BIOPESTICIDE REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis Cry1Ac Protein and the Genetic Material (Vector PV-GMIR9) Necessary for Its Production in MON 87701 (OECD Unique Identifier: MON 877Ø1-2) Soybean
[PC Code 006532]

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

September 2010

TABLE of CONTENTS

I. OVERVIEW 3

A. EXECUTIVE SUMMARY 3
B. USE PROFILE 4
C. REGULATORY HISTORY 5

II. SCIENCE ASSESSMENT 6

A. PRODUCT CHARACTERIZATION
B. HUMAN HEALTH ASSESSMENT
D. ENVIRONMENTAL ASSESSMENT 15
E. INSECT RESISTANCE MANAGEMENT (IRM) 33

III. TERMS AND CONDITIONS OF REGISTRATION 38

IV. REGULATORY POSITION FOR CRY1AC SOYBEAN 38

BIOPESTICIDE REGISTRATION ACTION TEAM

Office of Pesticide Programs:

Biopesticides and Pollution Prevention Division

Product Characterization and Human Health

John Kough, Ph.D.
Chris Wozniak, Ph.D.

Environmental Fate and Effects

Zigfridas Vaituzis, Ph.D.
Shannon Borges, M.S.

Insect Resistance Management

Alan Reynolds, M.S.
Jeannette Martinez, M.S.

Registration Support

Mike Mendelsohn

I. OVERVIEW

A. EXECUTIVE SUMMARY

Monsanto Company (hearafter, "Monsanto") has submitted an application to register the soybean product MON 87701, which expresses the *Bacillus thuringiensis* (*Bt*) subsp. *kurstaki* insecticidal protein Cry1Ac as a Plant Incorporated Protectant (PIP). Cry1Ac provides protection from feeding damage by target lepidopteran pests, including the velvetbean caterpillar (*Anticarsia gemmatalis*), soybean looper (*Pseudoplusia includens*), soybean axil borer (*Epinotia aporema*), and sunflower looper (*Rachiplusia nu*). Monsanto has applied for a FIFRA Section 3 registration for breeding and seed increase activities to support commercial production of MON 87701 in tropical and subtropical regions outside the U.S. and its territories.

Product Characterization

MON 87701, which was developed by *Agrobacterium*-mediated transformation of soybean (*Glycine max*) using the 2T-DNA plasmid vector PV- GMIR9, produces one *Bacillus thuringiensis* protein, Cry1Ac. This protein is intended to provide protection from feeding damage caused by a number of lepidopteran pests, such as the soybean looper, beet armyworm, green cloverworm and velvetbean caterpillar.

Mammalian Toxicity and Allergenicity Assessment

Cry1Ac protein is a δ-endotoxin from *B. thuringiensis* that has been used extensively in both microbial and plant-incorporated protectants as a means of insect pest management. An existing exemption from the requirement of a tolerance for Cry1Ac (CFR 40 Section 174.510; 72 FR 20435, April 25, 1997) in food and feed commodities precluded the need for a separate tolerance action in conjunction with this review of product characterization and human health assessment.

Environmental Assessment

Adverse effects to nontarget organisms, including birds, wild mammals, freshwater and marine/estuarine fish and invertebrates, nontarget insects, honey bees, soil invertebrates, and terrestrial and aquatic plants, are not anticipated. Horizontal gene transfer, gene flow, and the development of weediness are also not anticipated. Based on these findings and information on habitat requirements of federally-listed threatened and endangered lepidopteran species, the Agency also makes a No Effect determination for direct and indirect effects to threatened and endangered species and their habitats.

Insect Resistance Management

Given the low acreage permitted for MON 87701 (<15,000 total acres per year), it is unlikely that there will be a significant risk of resistance to the main soybean target pests in the United States, soybean looper and velvetbean caterpillar. This conclusion is further supported by the biology of the target insects, which do not overwinter in non-tropical areas and are polyphagous (feeding on a number of wild hosts and cultivated crops).

The use of MON 87701 on a limited basis (as described by Monsanto in their submissions) should not impact the natural refuge strategy in place for Bt cotton PIPs that express Cry1Ac. The total proposed acreage for MON 87701 would represent a small fraction of total soybean acres in each state and should not significantly reduce the amount of natural refuge. Further, MON 87701 will not be planted in states (i.e., Mississippi Delta states) with lower natural refuge.

The Agency is requiring adoption of a resistance monitoring plan, remedial action strategy, and annual sales reporting for MON 87701.

B. USE PROFILE

Active Ingredient Name: *Bacillus thuringiensis* Cry1Ac Protein and the Genetic Material (Vector PV-GMIR9) Necessary for Its Production in MON 87701 (OECD Unique Identifier: MON 877Ø1-2) Soybean

Trade and Other Name(s): MON 87701

OPP Chemical Codes: 006532

Basic Manufacturer: Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

Type of Pesticide: Plant-incorporated Protectant

Uses: Soybean. Limited to seed increase and to a total of 15,000 acres per year in the Atlantic Coast States within the Continental United States with no more than 1,000 acres per county per year.

Target Pests for Active Ingredient: Velvetbean caterpillar (*Anticarsia gemmatalis*), soybean looper (*Pseudoplusia includens*), soybean axil borer (*Epinotia apoerema*), and sunflower looper (*Rachiplusia nu*)

C. REGULATORY HISTORY

Monsanto previously submitted EUP requests for event MON 87701 in order to conduct field evaluations and gather data. Cry1Ac protein is a δ-endotoxin from *B. thuringiensis* and has been used extensively in both microbial and plant-incorporated protectants as a means of insect pest management. An existing exemption from the requirement of a tolerance for Cry1Ac (CFR 40 Section 174.510; 72 FR 20435, April 25, 1997) in food and feed commodities precludes the need for a separate tolerance action in conjunction with this review of product characterization and human health assessment.

On April 14, 2010, EPA announced its receipt of this application pursuant to FIFRA secion 3(c)(4) in the Federal Register and in regulations.gov under docket number EPA-HQ-OPP-2010-0023. No comments were received in response to this notice.

II. SCIENCE ASSESSMENT

A. PRODUCT CHARACTERIZATION

MON 87701 was developed by *Agrobacterium*-mediated transformation of soybean using the 2T-DNA plasmid vector PV- GMIR9 and produces *Bacillus thuringiensis* protein δ-endotoxin, Cry1Ac. This protein is intended to provide protection from feeding damage caused by a number of lepidopteran pests.

Transformation System and Genetic Elements:
PV- GMIR9 is a binary vector containing two separate transfer DNAs (2T-DNAs). The first T-DNA (T-DNA I) contains the *cry1Ac* gene under the direction of the RbcS4 (small subunit RuBISCO from *Arabidopsis thaliana*) promoter and termination sequences from T-7Sά (3' region of the *Sphas* gene of *Glycine max* encoding the 7Sά seed storage protein conglycinin). The chloroplast transit peptide coding sequence from *Arabidopsis thaliana* from *RbcS4* small subunit 1A was placed at the 5' end of the gene (N-terminus) to direct accumulation of the insecticidal protein to the chloroplast. In the Cry1Ac protein produced in MON 87701, 4 additional amino acids are present at the N-terminus of the protein as a result of the use of this targeting sequence.

The second T-DNA, designated as T-DNA II, contains the *cp4-epsps* gene under the direction of the Figwort Mosaic Virus 35S promoter. During transformation, both T-DNAs were inserted into the soybean genome independently. The *cp4-epsps* gene was used as the selectable marker (tolerance to glyphosate) to select transformed cells and plants. After the transformed cells and subsequent regenerated plants were identified, the *cp4-epsps* selectable marker gene was no longer needed. Therefore, traditional breeding and segregation were employed to isolate plants that contain only the *cry1Ac* expression cassette (T-DNA I), producing marker-free MON 87701 plants lacking the *cp4-epsps* selectable marker gene.

Analysis of the insert junction of the single copy T-DNA-I into MON 87701 genomic DNA indicated that the entire sequence, as present in T-DNA-I from PV- GMIR9, is intact in the final transformant. Sequencing of the insertion site indicated a loss of 32 bp as well as an addition of 14 bp at the junction with soybean genomic DNA. This has been previously noted in the literature regarding *Agrobacterium*-mediated integration events (Gorbunova and Levy, 1997; Salomon and Puchta, 1998).

Protein Characterization:
Protein characterization data demonstrate that the plant-produced Cry1Ac has biochemical and functional activities that are similar to those of the *E. coli*-produced protein that was used in several toxicity studies. The following techniques were used to characterize and compare the plant-produced and the *E. coli*-produced proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) / mass spectrometry, glycosylation analysis, N-terminal amino acid sequencing, and insecticidal activity bioassays. Glycoslyation analysis indicated that the protein is not glycoslyated. These analyses demonstrated the structural and

functional similarity between the plant-produced and the *E. coli*-produced Cry1Ac protein and justified the use of *E. coli*-produced protein in toxicity studies.

Soybean MON 87701 derived Cry1Ac protein contains an additional 4 amino acids derived from the chloroplast transit peptide (CTP1) sequence at the N-terminus. This addition is also found in the protein that was produced in *E. coli* for the oral toxicity study and the insect bioassays with corn earworm. It should be noted that the Cry1Ac protein is processed at both the N- and C- termini during trypsin activation of the protein in the insect gut. The CTP1 residual 4 amino acid residues would be cleaved off by protease action as part of that maturation process *in situ*. The equivalency of the protein used as a test substance and that as produced *in planta* were reviewed in this risk assessment and found to be acceptable.

Analytical Detection Methods:
An exemption from the requirement of a food tolerance is already in existence (since 1997) for this protein and an appropriate analytical detection method already reviewed and in place for Cry1Ac. Hence, there is no separate review of an analytical method in this risk assessment summary.

