BIOPESTICIDES REGISTRATION ACTION DOCUMENT

PLANT-INCORPORATED PROTECTANTS:

Bacillus thuringiensis Cry1Ac Protein and the Genetic Material Necessary for its Production [PC Code 006527]
and
Bacillus thuringiensis Cry1F Protein and the Genetic Material Necessary for its Production [PC Code 006528]

as expressed in
Event DAS-81419-2 Soybean (PIP product)
[OECD Unique Identifier: DAS-81419-2]

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

January 9, 2014

Bacillus thuringiensis Cry1Ac and Cry1F as DAS-81419-2 Soybean

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GLOSSARY OF ACRONYMS AND ABBREVIATIONS

APHIS	Animal and Plant Health Inspection Service (USDA)
bp	Base pairs
BPPD	Biopesticides and Pollution Prevention Division
BRAD	Biopesticides Registration Action Document
b.w.	body weight
CAS	Chemical Abstracts Service
40 CFR	Title 40 of the Code of Federal Regulations
cDNA	Copied (or Copy) DNA
cfu	colony forming units
dsRNA	Double-Stranded RNA
cos	Cosmid
^{o}C	Temperature in Centigrade or Celsius Degrees
DER	Data Evaluation Record
DNA	Deoxyribonucleic Acid
EPA	Environmental Protection Agency (the "Agency")
ELISA	Enzyme-Linked Immunosorbent Assay
EPA Reg. No.	EPA Registration Number
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA	Food Quality Protection Act
FR	Federal Register
g	Gram
IU	International Units
IRM	Insect Resistance Management
kg	kilogram
L	Liter
MRID No.	Master Record Identification Number
mg	Milligram
mL	Milliliter
µg	Microgram
MP	Manufacturing-Use Product
mRNA	Messenger RNA
NE	No Effect
ng	nanogram (10^{-9} gram)
NIOSH	National Institute for Occupational Safety and Health
OPP	Office of Pesticide Programs
OCSPP	Office of Chemical Safety and Pollution Prevention
OECD	Organisation for Economic and Community Development
PC	Pesticide Chemical
PCR	Polymerase Chain Reaction
PIP	Plant-Incorporated Protectant
pg	picogram (10^{-12} gram)
ppb	parts per trillion
ppm	parts per million
ppt	parts per trillion
PTGS	Post-Transcriptional Gene Silencing
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
T-DNA	transfer DNA from Agrobacterium
TGAI	Technical Grade of the Active Ingredient
USDA	United States Department of Agriculture
U.S. EPA (EPA)	United States Environmental Protection Agency
U.S. FDA	United States Food and Drug Administration

EXECUTIVE SUMMARY

The United States Environmental Protection Agency (U.S. EPA) proposes to register two new *Bacillus thuringiensis* (*Bt*) soybean plant-incorporated protectant (PIP) active ingredients as described in this Biopesticide Registration Action Document (BRAD).

On July 24, 2012, Dow AgroSciences LLC (Dow) submitted an application for a breeding/seed increase registration of a new transgenic soybean (*Glycine max*) PIP, Event DAS-81419-2 Soybean, EPA File Symbol 68467-EN. The proposed PIP is a new transformation event that expresses lepidopteran-active insecticidal proteins (also known as "Cry" proteins) derived from the soil bacterium *Bacillus thuringiensis* (*Bt*). The proposed new PIP expresses both the Cry1Ac and Cry1F proteins in soybean. Different events of Cry1Ac have been previously registered in corn, cotton, and soybean. Similarly, separate events containing Cry1F have been registered in corn and cotton. However, the expression of Cry1F and Cry1Ac proteins in Event DAS-81419-2 Soybean represents their first use together in soybean. Dow concurrently filed a petition for a permanent exemption from the requirement of a tolerance for Cry1F protein residues in or on soybean food and feed commodities.

On October 1, 2009, the EPA announced a new policy to provide a more meaningful opportunity for the public to participate in major registration decisions before they occur. According to this policy, the EPA intends to provide a public comment period prior to making a registration decision for, at minimum, the following types of applications: new active ingredients; first food uses; first outdoor uses; first residential uses; or any other registration actions for which the Agency believes there may be significant public interest.

