a host plant for this species nor do host-range considerations place habitat in or near cotton fields. The Kern primrose sphinx moth is the only endangered lepidopteran taxa known to occur in counties where cotton is grown. It is not really known with certainty whether the endangered lepidopteran may be adversely affected by Cry1Ab protein if exposed. Therefore, a 1986 USFWS formal consultation states that as a "reasonable and prudent alternative" the [crops with] anti-lepidopteran Bt Cry protein must not be within 1/4 mile of any habitats of endangered or threatened Lepidoptera species since these may be adversely affected if exposed to lepidopteran-active *B. thuringiensis* protein in the soil or through pollen consumption.

An examination of the endangered bird and bat species shows that their breeding habitats are mostly non-agricultural. Insectivorous bats do not prey on larvae. They rely on flying insects. Taking these, and other pertinent issues into consideration, it becomes apparent that reduction in the target pests of cotton would not have an effect on the food source of endangered birds and bats. Of those that do encroach on agricultural fields 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 remain abundant in Cry protein crop fields as opposed to non-Bt 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.

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

IV. Environmental Assessment Summary

From all of the required and voluntarily developed indicator and host range species test data on WideStrike™ cotton, including the supplementary field data, the EPA concludes that the levels of Cry1F and Cry1Ac protein in cotton will not pose unreasonable adverse effects to cotton agroecosystem flora and fauna. Available data also indicate that there should be minimal short term accumulation of Cry1F and Cry1Ac protein in agricultural soil. In addition, no adverse effect on listed endangered and threatened species listed by the US Fish and Wildlife Service is expected from the proposed WideStrike™ cotton registration.

Incidental exposure to sensitive larval stages of a non-target butterfly or moth to Cry1F or Cry1Ac may occur if MXB-13 pollen is present on host plants and is consumed. The likelihood of exposure is remote due to the insignificant outflow of pollen from cotton and the presence of other food sources which occur near cotton fields; thus, there is negligible risk from cropping of MXB-13. In excess of 300 different species of beneficial insects are know to inhabit cotton fields. Common arthropod predators and parasites of cotton fields represent orders that are insensitive to the Cry1 proteins. Additionally, these beneficial organisms are predominately predators and parasites and only in a few instances are plant product consumers. Therefore, direct risks to beneficial insects from exposure to Cry1F and

Cry1Ac expressed in MXB-13 are negligible. Risk from indirect exposure through tritrophic feeding on insect host/prey is also negligible due to the low levels of exposure anticipated in comparison to effect levels shown in testing of surrogates.
Analysis of the effect (selectivity and activity on non-targets) and exposure (exposure routes, concentrations and habitat for taxa of concern) indicates negligible ecological risks are posed by cropping of MXB-13 cotton expressing Cry1F and Cry1Ac ICPs.

At present, the Agency is aware of no identified significant adverse effects of Cry1F and Cry1Ac proteins on the abundance of non-target beneficial organisms in any population in the field, whether they are pest parasites, pest predators, or pollinators. Field census data submitted to the Agency show minimal to undetectable changes in the beneficial insect abundance or diversity. In cotton 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. In general invertebrate abundance studies in Bt crop fields do not show a shift in biodiversity, 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, annual insect monitoring of representative commercial fields will continue for long term biodiversity effects assessment.

The Agency believes that cultivation of WideStrike™ cotton may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, WideStrike™ cotton requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of nonspecific conventional chemical pesticides is known to have an adverse effect on non-target beneficial organisms found living in the complex environment of an agricultural field. Many of these beneficial organisms are important integrated pest management controls (IPM) for secondary pests such as aphids and leafhoppers. The overall result of cultivation of cotton expressing Cry proteins is that the number of chemical insecticide applications for non-target pest control is reduced for management of multiple pest problems.

The movement of transgenes from WideStrike™ cotton into weeds and other crops in the U.S., its possessions or territories has also been considered. The fate of Cry1F and Cry1Ac proteins in soils and indirect effects on soil biota have also been evaluated. Test data show that most of the Cry protein deposited into soil is quickly degraded, although a residual amount may persist in biologically active form for a longer period of time. It is also reported that detectable Bt Cry protein persistence exists in soils that have been exposed to repeat Bt spray applications. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil. Horizontal transfer of genes or toxins from transgenic plants to soil bacteria has not been demonstrated. Published studies of Bt Cry protein 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.

Conclusions:

This assessment finds no hazard to the environment at the present time from cultivation of Cry1F and Cry1Ac protein expressing cotton for a time-limited registration period.

V. Supplemental Data Needed for Long Term Environmental Hazard Assessment

The Agency has sufficient information to believe that there is no hazard from the proposed uses of WideStrike™ cotton to non-target wildlife, aquatic and soil organisms. However, the Agency is requesting additional, primarily long term effects data. The supplementary studies would provide additional weight to support the Agency's conclusions. Therefore, the Agency is requesting the following data (Table 3) to ascertain any possible adverse environmental effects from long term use of this product, as well as testing on more appropriate non-target invertebrates found in cotton fields. The Agency does not believe that this data requirement was reasonably foreseeable by the applicant at the time of application.

Table 3. Supplemental data:

Testing Category	Type of Data
Avian chronic exposure testing	The submitted avian dietary toxicity data are not sufficient to make a chronic avian hazard assessment from repeated exposure(s) to higher doses of Cry1F/Cry1Ac cotton. A six week broiler dietary study is needed to assess hazard to wild and domesticated fowl from chronic exposure to Cry1F/Cry1Ac protein. If necessary, Cry1F/Cry1Ac corn may be used in the study.
Non-target insect more appropriate for cotton fields	Conduct a maximum hazard dose laboratory toxicity test with *Orius insidiosus* (minute pirate bug).
Ecosystem effects	Submitting 2003 field survey data and conducting additional field surveys on large plots that have been planted with Cry1F/Cry1Ac cotton for at least three consecutive years. The large-scale field surveys should include sweep net and sticky traps sampling as well as sampling soil-dwelling arthropods with a method such as pitfall traps. Additional long range field studies should also be conducted based on recommendations of the August, 2002 SAP found in the conclusion section of the review of MRID No. 458084-19 above.
Soil fate/terrestrial expression studies	Additional long range soil persistence field studies should also be conducted including the parameters outlined by the August 2002 SAP found in the conclusion section of the summary in MRID No. 455568-01 above