Protein Expression:
Expression level data were provided for Cry1Ac in different plant tissues and at different growth stages. The data were produced using ELISA methods for this protein. Summary results are provided below in Table 1. Table 2 provides summaries of the product characterization studies and data provided.

TABLE 1. Cry1Ac protein levels in MON 87701 tissues

Tissue type[1]	Mean (SD)[2,4] (µg/g fwt)	Range[5] (µg/g fwt)	Mean (SD)[3,4] (µg/g dwt)	Range[5] (µg/g dwt)
OSL-1	30 (8.5)	12-40	220 (70)	110-350
OSL-2	38 (16)	18-80	260 (100)	130-500
OSL-3	34 (17)	14-77	240 (110)	94-480
OSL-4	53 (36)	15-110	340 (290)	78-960
Root	<LOD	<LOD	NA[6]	NA[6]
Forage	9.0 (8.8)	2.5-32	34 (36)	8.2-140
Seed	4.2 (0.73)	3.1-5.0	4.7 (0.79)	3.4-5.7
Pollen	2.3 (0.58)	1.8-3.1	NA	NA

Data from pp. 17 and 18 of 20, MRID 47841703

[1]Tissues were collected at the following growth stages: OSL-1: V3-V4; OSL-2: V6-V8; OSL-3: V10-V12; OSL-4: V14-V16; Root: R6; Forage: R6; Seed: R8, harvested at or dried to 10-15% moisture; Pollen: R2.

[2]Protein levels expressed as µg of protein/g of tissue on a fresh weight basis.

[3]Protein levels expressed as µg/g on a dry weight basis, calculated by dividing the fresh weight by the dry weight. conversion factors obtained from moisture analysis data. Moisture analysis was not performed for pollen.

[4]The mean and standard deviation were calculated across sites (n=15, except OSL-1 n=13 and pollen n=4).

[5]Minimum and maximum values were determined for each tissue type across sites.

[6]Protein levels <LOD on a fresh weight basis were not converted to dry weight values.

Table 2. Product Characterization Data Submitted

Study Type/Title	Summary	MRID #
Human Health and Environmental Assessment of the Plant-Incorporated Protectant *Bacillus thuringiensis* Cry1Ac Protein Produced in Insect-Protected Soybean MON 87701	MON 87701 is an insect-protected soybean that produces the Cry1Ac insecticidal protein derived from *Bacillus thuringiensis* subsp. *kurstaki*. Cry1Ac provides protection from feeding damage by target lepidopteran pests. Characterization studies have confirmed that the genetic modification in MON 87701 contains a single insert with the intended sequence, and that the insert is stable over multiple generations. The mean Cry1Ac content in MON 87701 tissues (dry weight basis) was determined to be 4.7 µg/g in harvested seed, 34 µg/g in forage, 220 to 340 µg/g in leaves, 2.3 µg/g in pollen/anthers (fresh weight basis), and below the limit of detection in roots. MON 87701 was effective against major lepidopteran pests of soybean in screenhouse and open field trials at multiple locations in the US and Argentina. The Cry1Ac protein has a history of safe use in cotton and corn products. No structurally relevant similarity exists between Cry1Ac and any known toxic or biologically active proteins or known allergens that would be harmful to human or animal health. MON 87701 is not expected to produce adverse effects in non-target organisms, including threatened or endangered species, based on tests with mice, two avian species, soil decomposers, and beneficial insects. Tests on aquatic species were not conducted since there is no meaningful, ecologically relevant exposure to aquatic organisms from soybean. A laboratory soil degradation study showed that 72% to 96% of the Cry1Ac in MON 87701 biomass incorporated into soil degraded within six months, indicating Cry1Ac is unlikely to persist or accumulate in the environment. **Classification: ACCEPTABLE**	47841701
Assessment of the Cry1Ac Protein Levels in Soybean Tissues Collected from MON 87701 Produced in U.S. Field Trials During 2007	MON 87701 is an insect-protected soybean that produces the Cry1Ac insecticidal protein derived from *Bacillus thuringiensis* subsp. *kurstaki*. Cry1Ac provides protection from feeding damage by the target lepidopteran pests. An enzyme linked immunosorbent assay method was used to determine the level of Cry1Ac protein expressed in MON 87701 tissues from plants grown in field trials conducted in Alabama, Arkansas, Georgia, Illinois, and North Carolina in 2007. The mean Cry1Ac levels across all sites were 4.7 µg/g dry weight in harvested seed, 34 µg/g dry weight in forage, and below the limit of detection (LOD) in roots. The mean Cry1Ac level in	47841703

Study Type/Title	Summary	MRID #
	leaves harvested during difference stages of the growing season ranged from 220-340 µg/g dry weight. The mean Cry1Ac level in pollen from the single site sampled was 2.3 µg/g fresh weight. **Classification: ACCEPTABLE**	
Characterization of the Cry1Ac protein purified from the harvested seed of MON 87701 soybean and comparison of the physicochemical and functional properties of the MON 87701-produced and *E coli*-produced Cry1Ac protein	The information and data presented are in accord with the conclusion of the study author that MON-87701-derived and *E. coli*-derived Cry1Ac are equivalent proteins. Relative molecular mass measurements derived through SDS-PAGE and fragment analysis through MALDI-TOF / MS clearly indicate that the proteins derived from *in planta* expression in MON 87701 and from expression in *E. coli* are of the same mass. N-terminal sequencing of the first 7 amino acids and their identity to the Cry1Ac sequence aided in verifying that the protein of interest was consistent with what is known of the insecticidal protein, along with the ambiguous cysteine residue present in accord with the use of the CTP1 peptide at the terminus. Reactivity to antiserum directed against Cry1Ac also indicates the sequence similarities between the two proteins and any products of catalysis as noted on the blots. An absence of glycosylation in both microbial and plant produced forms of Cry1Ac is as expected for this protein and further suggests the homology between the proteins from the two sources. The EC_{50} values for the two proteins, when tested against the corn earworm, identify the insecticidal activity of the two proteins as so similar in value as to be the same. The weight of evidence from the variety of testing parameters indicates that the two sources of the Cry1ac test substance protein are identical and interchangeable for the testing performed. **Classification: ACCEPTABLE**	47841704
Functional Equivalence of *E. coli*-Produced Full Length Cry1Ac Protein and the Cry1Ac Tryptic Core Protein	The functional activity of the 131.7 kDa full-length *E. coli*-produced Cry1Ac protein (protoxin) was compared to that of the 57.8 kDa *E. coli*-produced Cry1Ac tryptic core (toxin). The EC_{50} values from 18 previous dietary bioassays of corn earworm (*Helicoverpa zea*) exposed to the Cry1Ac tryptic core were compared to the EC_{50} values from 12 previous studies exposing corn earworm to full length Cry1Ac. To correct for differences in the molecular mass, the EC_{50} values (µg/mL) for each set of bioassays were converted to molar concentrations, and the means were compared statistically. There was no	47841706

Study Type/Title	Summary	MRID #
	statistically significant difference ($p = 0.39$) in the mean EC_{50} values for the tryptic core and full length Cry1Ac protein expressed on a molar basis, indicating that both forms are functionally equivalent in potency against susceptible Lepidoptera. Therefore, results of safety and efficacy studies using the tryptic core can be used to evaluate the safety and insecticidal activity of the full length protein. **Classification: ACCEPTABLE**	
Molecular Analysis of Insect-Protected Soybean MON 87701 – Amended Report for MSL0022176 (Amendment 3)	MON 87701 contains one copy of the insert at a single integration locus and all expression elements were present in the T-DNA I; MON 87701 does not contain detectable plasmid backbone or selectable marker sequences; and the integrity of the inserted *cry1Ac* expression cassette within the inserted sequences confirmed and identified the 5' and 3' insert to-genomic DNA junctions. The insertion site in conventional soybean confirmed the flanking genomic DNA is native to the soybean genome and was maintained through five generations of breeding. **Classification: ACCEPTABLE**	47873801

B. HUMAN HEALTH ASSESSMENT OF CRY1AC AS EXPRESSED IN MON 87701

Toxicological Profile

EPA has reviewed the available scientific data and other relevant information in support of this action and considered its validity, completeness and reliability and the relationship of this information to human health risk

The human health studies submitted for Cry1Ac are summarized in Table 3 below.

Table 3. Summary of Cry1Ac Human Health Data

Study Type/Title	Summary	MRID #
Immunodetection of Cry1Ac protein in MON 87701 ground seed following heat treatment.	Heating of MON 87701 ground seed in a manner that simulates baking results in the loss of immunodetectable Cry1Ac protein below the LOD, indicating a decrease ≥ 94% of the Cry 1Ac relative to the protein in unheated MON 87701 samples. **Classification: ACCEPTABLE**	47841705