Consistent with the policy of making registration actions more transparent, the proposed *Bt* Plant-Incorporated Protectant (PIP) product, DAS-81419-2 Soybean (EPA File Symbol 68467-EN), expressing both the Cry1Ac and Cry1F proteins, is subject to a 15-day comment period as a new active ingredient. The docket identification number associated with these registration actions, and accessed through either *http://www.regulations.gov* or *http://www.epa.gov/pesticides/regulating/registration-status.html*, is EPA-HQ-OPP-2013-0703. The following documents are available for comment in EPA-HQ-OPP-2013-0703:
(1) This draft Biopesticides Registration Action Document (BRAD);
(2) The December 30, 2013, Environmental Risk Assessment for PIP Event DAS-81419-2;
(3) The November 7, 2013, Review of Insect Resistance Management (IRM) Considerations for *Bt* soybean event DAS-84149-2
(4) The November 22, 2013, Review of Human Health and Product Characterization Data for Registration of *B. thuringiensis* Cry1Ac and Cry1F Proteins and the Genetic Material Necessary for their Production in DAS-81419-2 Soybean.
and
(5) a draft label for the PIP (EPA File Symbol 68467-EN)

While a final decision on registration is contingent upon review and consideration of public comments, EPA believes that, based upon the data and information submitted in support of the proposed *Bt* soybean Plant-incorporated Protectant (PIP) pesticide product, DAS-81419-2 Soybean, it is appropriate to issue the registration. The basis for this preliminary decision can be found in the risk assessments for the proposed *Bt* soybean Plant-Incorporated Protectant (PIP) pesticide product, DAS-81419-2 Soybean, which are characterized throughout this BRAD.

This BRAD presents EPA's evaluations of the data and information submitted by Dow for the proposed registration of:

1. EPA File Symbol 68467-EN: Event DAS-81419-2 Soybean expressing *Bt* Cry1Ac and *Bt* Cry1F insecticidal proteins.

The genes that are responsible for the *Bt* Cry1Ac and *Bt* Cry1F proteins, *cry1Ac* and *cry1F*, have been modified for their expression in plants. These proteins are referred to as Cry1Ac and Cry1F, respectively, in this BRAD. The event also includes the *pat* gene that produces Phosphinothricin Acetyltransferase (PAT) enzyme, an inert ingredient in the proposed product that confers tolerance of the soybean plant to the herbicide, glufosinate.

DAS-81419-2 was developed by *Agrobacterium*-mediated transformation of soybean using the 2T-DNA plasmid vector pDAB9582 and produces *Bacillus thuringiensis* protein δ-endotoxins, Cry1Ac and Cry1F. A single T-DNA insert containing each of the intact plant transcription units (PTUs) for the *cry1Fv3, cry1Ac(synpro),* and *pat* genes was integrated into soybean to create DAS-81419-2. This protein is intended to provide protection from feeding damage caused by a number of lepidopteran pests.

Dow applied for pesticide registration of Cry1Ac and Cry1F proteins as co-expressed in Event DAS-81419-2 soybean as a new PIP product, DAS-81419-2 Soybean, for use as a PIP in 2014, under section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Dow concurrently filed a petition for a permanent exemption from the requirement of a tolerance for Cry1F protein residues in or on soybean food and feed commodities. The EPA believes that, based upon its assessment of the data and information submitted by Dow, it is in the interest of the public and the environment to issue the registration proposed by Dow.

I. ACTIVE INGREDIENT OVERVIEW

Active Ingredients and Office of Pesticide Programs (OPP) Chemical Code and File Symbol:	*Bt* Cry1Ac insecticidal protein and the genetic material necessary for its production in DAS-81419-2 Soybean PIP product: 006527 *Bt* Cry1F insecticidal protein and the genetic material necessary for its production in DAS-81419-2 Soybean PIP product: 006528 EPA File Symbol 68467-EN. OECD Unique Identifier: DAS-81419-2
Applicant/ Manufacturer:	Dow AgroSciences LLC (Dow) 9330 Zionsville Road Indianapolis, In 46268
Type of Pesticide:	Plant-incorporated Protectant (PIP)
Use:	Insecticide
Target Pests:	Larval stage lepidopteran pests of soybean, such as soybean looper (*Pseudoplusia includens*), velvetbean caterpillar (*Anticarsia gemmatalis*), beet armyworm (*Spodoptera exigua*), cotton bollworm/corn earworm (*Helicoverpa zea*), green cloverworm (*Plathypena scabra*), and cutworms (*Agrotis* spp.)