Study Type/Title	Summary	MRID #
An Acute Study of Cry1AcProtein Administered by the Oral (Gavage) Route to Mice; Human Healthand Environmental Assessment	In an acute oral toxicity study, twenty male and twenty female fasted, approximately 8-10 week old CD-1 mice were given an oral dose of control Bovine serum albumin (BSA) or Cry1Ac Protein at doses of 1280 or 1290 mg protein/kg body weight, respectively. In an additional study phase, twenty male fasted, approximately 6 week old CD-1 mice were given an oral dose of control BSA or Cry1Ac Protein at dose of 1620 or 1460 mg protein/kg body weight, respectively. The control and test material were administered as two doses separated by approximately 4 hours. The animals were observed for 14 days. The Oral LD_{50} for female mice was > 1290 mg / kg BW and for male mice it was > 1460 mg / kg BW. Based on the results of this study, Cry1Ac Protein is in EPA Toxicity Category III. All animals survived the study. No test material-related clinical signs, body weight effects, and food consumption effects were noted during the study. No test material-related gross abnormalities were noted in any animal at necropsy. **Classification: ACCEPTABLE**	47841707
Assessment of the *in vitro* digestibility of the Cry1Ac protein in simulated gastric and simulated intestinal fluids	The data presented in this evaluation of protein digestibility in simulated gastric and simulated intestinal fluids clearly indicate a trend toward digestion of the Cry1Ac protein in a rapid manner. Within the limits of detection of the colloidal Coomassie Brilliant Blue protein binding dye (between 10 - 20 ŋg) following SDS-PAGE, the Cry1Ac protein was degraded (i.e., not visible) within 30 sec of treatment with SGF. Treatment with trypsin (SIF) results in a peptide fragment representing the tryptic core of Cry1Ac. This tryptic core fragment is typical of δ-endotoxins from *B. thuringiensis*. With sequential treatment of SGF and SIF, Cry1Ac was rapidly degraded suggesting that under conditions typical of a monogastric mammalian stomach, the Cry1Ac protein would not enter the small intestine in intact form, but rather as small, peptide fragments. Similar results were obtained using Western blotting techniques with chemiluminescent detection; Cry1Ac protein was degraded within 30 seconds of SGF treatment with a limit of detection of approximately 1 ŋg. A small stained band of approximately 4 kDa was noted on the SDS-PAGE gel following SGF digestion. This band was examined by N-terminal sequencing and determined to consist of two peptides from Cry1Ac. Following treatment with SIF for 5 min, this band of two peptides was no longer detected. The study results	47841708

Study Type/Title	Summary	MRID #
	show that full-length Cry1Ac protein is rapidly digested in the gastrointestinal digestive system. **Classification: ACCEPTABLE**	
Bioinformatics evaluation of the Cry1Ac protein present in MON 87701 soybean utilizing the AD8, TOXIN6, and PROTEIN databases	No significant sequence homology between any sequential MON 87701 Cry1Ac protein sequences and known allergens, toxins, or reactive proteins was found as based upon FASTA alignment procedures and an 8 amino acid sliding window comparison. **Classification: ACCEPTABLE**	47841709

HUMAN HEALTH ASSESSMENT Cry1Ac in MON 87701

.

Mammalian Toxicity and Allergenicity Assessment

Monsanto has submitted acute oral toxicity data demonstrating the lack of mammalian toxicity at high levels of exposure (ingestion) to the pure Cry1Ac protein. These data demonstrate the safety of the product at a level well above maximum possible consumption levels that are reasonably anticipated as expressed in the crop. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity testing and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR Sec. 158.740(b)(2)(i)). For microbial products, further toxicity testing (Tiers II & III) and residue data are triggered by significant adverse acute effects in studies, such as the acute oral toxicity study, to verify the observed adverse effects and clarify the source of these effects.

An acute oral toxicity study in mice (MRID 47841707) indicated that Cry1Ac is non-toxic to humans and other mammals. Three groups of ten male and ten female mice were dosed by oral gavage with 1290 or 1460 mg/kg bodyweight of microbially-produced Cry1Ac protein. A negative control group was also included in the study: bovine serum albumin protein control. There were no significant differences between the test and control groups; therefore, the Cry1Ac protein does not appear to cause any significant adverse effects at an exposure level of > 1460 mg/kg bodyweight.

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 acute effects were shown to be caused by Cry1Ac, even at relatively high dose levels, the Cry1Ac protein is not considered toxic. Further, amino acid

sequence comparisons showed no similarities that would raise a safety concern between the Cry1Ac protein and known toxic proteins in protein databases.

Since Cry1Ac is a protein, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including *in vitro* digestibility in simulated gastric fluid (SGF) followed by simulated intestinal fluid (SIF), and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for Cry1Ac follows:

1. Source of the trait. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of Cry1Ac with known allergens showed no significant overall sequence similarity or identity at the level of eight contiguous amino acid residues.
3. Digestibility. The Cry1Ac protein was digested within 30 seconds in simulated gastric fluid containing pepsin. Small peptides remaining following gastric simulated digestion were completely degraded to amino acid residues in SIF upon contact.
4. Glycosylation. Cry1Ac expressed in soybean was shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for Cry1Ac to be a food allergen is minimal.

Although Cry1Ac was only shown not to be glycosylated in corn, it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it must contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms of protein glycosylation are similar in different plants (Lerouge et al., 1998).

Overall Safety Conclusions

The continued use of Cry1Ac protein in plant expression systems (plant-incorporated protectants) and in microbial biopesticides is fully supported by the information presented in this registration submission relative to human and animal health concerns. The lack of mammalian toxicity and allergenicity effects following thorough examination of pertinent information, as well as the efficacy noted in the insect bioassays, indicates that the specificity of the δ-endotoxin Cry1Ac as an insect management mechanism is safe as proposed for use in soybean intended for cultivation and human and animal consumption.

Endocrine Disruptors

As required under FFDCA section 408(p), the Agency 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 the Agency 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, the Agency 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 Cry1Ac protein is not among the group of 58 pesticide active ingredients on the initial list to be screened under the EDSP. Under FFDCA section 408(p), the Agency must screen all pesticide chemicals. Accordingly, the Agency anticipates issuing future EDSP orders/data call-ins for all pesticide active ingredients.

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/*.

REFERENCES:

Gorbunova, V. and A.A Levy, (1997) Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. *Nucleic Acids Res.*, 25:4650–4657.

Lerouge, P. Cabanes-Macheteau, M., Rayon, C., Fichette-Lainé, A-C., Gomord, V., and Faye, L., (1998) N-Glycoprotein biosynthesis in plants: recent developments and future trends. Plant Molecular Biology 38: 31-48.

Salomon, S. and H. Puchta (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. The EMBO Journal, 17:6086–6095.

Sjoblad, Roy D., et al., (1992) Toxicological Considerations for Protein Components of Biological Pesticide Products. Regulatory Toxicology and Pharmacology 15:3-9.

C. ENVIRONMENTAL ASSESSMENT for MON 87701

Overall Conclusions

Adverse effects to nontarget organisms, including birds, wild mammals, freshwater and marine/estuarine fish and invertebrates, nontarget insects, honey bees, soil invertebrates, and terrestrial and aquatic plants, are not anticipated. Horizontal gene transfer, gene flow, and the development of weediness are also not anticipated. Based on these findings and information on habitat requirements of federally-listed threatened and endangered lepidopteran species, the Agency also makes a No Effect determination for direct and indirect effects to threatened and endangered species and their habitats.

1. Nontarget Organism Tiered Testing and Risk Assessment Process for PIPs

The paragraphs below describe the process and rationale developed by BPPD for evaluating hazard of PIPs to nontarget organisms. This process is described in several of BPPD's documents and is presented again here as background information.

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

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

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

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

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

The Tier I screening maximum hazard dose (MHD) approach to environmental hazard assessment is based on some factor (whenever possible >10) times the maximum amount of active ingredient expected to be available to terrestrial and aquatic non-target organisms in the environment (EEC).[3] Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels that 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 30 animals or three groups of 10 test animals at the screening test concentration. The Guidelines further state that the duration of all Tier I tests should be approximately 30 days.

---

[2] OPPTS Testing Guidelines, Series 850 and 885 website: http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/885Microbial_Pesticide_Test_Guidelines/Series

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

Some test species, notably non-target insects, may be difficult to culture and the suggested test duration has been adjusted accordingly. Control and treated insects should be observed for at least 30 days, or in cases where an insect species cannot be cultured for 30 days, until negative control mortality rises above 20 percent.

Failing the Tier I (10 X EEC) screening at the MHD dose does not necessarily indicate the presence of an unacceptable risk in the field but it triggers the need for additional testing.[4] A less than 50% mortality effect at the MHD is taken to indicate minimal risk. 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 OPPTS Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} and to quantify the hazard. In the definitive testing, the number of doses and test organisms evaluated must be sufficient to determine an LD_{50} value and, when necessary, the Lowest Observed Effect Concentration (LOEC), No Observed Adverse Effect Level (NOAEL) , or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. In the final analysis, a risk assessment is made by comparing the LOAEC to the EEC; when the EEC is lower than the LOAEC, a no risk conclusion is made. These tests offer greater environmental realism, but they may have lower statistical power. Appropriate statistical methods, and appropriate statistical power, must be employed to evaluate the data from the definitive tests. Higher levels of replication, the number of test species, 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} >10 X EEC) are sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. It is also notable that the recommended >10X EEC maximum

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

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

hazard dose level is a highly conservative factor. The published EPA Level of Concern [LOC] is 50% mortality at 5X EEC (US EPA, 1998).[6]

Validation: The tiered hazard assessment approach was developed for the EPA by the American Institute of Biological Sciences (AIBS) and confirmed in 1996 as an acceptable method of environmental hazard assessment by a FIFRA Scientific Advisory Panel (SAP) on microbial pesticides and microbial toxins. The December 9, 1999, SAP agreed that the Tiered approach was suitable for use with Plant-Incorporated Protectants (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 GM crop fields should be conducted. Testing of *Bt* Cry proteins on species not closely related to the target insect pest was not recommended, although it is still performed to fulfill the published EPA non-target species data requirements. In October 2000, another SAP also recommended that field testing should be used to evaluate population-level effects on non-target organisms. The August 2002 SAP, and some public comments, generally agreed with this approach, with the additional recommendation that indicator organisms should be selected on the basis of potential for field exposure to the subject protein (US EPA, 2000, 2001a, 2002, and 2004).

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. But, the 30 day test duration requirement does amount to subchronic testing when performed at field exposure test doses. Proteins also 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 1 testing for short-term hazard assessment will be sufficient for most studies submitted in support of PIP registrations. But, if long range adverse effects must be ascertained, then higher-tier 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

[6] The established peer and EPA Science Board reviewed guidance on screening test levels of concern is 50% mortality at 5X environmental concentration. The appropriate endpoints in high dose limit/screening testing are based on mortality of the treated, as compared to the untreated (control) non-target organisms. A single group of 30 test animals may be tested at the maximum hazard dose.