Mode of action:

Insecticides formulated with microbial *Bt* have been in use worldwide since 1961. These microbial active ingredients are the source of the genes that express the Cry1Ac and Cry1F proteins in the proposed *Bt* Soybean PIP. There is no mechanistic evidence that *Bt* proteins pose risks for humans or mammals during their use as pesticides or as PIPs (OECD, 2007). When Cry proteins are ingested by an insect, they are dissolved under the alkaline conditions specific to the insect's gut, and then activated upon cleavage by the insect's midgut enzymes (proteases). These activated Cry proteins must bind specifically to glycoprotein or glycolipid receptors on microvillar membrane of the insect's midgut to initiate the pore-forming process that ultimately kills the insect. The fragment of the protein that is toxic to insects then interacts with specific high-affinity receptors on the microvilli of the target insect's midgut epithelium (stomach), in particular brush border membrane vesicles (BBMV) (Ferré and Van Rie, 2002). Such receptors are not present in vertebrate species. Cry proteins must enter the cell membrane and form a pore.

Although receptor binding appears to be essential for the insecticidal activity of the crystal proteins, binding by itself may not lead to toxicity. It appears that the initial protein-receptor interaction or binding is a reversible process, while the irreversible insertion of at least part of the Cry protein (the fragment that is toxic to insects) into the cell membrane is responsible for forming the pore. Consequently, the ion gradient across the membrane is compromised, and the columnar cells of the midgut swell and lyse osmotically. Lysis leads to disruption of the gut epithelium, resulting in starvation, and insect death (Ferré and Van Rie, 2002).

II. REGULATORY BACKGROUND

Applications for use of Cry1Ac and Cry1F expressed in DAS-81419-2 Soybean PIP

A. Cry 1Ac and Cry1AF proteins

1. Registration Application and Permanent Tolerance Exemption

a. Cry1Ac and Cry1F PIP FIFRA Section 3 Registration Application

On November 22, 2013, EPA published a Notice of Receipt in the Federal Register (78 FR 70043), announcing that Dow submitted an application to register a new active ingredient, *Bt* Cry1Ac and Cry1F insecticidal proteins expressed in soybean (DAS-81419-2 Soybean; EPA File Symbol 68467-EN), not included in any currently registered pesticide products. No public comments were received in response to this publication.

b. Cry1Ac protein - Permanent Tolerance Exemption

Residues of Cry1Ac protein are exempt from the requirement of a tolerance when used as PIPs in all food commodities (40 CFR § 174.510). EPA granted an exemption from tolerance for Cry1Ac protein in all food and feed commodities on April 11, 1997 (62 FR 17722). The tolerance exemption was published in the Code of Federal Regulations (40 CFR §180.1155). In October 2001, EPA completed a reassessment of this tolerance exemption considering all of the existing data, public literature, and public comments. The reassessment determined that the tolerance exemption met all the required scientific and regulatory standards. This tolerance exemption for the Cry1Ac protein is not event-specific and applies to all Cry1Ac protein and any other event producing the Cry1Ac protein that might be found in the food supply. On April 25, 2007, the tolerance exemption for Cry1Ac protein was reassigned to 40 CFR § 174.510 (72 FR 20435). Based on this existing tolerance exemption, a petition to establish a tolerance or tolerance exemption was not required for the Cry1Ac protein expressed in this soybean PIP.

c. Cry1F protein - Permanent Tolerance Exemption

Concurrently with the FIFRA section application, Dow submitted a petition (PP2F8066) to establish a permanent exemption from the requirement of a tolerance for residues of *Bacillus thuringiensis* Cry1F protein in or on soybean. EPA published a Notice of Filing of the petition in the Federal Register on November 22, 2013 (78 FR 70007) and the public was given a 30-day comment period. No public comments were received in response to this publication.

Event DAS-81419-2 Soybean also expresses the PAT enzyme that is exempt from the requirement of a tolerance when used as a PIP inert ingredient in all food commodities (40 CFR §174.522).

III. RISK ASSESSMENT SUMMARIES

EPA assessed the data submitted by Dow in 2012 for the proposed PIP soybean registration and is presenting summaries of the product characterization and human health risk assessments in Appendix A (Cry1Ac and Cry1F expressed in soybean DAS-81419-2 Soybean). Summaries of ecological and environmental risk assessments and Insect Resistance Management (IRM) for the PIP product are presented in Appendices B and C, respectively. In its assessment, the EPA relied upon data and other information submitted by the applicant and general knowledge of best available scientific technology.