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, the Agency has required field studies on long term invertebrate population/community and Cry protein accumulation in soils as a condition of registration due to the lack of baseline data on the potential for long-term environmental effects from the cultivation of PIP-producing plants.

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. Nontarget Organism Risk Assessment for Cry1Ac as Expressed in MON 87701 Soybeans

a. Exposure Assessment

Two separate SAP reports (October 2000 and August 2002) recommended that non-target testing of *Bt* Cry proteins should focus on invertebrate species exposed to the crop being registered. Following SAP recommendations, the EPA determined that non-target organisms with the

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

greatest exposure potential to Cry proteins in transgenic crop fields are beneficial insects, especially those that feed on pollen and nectar, and soil invertebrates. While EPA's risk assessments of *Bt* PIP crops have focused primarily on these taxa, BPPD recognizes that exposure to other nontarget organisms can occur and has required testing on representative species.

The EPA risk assessment is centered only on adverse effects at the field exposure rates, which for PIPs are typically based on protein expression levels within the plant. Although it is recommended that non-target testing be conducted at a test dose 10X the EEC whenever possible, the test 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. BPPD may also allow for testing at lower doses in cases where many species are tested or tests are very sensitive, although the concentration used must exceed the EEC. For the purposes of the non-target organism studies submitted in support of MON 87701 soybeans, the test material dose levels were based on an estimated concentration of Cry1Ac protein expressed in the tissue(s) to which the assessed taxa are likely to be exposed. For nontarget insects and honeybees, tests were performed at concentrations of 19X to 322X the expression level in MON 87701 pollen. For soil invertebrates, Monsanto tested at 15X to 18X the estimated soil concentration based on the expected amount resulting from plant incorporation in the soil. For birds, tests were performed using raw soybeans. Details of these dosing amounts are discussed further in the summary of the toxicity studies presented below.

b. Ecological Effects Data

As discussed above, in the absence of PIP-specific risk assessment data requirements, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins. These requirements include testing on birds, mammals, nontarget insects, honey bee, plants, and aquatic species, and information has been submitted to address these requirements. Limit dose testing and dietary studies were performed on representative organisms from several taxa in support of the MON 87701 Soybean Section 3 registration. As stated above, BPPD's risk assessments focus heavily on beneficial nontarget invertebrates, since they are most closely related to organisms susceptible to the insecticidal action of *Bt* toxins. The Cry1Ac protein is meant to target species within the order Lepidoptera (moths and butterflies). *Bt* toxins are known typically to have a limited host range; however, to address any unforeseen change in activity spectrum as a result of laboratory protein synthesis and to fulfill the published registration data requirements EPA requires that test species used for non-target insect evaluations should include several invertebrate species that are not related to the target pests, as well as tests on birds and other vertebrates as appropriate. Earthworm studies are also recommended. Data to evaluate the toxicity of Cry1Ac on several species of invertebrates were submitted, including the lady beetle, honey bee, and earthworm.

Since exposure may also occur to other nontarget organisms, EPA has received data to comply with the Agency's published non-target data requirements on other nontarget organisms. A

study to determine soil degradation of Cry1Ac has also been submitted, as well as a description of the MON 87701 soybean Cry1Ac in soil. The individual results for nontarget organism and soil degradation testing for Cry1Ac are summarized in Table 1. The studies are described in more detail within the risk assessment conclusions below, and full reviews of each study for each event can be found in the individual Data Evaluation Records.

The October 2000 SAP recommended that while actual plant material is the preferred test material, bacteria derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume soybean plant tissue and where large amounts of Cry protein are needed for maximum hazard dose testing. In support of the MON 87701 soybean registration, test substances used in the submitted studies included bacterially produced purified Cry1Ac protein. Comparative analyses have been performed to verify the equivalence of the bacterially produced and purified Cry1Ac and the Cry1Ac protein produced in MON 87701 soybean plants (MRID 47841701). These studies show that the bacteria produced protein is equivalent to those produced by MON 87701 soybean plants.

Table 4. Summary of environmental effects studies for Cry1Ac as expressed in MON 87701 Soybean

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
Avian dietary testing, broiler chicken, *Gallus domesticus*	885.4050	MON 87701 Soybean meal	A 42-day dietary study showed no adverse affects to broiler chickens when fed a diet composed of approximately 30% MON 87701 soybean meal containing Cry1Ac. The presence of active Cry protein in the test material was not confirmed. **Classification: Unacceptable**	47841712
Avian dietary testing, Northern bobwhite, *Colinus virginianus*	885.4050	Raw ground MON 87701 soybean	An 8-day dietary study showed no adverse affects to Northern bobwhite fed a diet composed of 20% raw ground MON 87701 soybean. This exposure level is consistent with previously accepted studies for other PIPs. **Classification: Acceptable**	47841711
Non-target insect testing, ladybird beetle, *Coleomegilla maculata*	885.4340	Microbially-produced Cry1Ac	No adverse effects on survival, development to adult, or mean adult weight were observed in *Coleomegilla maculata* larvae exposed to 60 µg Cry1Ac protein/g of diet for 20 days. The LC_{50} was determined to be >60 µg Cry1Ac/g of diet and the NOAEC is ≥ 60 µg Cry1Ac/g of diet. **Classification: Acceptable**	47841715
Non-target insect testing, parasitic wasp, *Pediobius foveolatus*	885.4340	Microbially-produced Cry1Ac	No adverse effects on survival were observed in adult *Pediobius foveolatus* fed 250 µg Cry1Ac protein/g of diet in a 21-day laboratory bioassay. The nominal LC_{50} was determined to be >250 µg Cry1Ac/g diet and the NOAEC is ≥250 µg Cry1Ac/g diet. **Classification: Acceptable**	47841716
Honeybee testing, Honeybee larvae, *Apis mellifera*	885.4380	Microbially-produced Cry1Ac	No adverse effects on survival were observed in honey bee larvae treated in natal cells with 410 µg/mL (4.1 µg/cell) solution of Cry1Ac protein. The LC_{50} was determined to be > 410 µg Cry1Ac/mL or >4.1 µg/cell.	47841713

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
			Classification: Acceptable	
Honeybee testing, Honeybee adult, *Apis mellifera*	885.4380	Microbially-produced Cry1Ac	No adverse effects on survival attributable to the test material were observed in honey bee adults fed 175 µg Cry1Ac protein/mL for 10 days. The LC_{50} was determined to be > 175µg Cry1Ac/mL and the NOAEC was determined to be ≥175 µg Cry1Ac/mL. **Classification: Acceptable**	47841714
Earthworm toxicity, *Eisenia foetida*	850.6200	Microbially-produced Cry1Ac	No adverse effect on survival or burrowing time were observed in adult earthworms exposed to 250 mg Cry1Ac protein/kg dry soil for 14 days. The LC_{50} was determined to be >250 mg Cry1Ac protein/kg dry soil and the NOAEC was determined to be ≥250 mg Cry1Ac protein/kg dry soil. **Classification: Acceptable**	47841717
Aerobic soil degradation	885.5200	Lypohilized MON 87701 soybean plants	Based on the measured bioactivity in corn earworm (*Helicoverpa zea*), the DT_{50} for Cry1Ac in three different soils was ranged from 0.4 –13 days. **Classification: Acceptable**	47841718

3. Risk Assessment Conclusions for MON 87701 Soybeans

a. Effects to Nontarget Wildlife, Invertebrates, and Plants

1. Avian Wildlife

The primary routes of exposure of birds to Cry1Ac expressed in MON 87701 soybean plants are expected to occur through consumption of MON 87701 plant material (e.g., seeds) and invertebrates that feed on MON 87701 soybean plant material. Exposure via consumption of soybean seeds may be limited, however, by compounds that interfere with digestion and nutrient uptake, such as trypsin inhibitors. Other routes of exposure (e.g., inhalation) are not expected to be significant, since the Cry1Ac protein is contained primarily within the plant.

The registrant has submitted two toxicity studies for use in determining risks to birds. In a 42-day feeding study, broiler chickens (MRID 47841712) exposed in a diet containing approximately 30% processed soybean meal showed no adverse effects. We note, however, that soybean meal processing involves heat, which could inactivate the Cry1Ac protein. Given that confirmation of the presence of the Cry protein in the diet was not performed, it is unknown whether the test animals were exposed to Cry1Ac, so this study may not be reliable for risk assessment. Confirmation of the presence of the Cry1Ac in the diet would resolve this uncertainty. An 8-day feeding study with Northern bobwhite (MRID 47841711) is available, in which the birds were fed a diet of 20% raw ground soybeans. As mentioned above, consumption of raw soybeans by birds can have detrimental effects; however, the inclusion of raw soybean in the diet at this level without significant effects was supported by an additional non-guideline study (MRID 47896601). This study shows that effects are not expected when birds consume a

diet containing 20% MON 87701 soybeans. Additionally, no adverse effects were observed in a study of birds exposed to Cry1Ac expressed in Monsanto's Bollgard cotton (USEPA 2001b) tested an exposure of 10% cottonseed in the diet. The level of exposure to the birds in these studies is consistent with the Agency's previous assumptions of EECs of PIPs for birds (e.g., USEPA 2001b, USEPA 2005a, USEPA 2005b, USEPA 2008), and based on those assumptions adverse effects to birds are not anticipated. A refined estimate of exposure to birds in the field for soybeans has not been determined (e.g., based on their potential avoidance of soybean seeds and/or consumption of other foods, such as invertebrates). Therefore, a feeding test at a higher concentration of Cry1Ac or a limit dose test at the MHD would increase the certainty of risk conclusions.

Cry1Ac has been registered as a PIP in several crops with no identified concerns for birds, and it has a history of use with no reported incidents involving birds in the field, including use in a crop that is typically ingested by birds (corn). Therefore, based on this history, current toxicity data, and the BPPD's assumptions of avian exposure, adverse effects to birds as a result of the registration of MON 87701 soybeans are not expected

2. Wild Mammals

Wild mammals could be exposed to Cry1Ac produced by MON 87701 soybean plans by consumption of plant material and invertebrates that feed on MON 87701 soybean plant material. As with birds, these are expected to be the primary sources for exposure.

Data are available with which to determine the risk of Cry1Ac from MON 87701 soybeans to wild mammals. The results from the acute oral toxicity studies showed no significant toxicity to mice from acute oral testing with the Cry1Ac protein, with a NOAEL that exceeds 1290 mg/kg body weight for females and 1460 mg/kg for males (MRID 47841707). These data are sufficiently representative of toxicity that would be expected in wild mammals, and based on the results of this study risk to wild mammals resulting from exposure to Cry1Ac expressed in MON 87701 soybean is not expected.

3. Freshwater Animals

The Agency has previously determined that the exposure of aquatic systems to registered Cry proteins produced in their respective crops is minimal. Off-field pollen movement is a possible mechanism by which Cry proteins may be transported to aquatic habitats. But, given that soybeans are primarily self-pollinated and cultivated soybean plants release pollen prior to flower opening (Abud et al. 2007, Caviness 1966), off-site movement of pollen in this case is not expected. The Agency has concluded that plant litter from both *Bt* corn and *Bt* cotton crops are not deposited in amounts high enough to result in adverse effects (USEPA 2001b). Moreover, given that soybean produces less above-ground residue under cultivation (Green and Blackmer 1995), this conclusion is also made for MON 87701 soybeans. Monsanto submitted data waiver rationales that are consistent with the Agency's position on aquatic exposure with *Bt* PIPs.

Based on the expected lack of exposure, risk to freshwater fish as a result of the registration of Cry1Ac in MON 87701 soybeans is not expected. While exposure is expected to be very low in aquatic habitats, and effects on freshwater invertebrates is not expected, a published laboratory study with lepidopteran-active Cry proteins has revealed that the leaf shredding (caddis fly) trichopteran, *Lepidostoma liba*, had 50% lower growth rate when fed *Bt* corn litter (Rosi-Marshall, et al. 2007). Two previous field study reports by the same authors did not find adverse effects on head stream invertebrates. Because of concerns raised in this study, EPA has been requiring registrants of *Bt* PIPs to submit a 7-10 day freshwater invertebrate toxicity study for each PIP to reduce uncertainty regarding this issue.

4. Estuarine and marine animals

BPPD typically waives the requirement of studies with estuarine/marine animals for *Bt* Cry proteins because of an expected lack of exposure in these environments. Therefore, data were not required for Cry1Ac for the registration of MON 87701 soybeans, and based on expected lack of exposure, adverse effects to these species are not anticipated.

5. Terrestrial and aquatic plant species

BPPD typically waives nontarget plant testing for *Bt* Cry proteins, since the active ingredient is an insect toxin (*Bt* δ-endotoxin) that has never shown any toxicity to plants. Therefore, BPPD has concluded that adverse effects of Cry1Ac expressed in MON 87701 soybeans to terrestrial and aquatic plants are not anticipated.

6. Invertebrate species

BPPD assumes that nontarget insects receive exposure primarily through consumption of MON 87701 soybean pollen and/or nectar, though they may also consume pest insects that feed on soybean plant tissue or occasionally consume other soybean plant tissues themselves. The principal route of exposure to soil-dwelling invertebrates, such as collembola, earthworms, and rove beetles, is assumed to be consumption of decomposing plant tissue, and also possibly plant exudates, in soil during feeding.

Monsanto submitted studies with which to determine the effects of Cry1Ac expressed in MON 87701 to nontarget insects and soil invertebrates. Studies were submitted with a lady bird beetle and a parasitic wasp species, each of which showed that adverse effects did not occur to the test insects at >60 µg/g diet and >250 µg/g diet, respectively. Assuming a maximum expected environmental concentration of 3.1 µg/g diet (see MRID 47841701) based on the estimated expression level in pollen, adverse effects would not be expected at concentrations up to 19X and 80X this assumed maximum EEC for lady bird beetle and parasitic wasp, respectively. Additionally, a study was submitted with earthworms that showed no effects on mortality or burrowing time resulting from exposure to Cry1Ac present in artificial soil at approximately 18X

maximum EEC. The maximum EEC of the soil was determined based on incorporation of entire soybean plants into the top six inches of soil, and is described in MRID 47841717.

Two studies that tested the effect of Cry1Ac expressed in MON 87701 soybean in honey bee were also submitted. One study tested the effects in honey bee larvae at 410 µg Cry1Ac/mL test solution or 4.1 µg Cry1Ac/cell (larva). This study tested effects at 132X EEC based on the concentration of the test solution. No adverse effects were observed in the larvae in this study, and the number emerging to adults in the group exposed to Cry1Ac was not significantly different from the controls. The other bee study tested the effects of oral exposure to Cry1Ac in adult bees. In this study, adult bees were fed a solution containing 175 µg Cry1Ac/mL test solution (56X EEC). The test solution contained a buffer, which was shown to cause a decrease in survival. A decrease in survival was also observed in the group exposed to Cry1Ac, but these effects were determined to be statistically similar to those in the buffer control group. Therefore, the effects observed on adult honey bees in the Cry1Ac group were determined to be due to the buffer and not the Cry1Ac.

The Agency has additional information for nontarget insects on Cry1Ac submitted to support the registration of Bollgard cotton. The Cry1Ac protein produced in MON 87701 soybeans is identical to the Cry1Ac produced in Bollgard cotton, with the exception of four additional N-terminal amino acids derived from a chloroplast transit peptide targeting sequence that is not expected to impact activity or toxicity because of excision of the N-terminus during prototoxin activation (MRID 47841701). Studies with the Cry1Ac protein produced in Bollgard additionally showed no adverse effects in two Collembola spp. (*Folsomia candida* and *Xenylla grisea*) and minute pirate bug (*Orius albidipennis*) (USEPA 2001b).

Based on the studies submitted for MON 87701, as well as other information for Cry1Ac, risk to nontarget insects, soil invertebrates, and honey bees resulting from registration of Cry1Ac in MON 87701 soybean is not expected.

b. Fate of Cry1Ac in Soil

A study was submitted to show that Cry1Ac expressed in MON 87701 soybean plants degrades quickly and does not accumulate in soil (MRID 47841718). Lyophilized MON 87701 soybean plants (leaves + stems + roots), containing 63 µg of Cry1Ac/g of soybean biomass were incorporated into soils collected in three locations with soybean-growing regions. Soils were incubated for 181 days, and samples collected weekly were subjected to susceptible insect (corn earworm [*Helicoverpa zea*]) bioassays and ELISA to determine Cry1Ac content and bioactivity. Both the ELISA and the bioassays showed that Cry1Ac degraded in all three soils, with the most rapid degradation occurring during the first 16 days. At test end, 72% to 96% of the Cry1Ac had degraded regardless of soil texture, pH, clay content, or the method used to analyze the soil (ELISA or bioassay), and the DT_{50} of Cry1Ac in the soils was ranged from 0.4 – 13 days. These results indicate that Cry1Ac from MON 87701 breaks down rapidly in soil and is unlikely to persist or accumulate in the environment after continuous cultivation. Because currently

registered PIPs have not been shown to persist in soil, the Agency has determined that no additional long-term field studies are required for MON 87701 soybeans.

c. 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). 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, the soil degradation study discussed above shows that the Cry1Ac protein from MON 87701 plants degrades quickly in soil.

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

d. Horizontal Transfer of Transgenes from *Bt* Crops to Soil Organisms

The EPA has evaluated the potential for horizontal gene transfer (HGT) from *Bt* crops to soil organisms and has considered possible risk implications if such a transfer were to occur. Genes that have been engineered into *Bt* crops are mostly found in, or have their origin in, soil-inhabiting bacteria. Soil is also the habitat of anthrax, tetanus and botulinum toxin-producing bacteria. Transfer of these genes and/or toxins to other microorganisms or plants has not been detected. Furthermore, several published experiments, 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 and the fact that the *Bt* toxins engineered into MON 87701 were derived from soil-inhabiting bacteria, the EPA has concluded that HGT of transgenes found in Cry1Ac producing soybean is not expected.

e. Gene Flow and Weediness Potential

Movement of transgenes from crop plants into weeds is a significant concern, due to uncertainty regarding the effect that a new pest resistance gene may have on plant populations in the wild. Under FIFRA, the Agency has reviewed the potential for gene capture and expression of Cry proteins in commercial *Bt* soybeans by wild or weedy relatives of cotton in the United States, its possessions or territories.

An Experimental Use Permit (EUP) registration was granted for MON 87701 soybeans in 2007. At that time, BPPD examined the potential for gene flow and the weediness potential of MON 87701 soybean. Since no known wild or weedy relatives exist in the U.S. with which *Glycine max* (L.) Merr. can form viable hybrids in nature, and because soybean is not weedy in character or invasive, BPPD determined that there is no significant risk of gene capture and expression of any *Bt* endotoxin by wild or weedy relatives of soybean in the U.S., its possessions or territories (BPPD 2007).

f. Impacts on Endangered Species

Because of the selectivity of Cry1Ac for lepidopteran species and lack of evidence of effects on other nontarget species, the Agency has investigated concerns for federally-listed threatened and endangered insect species in the order Lepidoptera. Because soybean pollen is not expected to move beyond the planted soybean field and its immediate margins, as discussed above, any exposure to lepidopterans would be expected to occur within those areas.