The classifications that are found for each data submission are assigned by Agency science reviewers and are an indication of the usefulness of the information contained in the documents for risk assessment. A rating of "ACCEPTABLE" indicates the study is scientifically sound and is useful for risk assessment. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. The studies may have certain aspects determined not to be scientifically acceptable ("SUPPLEMENTAL: UPGRADABLE"). If a study is rated as "SUPPLEMENTAL: UPGRADABLE," the Agency always provides an indication of what is lacking or what can be provided to change the rating to "ACCEPTABLE." If there is simply a "SUPPLEMENTAL" rating, the reviewer will often state that the study is not required by the current 40 Code of Federal Regulations (CFR) part 158. Both "ACCEPTABLE" and "SUPPLEMENTAL" studies may be used in the risk assessment process as appropriate. An "UNACCEPTABLE" rating indicates that new data need to be submitted.

For the acute toxicity data requirements, toxicity categories are assigned based on the hazard(s) identified from studies and/or other information submitted to the Agency in support of a pesticide registration. The active ingredient or particular product is classified into Toxicity Category I, II, III, or IV, where Toxicity Category I indicates the highest toxicity and Toxicity Category IV indicates the lowest toxicity.

A. Product Characterization

All product characterization data requirements for the *Bt soybea*n PIP containing the Cry1Ac and Cry1F proteins and their co-expressed traits in DAS-81419-2 Soybean have been *satisfied*. The soybean event developed by Dow to express these PIPs is:

> Event DAS-81419-2 Soybean: co-expresses Cry1Ac and Cry1F proteins, derived from *Bacillus thuringiensis* (*Bt).* It was developed by *Agrobacterium*-mediated transformation of soybean using the 2T-DNA plasmid vector pDAB9582 and produces *Bacillus thuringiensis* protein δ-endotoxins, Cry1Ac and Cry1F. A single T-DNA insert containing each of the intact plant transcription units (PTUs) for the *cry1Fv3, cry1Ac(synpro),* and *pat* genes was integrated into soybean to create DAS-81419-2.

DAS-81419-2 Soybean co-expresses Cry1Ac and Cry1F proteins to provide protection against feeding damage by lepidopteran insect larvae.

For summaries of the data evaluated for product characterization for these PIPs, see Appendix A. The summaries include discussions of the following:

- Product Characterization
- Transformation System and Genetic Elements
- Protein Characterization
- Analytical Detection Methods
- Protein Expression

As discussed in the summaries, Cry1Ac and Cry1F proteins are expressed at very low parts per million (ppm) levels in various parts of the transgenic soybean plant and are shown to be equivalent to the microbially expressed proteins

B. Human Health Assessment

1. Toxicological Profile

EPA reviewed the available scientific data and other relevant information submitted in support of this action to register Cry1Ac and Cry1F as the PIP DAS-81419-2, and considered its validity, completeness and reliability, and the relationship of this information to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children.

All toxicology data requirements for the *Bt* soybean PIP containing Cry1Ac and Cry1F proteins co-expressed in DAS-81419-2 Soybean have been *satisfied***.** Acceptable Tier I mammalian toxicology data/information support the proposed registration of the active ingredients. Furthermore, Tier II and Tier III studies were not required for either Cry1Ac or Cry1F proteins, based on the lack of acute toxicity or pathogenicity in the Tier I studies. Refer to Tables in Appendix A for brief summaries of data evaluated to satisfy toxicity and allergenicity data requirements for these PIPs.

Acute toxicity

Cry1Ac and Cry1F proteins

The following summarizes the final determinations regarding acute toxicology data requirements for Cry1Ac and Cry1F proteins. See Appendix A in this BRAD for more detailed summaries.

Cry1Ac

Previously submitted acute oral toxicity data demonstrated a lack of mammalian toxicity at high levels of exposure (ingestion) to the pure Cry1Ac protein. These data demonstrate the safety of this protein 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 No. 45542313) indicated that Cry1Ac is non-toxic to humans and other mammals. 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 all food and feed commodities precluded the need for a separate tolerance action in conjunction with this review of product characterization and human health assessment.