Exposure could occur via direct consumption of MON 87701 soybean plants or consumption of MON 87701 pollen that falls on non-soybean plants within the soybean field and its immediate margins. However, exposure to significant amounts of Cry1Ac via pollen consumption is not likely. Little pollen is expected to be released from soybean flowers, since soybean plants are primarily self-pollinated and anthers usually dehisce and release pollen before flowers open. Airborne pollen concentrations have been measured at very low levels within soybean fields (mean of 0.18 grains/cm^2/day) (Yoshimura et al. 2006). To examine the potential exposure from pollen falling on food plants, BPPD performed a similar analysis as that used in Hellmich et al. (2001), which was also employed in the Bt crops reassessment BRAD (USEPA 2001b) and the assessment of nontarget lepidopteran risk for WideStrike cotton (USEPA 2005a). Based on the most sensitive EC_{50} value reported for CEW (used in confirmatory tests) in nontarget organism studies submitted for MON 87701 soybean (0.0022 µg Cry1Ac/mL diet), an expression level of 3.1 µg Cry1Ac/g for pollen, and assumptions of leaf consumption and pollen weight as given in Hellmich et al. (2001), BPPD determined that an estimated number of pollen grains resulting in the EC_{50} is 3.51 grains/day. Based on the assumed daily food consumption, 18.49 grains pollen/cm^2/day must be present on potential food plants to result in an amount equal to the EC_{50} for the sensitive species. This amount is 100X the mean levels of pollen measured in soybean fields. The Hellmich et al. analysis was performed for corn. Corn pollen is recognized as being relatively heavy; however, since corn is wind pollinated, soybean pollen is likely to be heavier. However, even if a soybean pollen grain weighs 5x that of corn pollen, the estimated number of pollen grains resulting in the EC_{50} would still be 20x the amount of pollen measured in soybean fields. These are expected to be conservative estimates, since higher pollen weights for corn are reported (e.g., 2.0 million grains/gram [Mascarenhas et al. 1984], 3.5 million grains/gram [Rosi-Marshall et al. 2007]). Additionally, based on the mean amount of pollen measured in soybean fields, and assuming a 14 day flowering period for a soybean field, 2.52 grains/cm^2 would be expected on potential food plants at the end of the flowering period if they are entirely

undisturbed and no other organisms consume the pollen, and the amount needed to result in the equivalent EC_{50} would not be attained. Based on this analysis, BPPD concludes that exposure resulting from pollen falling on potential non-soybean food plants in the field and immediate margins is not sufficient to cause effects in listed lepidopterans. Therefore, any significant exposure would have to occur through consumption of the MON 87701 soybean plants on the field.

A search of EPA's LOCATES database indicates that three species of listed lepidopterans are present in U.S. counties in which soybeans are grown. These are the Karner blue butterfly (*Lycaeides melissa samuelis*), St. Francis's Satyr Butterfly (*Neonympha mitchellii fransisci*), and Mitchell's Satyr Butterfly (*Neonympha mitchellii mitchellii*).

The potential effects of *Bt* PIPs in corn on the Karner blue butterfly was extensively analyzed in BPPD's *Bt* Crops Reassessment (USEPA 2001b). This species requires a specific wild lupine species (*Lupinis perrenis*) as a site of oviposition and larval food source, and the concern for exposure in the case of *Bt* corn resulted from the possibility of pollen falling on this required plant species. As shown in the analysis above, soybean pollen is not expected to be deposited on plants in or around soybean fields in amounts sufficient to cause effects in sensitive lepidopterans. Additionally, EPA has previously reviewed information on the proximity of Karner blue habitats, and none are known to exist immediately adjacent to agricultural fields ("adjacent to" was defined as 0-3 m for corn fields, which is reasonably applied to soybean fields) (USEPA 2001b). Therefore, effects to the Karner blue butterfly via consumption of pollen on its food/host plant are not expected. Additionally, because the Karner blue butterfly has an obligate relationship with a specific host plant, it will not be exposed to Cry1Ac via direct consumption of MON 87701 soybeans. Therefore, effects to this species as a result of the proposed registration of MON 87701 soybeans are not expected.

Mitchell's satyr butterfly has a strict reliance on fen habitats, which are characterized as stable, highly alkaline, and nutrient poor wetland habitats. Fen habitats can occur near agricultural areas; however this species is considered to be a strict habitat specialist (USFWS 1998, USFWS 2009). Szymanski et al. (2004) describes habitat for this species as "mosaics of open shrubby and forested communities and associated ecotones." They also note that all active habitats for this species are sedge-dominated, and contain scattered deciduous or coniferous shrubs, and observed that individuals stay within their habitat patch. Edge habitat and scattered shrubs appear to be crucial habitat components for these species, and vegetation structure is as important as the presence of suitable host plants (Lee 2000, Shuey 1997). The species has a strong preference for habitats with dense stands of sedges (USFWS 1998), and meadows and fens without shrubs are considered to be unsuitable habitat (Shuey 1997). In a study by Barton and Bach (2005) captures of adults at their study sites showed clear preference for edge and scattered shrub cover and avoidance by this species of open areas and habitats other than suitable fen habitats, including agricultural fields. Host plants have been determined to be almost certainly sedges, and *Carex stricta* is considered likely to be their primary host plant (USFWS 1998, USFWS 2009), although they have been observed ovipositing on other small (<5 cm)

herbaceous plants (Lee 2000, Szymanski et al. 2004), reported to be located under dense stands of sedge (Szymanski et al. 2004). Sources cited within Barton and Bach (2005) observed ovipositioning within 1 m of the nearest tree or shrub. The species is also considered to be sedentary and a weak flyer (USFWS 1998, USFWS 2009, Szymanski et al. 2004, Lee 2000), although some populations may be less sedentary than others (Barton and Bach 2005, USFWS 1998). Corridors containing suitable habitat are considered to be critical for dispersal and conservation (Barton and Bach 2005, Szymanski et al. 2004), so an open, agricultural field is not likely to be used for this purpose. Adults are generally not observed feeding, and the butterflies do not live for more than a few weeks in this life stage. U.S. Fish and Wildlife Service (FWS) documents describing habitat for this species do not associate it with agricultural fields (USFWS 1998, USFWS 2009), though the extent to which such habitats have been surveyed for this species is not reported. Mitchell's satyr butterfly is recognized as a strict specialist of fen habitats that cannot be provided by soybean cultivation. Based on the life history described in the documents and studies examined, BPPD concludes that the occurrence of Mitchell's satyr in MON 87701 soybean fields is extremely unlikely and its use of MON 87701 soybean plants as ovipositioning sites or larval food plants will not occur. Therefore, effects to this species as a result of the registration of MON 87701 soybeans are not anticipated.

The St. Francis's satyr butterfly is closely related to the Mitchell's satyr, but occupies bog habitats that tend to be more transitory compared to fens. This species is also considered to be a strict habitat specialist, and is most closely associated with *Carex* spp. The St. Francis's satyr has habits similar to those described for Mitchell's satyr above, in that it relies heavily on early successional habitats dominated by sedges, is sedentary, and is a weak flyer. All occupied habitats have been created by some kind of disturbance (e.g., beaver activity, fire). Dispersal has been recorded rarely over unsuitable habitats. Dispersing individuals show a strong tendency to fly along streams, remaining within riparian vegetation, and have been observed almost always turning back to the colony once the edge of the wetland is reached. Movements >1 km are probably rare (NatureServe 2009). The St. Francis's satyr butterfly exists in limited number of small populations (one metapopulation). It is currently known to occupy only a small area (10-20 ha) within Department of Defense (DOD) lands in the sandhills region of North Carolina (Bartel and Sexton 2009, Haddad et al. 2008, Kuefler et al. 2008, USFWS 1996), which substantially restricts its potential for exposure. Since this species is recognized as a strict habitat specialist, and because it has not been detected outside of DOD lands, despite surveys of surrounding areas, cultivation of MON 87701 soybean plants will not result in effects to the St. Francis's satyr.

Based on the above analysis, the Agency determines that there will be no direct effect to listed lepidopteran species as a result of the cultivation of MON 87701 soybeans as proposed. Obligate relationships between insectivorous listed species with lepidopterans that are expected to be found in soybean fields, especially pest species that feed on MON 87701 plants, are not currently known. Since the Cry1Ac in MON 87701 soybeans targets only lepidopteran insects, loss of the pest insects as a result of MON 87701 are expected to be offset by the presence of other insects that could act as food sources for listed species, including beneficial insects that are known not to

be affected by Cry1Ac. Effects on species other than insects have also been determined to be very unlikely because of the specificity of Cry1Ac. Therefore, no effects to listed species due to indirect effects or effects on habitat are anticipated to occur.

4. Data Needed to Confirm MON 87701 Soybean Non-Target Hazard Assessment

BPPD has sufficient information to believe that there is no risk from the proposed uses of MON 87701 soybean to non-target wildlife and terrestrial invertebrates, fish and aquatic invertebrates, plants and soil organisms. As indicated above, the Agency has been alerted to possible concerns of effects of *Bt* PIPs on freshwater aquatic invertebrates. As a result, the Agency is requiring registrants of *Bt* PIPs to submit a 7 -10 day freshwater invertebrate toxicity study to reduce uncertainty with this issue. Consistent with this determination, this study must be submitted to support a full commercial registration of Cry1Ac in MON 87701 soybeans.

CONCLUSION

The environmental risk assessment indicates that, based on information submitted to support this proposed registration, as well as previous assessments for Cry1Ac, cultivation of MON 87701 soybeans as intended will not result in unreasonable adverse environmental effects. The Agency additionally concludes that MON 87701 soybeans will have no direct or indirect effect on endangered and/or threatened species listed by the FWS and the National Marine Fisheries Service or their habitats.

References

Abud, S., P.I.M. de Souza, G.R. Vianna, E. Leonardecz, C.T. Moreira, F.G. Faleiro, J.N. Junior, P.M.F.O Monteiro, E.L. Rech, and F.J.L. Aragao. 2007. Gene flow from transgenic to nontransgenic soybean plants in the Cerrado region of Brazil. Genetics and Molecular Research 6:445-452.

Bartel, R.A. and J.O. Sexton. 2009. Monitoring habitat dynamics for rare and endangered species using satellite images and niche-based models. Ecography 32:888-896.

Barton, B.J. and C.E. Bach. 2005. Habitat use by the federally endangered Mitchell's satyr butterfly (*Neonympha mitchellii mitchellii*) in a Michigan prairie fen. American Midland Naturalist 153:41-51.

BPPD. 2007. Ecological risk assessment for Monsanto's MON 87701 *Bt* soybean EUP. Memorandum from T. Milofsky to M. Hunter.

Caviness, C.E. 1966. Estimates of natural crosspollination in Jackson soybeans in Arkansas. Crop Science 6:211-212.

Green. C.J. and A.M. Blackmer. 1995. Residue decomposition effects on nitrogen availability to corn following corn or soybean. Soil Science Society of America Journal 59:1065-1070.

Haddad, N.M., B. Hudgens, C. Damiani, K. Gross, D. Kuefler, and K. Pollock. 2008. Determining optimal population monitoring for rare butterflies. Conservation Biology 22:929-940.

Hellmich, R.L., B.D. Siegfried, M.K. Sears, D.E. Stanley-Horn, M.J. Daniels, H.R. Mattila, T. Spencer, K.G. Bidne, and L.C. Lewis. 2001. Monarch larvae sensitivity to *Bacillus thuringiensis*-purified proteins and pollen. Proceedings of the National Academy of Sciences 98:11925-11930.

Kuefler, D., N.M. Haddad, S. Hall, B. Hudgens, B. Bartell, E. Hoffman. 2008. Distribution, population structure, and habitat use of the endangered Saint Francis satyr butterfly, *Neonympha mitchellii fransisci*. American Midland Naturalist 159:298-320.

Lee, Y. 2000. Special animal abstract for *Neonympha mitchellii mitchellii* (Mitchell's satyr butterfly). Michigan Natural Features Inventory, Lansing, MI. 4pp.

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

Mascarenhas, N.T., D. Bashe, A. Eisenberg, R.P. Willing, C.M. Xiao, and J.P. Mascarenhas. 1984. Messenger RNAs in corn pollen and protein synthesis during germination and pollen tube growth. Theoretical and Applied Genetics 68:323-326.

National Academy of Science (NAS). 2000. *Environmental Effects of Transgenic Plants: The Scope and Adequacy of Regulation* is available from the National Academy Press, 2101 Constitution Avenue, N.W., Lockbox 285, Washington, DC 20055; (800) 624-6242 or (202) 334-3313 (in the Washington metropolitan area); http://www.nap.edu.

NatureServe. 2009. NatureServe Explorer: An online encyclopedia of life [web application]. Version 7.1. NatureServe, Arlington, Virginia. Available http://www.natureserve.org/explorer. (Accessed: July 15, 2010).

Romeis, J., Meissle, M. and Bigler, F. 2006. Transgenic Crops expressing *Bacillus thuringiensis* toxins and biological control. *Nature Biotechnology* 24: 63-71.

Rosi-Marshall E. J., J. L. Tank, T. V. Royer, M. R. Whiles, M. Evans-White, C. Chambers, N. A. Griffiths, J. Pokelsek, and M. L. Stephen. 2007. Toxins in transgenic crop byproducts

may affect headwater stream ecosystems. Proceedings of the National Academy of Sciences 104:16204–16208.

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

Shuey, J.A. 1997. Conservation status and natural history of Mitchell's Satyr, *Neonympha mitchellii mitchellii* French (Insecta: Lepidoptera: Nymphalidae). Natural Areas Journal 17:153-163. (abstract)

Szymanski, J., J.A. Shuey, and K. Oberhauser. 2004. Poulation structure of the endangered Mitchell's Satyr, *Neonympha mitchellii mitchellii* (French): implications for conservation. American Midland Naturalist 152:304-322.

United States Environmental Protection Agency (USEPA) (1998). "Guidelines for Ecological Risk Assessment." EPA 630/R-95-002F. Washington, DC, USA. [Federal Register, May 14, 1998. 63(93): 26846-26924.]

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

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

USEPA. 2001b. Biopesticides Registration Action Document: *Bacillus thuringiensis* (*Bt*) Incorporated Plant Protectants. Washington, DC. http://www.epa.gov/opp00001/biopesticides/pips/bt_brad.htm

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

USEPA. 2004. SAP Report No.2004-05. Product characterization, human health risk, ecological risk, and insect resistance management for *Bt* cotton products. Dated August 19, 2004. http://www.epa.gov/scipoly/sap/meetings/2004/june/final1a.pdf

USEPA. 2005a. Biopesticides Registration Action Document: *Bacillus thuringiensis* var.

aizawai Cry1F and the Genetic Material (From the Insert of Plasmid PGMA281) Necessary for its Production in Cotton and *Bacillus thuringiensis* var. *kurstaki* Cry1Ac and the Genetic Material (From the Insert of Plasmid PMYC3006) Necessary for its Production in Cotton. Washington, DC. http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006512-006513.pdf

USEPA. 2005b. Biopesticides Registration Action Document: *Bacillus thuringiensis* Cry2Ab2 Protein and its Genetic Material Necessary for its Production in Cotton. Washington, DC. http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006487.pdf

USEPA. 2008. Biopesticides Registration Action Document: *Bacillus thuringiensis* Cry1A.105 and Cry1Ab2 Insecticidal Proteins and the Genetic Material Necessary for Their Production in Corn. Washington, DC. http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006514-006515.pdf

U.S. Fish and Wildlife Service (USFWS). 1996. Saint Francis' Satyr Recovery Plan. Atlanta, GA. http://ecos.fws.gov/docs/recovery_plan/960423.pdf

USFWS. 1997. Recovery Plan for Mitchell's Satyr Butterfly (*Neonympha mitchellii mitchellii* French), Ft. Snelling, MN.

USFWS. 2009. Mitchell's Satyr Butterfly Spotlight Species Action Plan. Ft. Snelling, MN. http://ecos.fws.gov/docs/action_plans/doc3038.pdf

Yoshimura, Y., K. Matsuo, and K. Yasuda. 2006. Gene flow from GM glyphosate-tolerant to conventional soybeans under field conditions in Japan. Environmental Biosafety Research 5:169-173.

D. INSECT RESISTANCE MANAGEMENT (IRM)

1. Overall Conclusions

a) Given the low acreage projected by Monsanto for MON 87701 (<15,000 total acres per year), it is unlikely that there will be a significant risk of resistance to the main soybean target pests in the United States, soybean looper and velvetbean caterpillar. This conclusion is further supported by the biology of the target insects, which do not overwinter in non-tropical areas and are polyphagous (feeding on a number of wild hosts and cultivated crops).

b) The use of MON 87701 on a limited basis should not impact the natural refuge strategy in place for Bt cotton PIPs. The total proposed acreage for MON 87701 would represent a small

fraction of total soybean acres in each state and should not significantly reduce the amount of natural refuge. Further, MON 87701 will not be planted in states (i.e., Mississippi Delta states) with lower natural refuge.

c) BPPD is requiring adoption of a resistance monitoring plan, remedial action strategy, and annual sales reporting for MON 87701.

2. Insect Resistance Management Assessment

Based on the materials submitted to support registration, BPPD does not have sufficient information to completely assess the risk of resistance to the primary target pests for a full commercial registration of MON 87701 soybean. However, Monsanto's proposal to limit the overall acreage and distribution of MON 87701 in the U.S. should be sufficient to mitigate potential resistance development.

a. Target Pests

Soybean looper (SL, *Pseudoplusia includens*) and velvetbean caterpillar (VBC, *Anticarsia gemmatalis*) are the primary target pests of MON 87701 in the U.S., but only cursory information on pest biology was provided in the submission. These insects have not been targeted by other registered Bt crops (e.g. Bt cotton or Bt corn) and have not been assessed as part of any other IRM plan. The other two insects targeted by MON 87701, soybean axil borer (*Epinotia apoerema*), and sunflower looper (*Rachiplusia nu*), have South American distributions and do not occur in the U.S.

Soybean looper occurs throughout the U.S. where soybeans are grown, but overwinters only in tropical regions migrating annually from Mexico, Central America, and the Caribbean (Pedigo, 1999). The insect is multivoltine with two (northern regions) to seven (southern states) generations per season. In addition to soybean, SL is known to feed on a wide range of vegetables, field crops (including corn and cotton), and weeds (Capinera, 2005).

Velvetbean caterpillar, similar to SL, is a tropical species that migrates annually northward into the U.S. It can range as far north as New England, but overwinters only in the southern end of the Florida peninsula (Pedigo, 1999; Barbara, 2008). VBC is also polyphagous and feeds on a number of field crops (soybean, alfalfa, cotton, peanut, other legumes) and weeds (Barbara, 2008).

b. IRM Risk Considerations

To assess the risk of resistance for new PIPs, BPPD typically considers (in addition to pest biology) information on dose expression and simulation modeling. Regarding the dose of MON 87701, Monsanto referenced a published article (McRae et al., 2005) that concluded the tested soybean cultivar (TIC107 with a *cry1A* gene) exhibited high dose control against the target pests.

A copy of this article was not provided in the submission, though BPPD was able to obtain a reprint. This research showed that the tested soybean line appears to meet the criteria for high dose using two of the techniques established by the 1998 SAP, including serial dilutions with lyophilized plant tissue (technique #1) and field trials to demonstrate high efficacy against potential heterozygotes (technique #3). But, BPPD cannot verify the high dose claims from the article because it is unclear whether the tested variant (TIC107) and toxin (a Cry1A toxin described as being "similar" to Cry1Ac) are equivalent to MON 87701.

Simulation modeling was not conducted for MON 87701 and a refuge-based resistance management strategy was not proposed by Monsanto. Rather, the company has indicated that a number of factors should reduce the risk of resistance so that a formalized refuge strategy is unnecessary. These factors include the distribution of the target pests (SL and VBC), the abundance of natural refuge for these insects, and anticipated limited plantings of MON 87701 for seed increase purposes.

BPPD believes that additional information on pest biology, dose, simulation modeling, and cross resistance would be necessary to assess the risks for an unlimited commercial registration of MON 87701. But, for a limited "seed increase" registration, BPPD agrees with Monsanto's rationale regarding the low risk of resistance for the target pests.

Biological attributes of SL and VBC reduce the overall likelihood of resistance development. Both insects are polyphagous and feed on numerous other wild and cultivated hosts, which should provide a source of natural refuge to reduce selection pressure for Cry1Ac resistance. Monsanto's submission also indicated that soybeans have relatively small acreage (ca. 13% of U.S. soybean acres) in the southeast, where these two insects are most prevalent. Further, SL and VBC overwinter only in tropical areas (e.g., Florida in the U. S.); populations migrate annually from the Caribbean, Mexico and Central America northward through the U.S. Because of this, it is unlikely that any resistant individuals that might evolve during the growing season north of tropical areas would be able to successfully overwinter and propagate the resistant trait to future generations.

Monsanto suggested in their submission that the proposal for MON 87701 is similar to the requirements in place for "breeding and seed multiplication activities" with other registered PIPs. Seed production is often not compatible with refuges due to the need for purity in seed stock. For IRM purposes, the seed activity is geographically limited to reduce the selection pressure for resistance. To illustrate, an annual per county limit of 20,000 acres for seed production has been imposed for Bt corn PIPs. Also, refuges have not generally been used with PIP Experimental Use Permits because of smaller and dispersed acreages.

Based on the proposed acreage-limited (seed increase) registration and in consideration of the biological aspects of the target pests discussed above, BPPD concludes the risk of resistance to Cry1Ac should be low for SL and VBC. Therefore, a refuge strategy is not recommended at this time for MON 87701. BPPD notes that Monsanto's submission provided an estimate of the

geographic limitations for MON 87701. Projected acreage is not expected to exceed 15,000 (U.S.) total and 1,000 per county. A follow-up submission from Monsanto (April 8, 2010 letter) indicated that a maximum of 15,000 acres would be planted in Atlantic Coast states (Maryland through Georgia). Monsanto's submission also indicated, however, that "MON 87701 plantings....will be initially limited to breeding and seed multiplication activities..." (emphasis added). Should any request be made to expand the scope of MON 87701 to a commercial registration (with large or unlimited acreage), BPPD will require a formal IRM assessment to better evaluate the resistance risk and develop appropriate mitigation measures. Such a review should include appropriate data for dose expression, simulation modeling, and cross resistance.

c. Natural Refuge Considerations

In addition to the primary target pests of soybean, BPPD is also concerned about other insect pests that are targeted by Bt cotton but may also be exposed to Bt soybean. These insects, tobacco budworm (TBW, *Heliothis virescens*) and cotton bollworm (CBW, *Helicoverpa zea*), are similar to SL and VBC in that they are highly polyphagous and are known to exploit numerous crop and wild plant hosts. Cry1Ac is expressed in both MON 87701 and Bt cotton (Bollgard and WideStrike varieties).

The established resistance management plan for Bt cotton involves the use of "natural refuge" in which non-PIP hosts (such as weeds and other non-cotton cultivated crops) provide sources of susceptible insects to dilute any potential resistance genes arising from transgenic cotton. One of the non-cotton crops included in calculations of natural refuge is soybean. Should a significant portion of the soybean crop be devoted to a Bt variety, the potential amount of natural refuge available to TBW and CBW could be reduced.

In response to BPPD's concerns, Monsanto (April 8, 2010 Submission) provided a rationale for low resistance based on geographic limitations for MON 87701. The company intends to plant limited acres (<15,000) in areas with high amounts of natural refuge. While Monsanto did not provide revised natural refuge calculations or modeling to support MON 87701, BPPD agrees that there should be low resistance risk potential for TBW and CBW based on the low acreage projections. The areas intended for plantings of MON 87701 have high proportions (>90%) of natural refuge including numerous wild hosts and cultivated crops (see reviews in BPPD 2006, 2007). As described in Monsanto's submission, 15,000 acres of Bt soybean in these areas would represent only a small fraction of total soybean acres -- to illustrate, if all 15,000 acres were planted in one state and assuming the lowest USDA acreage figure (430,000), Bt soybean would represent 3.5% of total cultivation. Given the small overall amount of MON 87701 projected for planting, it is unlikely that the amount of natural refuge available to TBW and CBW will be significantly reduced. Further, even if natural refuge was lowered due MON 87701 there would still be abundant sources of other plants hosts (e.g., peanuts, tobacco, vegetable crops, and weeds) such that it is unlikely the resistance risk of TBW and CBW to Cry1Ac would appreciably increase.

BPPD's assessment of the impact of MON 87701 on Bt cotton natural refuge is contingent upon the acreage projections provided by Monsanto (as discussed in the previous section). A full commercial registration of MON 87701 without acreage limitations would require a more detailed assessment that likely would include revised natural refuge calculations and simulation modeling.

d. Stewardship Activities

BPPD recommends that other aspects of IRM including resistance monitoring, a remedial action plan, and annual sales reporting be implemented for MON 87701. A full resistance monitoring plan with pest sampling and detection bioassays may not be warranted for a limited registration; rather an approach based on investigations and follow-ups on reports of unexpected pest damage may be appropriate. A remedial action plan, in the event of document resistance, could mimic those in place for Bt corn or cotton or could rely on a "stop sale" approach if resistance develops. An annual sales report detailing total acreage by state is also recommended. Provided MON 87701 is registered without a structured refuge requirement, a compliance assurance plan and grower education program should not be necessary.

References

Barbara, K.A., 2008. University of Florida, Department of Entomology and Nematology informational web page for velvetbean caterpillar (*Anticarsia gemmatalis*): http://entnemdept.ufl.edu/creatures/field/velvetbean.htm. Publication Number: EENY-151.

BPPD, 2006. Review of data and support materials submitted by Monsanto to amend the insect resistance management requirements for Bollgard II Bt cotton. A. Reynolds and S. Matten memorandum to L. Cole, May 16, 2006.

BPPD, 2007. Scientific review of a proposal by Monsanto to replace structured refuge with natural refuge for Bollgard II Bt cotton (resubmitted amendment request to address the concerns of the FIFRA SAP). A. Reynolds and S. Matten memorandum to M. Mendelsohn, May 3, 2007.

Capinera, J.L., 2005. University of Florida, Department of Entomology and Nematology informational web page for soybean looper (*Pseudoplusia includens*): http://entnemdept.ufl.edu/creatures/veg/leaf/cabbage_looper.htm. Publication Number: EENY-116.

MacRae T.C., M.E. Baur, D.J. Boethel, B.J. Fitzpatrick, A.G. Gao, J.C. Gamundi, L.A. Harrison, V.T. Kabuye, R.M. McPherson, J.A. Miklos, M.S. Paradise, A.S. Toedebusch, A. Viegas, 2005. Laboratory and field evaluations of transgenic soybean exhibiting high-dose

expression of a synthetic *Bacillus thuringiensis* cry1A gene for control of Lepidoptera. *J Econ Entomol.* 98 (2): 577-87.

Pedigo, L.P., 1999. Entomology and Pest Management (Third Edition). Prentice Hall: New Jersey. 691 pp.

Scientific Advisory Panel (SAP), 1998. Transmittal of the final report of the FIFRA Scientific Advisory Panel Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management, Meeting held February 9 and 10, 1998. Report dated April 28, 1998. Available at http://www.epa.gov/scipoly/sap/meetings/1998/0298_mtg.htm

III. TERMS AND CONDITIONS OF REGISTRATION

1) The subject registration will automatically expire on midnight September 30, 2013.

2) The subject registration is limited to seed increase and to a total of 15,000 acres per year in the States of Georgia, South Carolina, North Carolina, Virginia, and Maryland with no more than 1,000 acres per county per year.

3) Monsanto must submit IRM monitoring and remedial action plans to EPA for approval by January 31, 2011 and reports on such annually by August 31st.

4) Monsanto must provide EPA annual reports on the acreage and States where MON 87701 has been grown by January 31st.

5) While exposure is expected to be very low in aquatic habitats, and effects on freshwater invertebrates are not expected, Monsanto must do the following to extend the expiration date of this registration. Namely, Monsanto must submit a 7-10 day freshwater invertebrate toxicity study or otherwise adequately address aquatic invertebrate issues raised by Rosi-Marshall, et al. in 2007 regarding the leaf shredding (caddis fly) trichopteran, *Lepidostoma liba.*

IV. REGULATORY POSITION FOR Cry1Ac Soybean

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. Monsanto has submitted or cited data sufficient for EPA to determine that an unconditional time-limited registration of *Bacillus thuringiensis Cry1Ac Protein and the Genetic Material (Vector PV-GMIR9) Necessary for Its Production in MON 87701 (OECD Unique Identifier: MON 877Ø1-2) Soybean* under FIFRA 3(c)(5) will not result in unreasonable adverse effects to the environment. Monsanto submitted and/or cited satisfactory data pertaining to the proposed use. The human

health effects data and nontarget organism effects data are considered sufficient for the period, limited acreage, and geographic limitations of the unconditional registration. These data demonstrate that no foreseeable human health hazards or ecological effects are likely to arise from the use of the product and that the risk of resistance developing to Cry1Ac protein, during the limited registration period is not expected to be significant.

The expiration date of this registration has been set to September 30, 2013. The registration is limited to seed increase and to a total of 15,000 acres per year in the States of Georgia, South Carolina, North Carolina, Virginia, and Maryland with no more than 1,000 acres per county per year.