Cry1F

An acute oral toxicity study in mice (MRID No. 45542314) indicated that Cry1F is non-toxic to humans and other mammals. Cry1F protein is a δ-endotoxin from *B. thuringiensis* that has been used extensively in plant-incorporated protectants as a means of insect pest management. An existing exemption from the requirement of a tolerance for Cry1F (CFR 40 Section 174.504; 72 FR 20434, Apr. 25, 2007) in cotton and corn (CFR 40 Section 174.520; 72 FR 20435 Apr. 25, 2007), precluded the need for a separate tolerance action in conjunction with this review of product characterization and human health assessment. Cry1F was previously granted an exemption from the requirement of a tolerance for expression in cotton (CFR 40 Section 174.504) and maize (CFR Section 174.520); a separate petition for an exemption from the requirement of a tolerance for Cry1F as expressed in soybean (PP2F8066) is included with the submission from Dow for registration of DAS-81419-2 soybean.

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 and Cry1F; even at relatively high dose levels, the Cry1Ac and Cry1F proteins are not considered toxic to humans and other mammals. Further, amino acid sequence comparisons showed no similarities that would raise a safety concern between the Cry1Ac and Cry1F proteins and known toxic proteins in protein databases

Hypersensitivity Incidents: Reporting is required when incidents occur. No hypersensitivity incidents, including immediate-type or delayed-type reactions in humans or animals, occurred during the manufacture of *Bt* Cry1Ac and Cry1F proteins as DAS-81419-2 Soybean PIP. Should any future hypersensitivity incidents occur, they must be reported to the Agency.

Allergenicity Assessment

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. (MRID No. 45542319) The Cry1Ac protein was digested rapidly in simulated gastric fluid containing pepsin. Small peptides remaining following gastric simulated digestion were completely degraded to amino acid residues in SIF upon contact (US EPA 2005, 2010a).
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.

Since Cry1F 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 Cry1F 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 Cry1F with known allergens showed no significant overall sequence similarity or identity at the level of eight contiguous amino acid residues.
3. Digestibility. (MRID No. 45542318) The Cry1F protein was digested rapidly (< 5 min) in simulated gastric fluid containing pepsin. Small peptides remaining following gastric simulated digestion were completely degraded to amino acid residues in SIF upon contact (US EPA 2005, 2010b).
4. Glycosylation. Cry1F expressed in soybean was shown not to be glycosylated.
5. Conclusion: Considering all of the available information, EPA has concluded that the potential for Cry1F to be a food allergen is minimal.

Cry1Ac and Cry1F were shown not to be glycosylated in extracts of DAS-81419-2 soybean; it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it needs to 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).

Considering all of the available information, EPA has concluded that the potential for Cry1Ac and Cry1F to be food allergens is minimal.

Tolerance Exemptions

Permanent tolerance exemptions have been established for residues of Cry1Ac and PAT, as discussed in Chapter II under REGULATORY BACKGROUND, above.

EPA's reviews of the toxicology data support granting a permanent exemption from tolerance for residues of Cry1F protein in/on soybean food/feed commodities under section 408 of the Federal Food, Drug, and Cosmetic Act (FFDCA).
On the basis of evaluations of all relevant data submissions for Cry1F, EPA has determined that the data support amendment of 40 CFR §174 to establish a permanent tolerance exemption for residues of Cry1F in/on soybean food and feed commodities.

2. 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.

Cry1Ac and Cry1F proteins are 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/*.

3. Food Quality Protection Act (FQPA) Considerations

a. Aggregate Exposures

In examining aggregate exposure, section 408 of FFDCA directs EPA to consider available information concerning exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

EPA considered available information on the aggregate exposure levels of consumers (including major identifiable subgroups of consumers) to the PIP residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the PIP residue, and exposure from non-occupational sources.

As previously discussed, data indicated that Cry1F protein is non-toxic to humans and other mammals, and Cry1F protein was shown to be rapidly digested *in vitro.* Exemptions from the requirement of a tolerance already have been established for *Bacillus thuringiensis* Cry1F protein in cotton (40 CFR § 174.504) and corn (40 CFR § 174.520). The EPA has considered dietary exposure under the tolerance exemption and all other exemptions in effect for the PIP residue and exposure from non-occupational sources. When Cry1F protein is used as a PIP in soybean, it is expressed at very low levels in the plant. Humans may be exposed to extremely low levels in the diet. There is also a very remote possibility that Cry1F protein can get in the water supply the same way that other proteins in crop debris can migrate into the ground, and, possibly, drinking water. Because such potential dietary exposure from soybean or drinking water is expected to be several orders of magnitude lower than the amounts of these proteins shown to have no toxicity in mammalian tests, EPA concludes that negligible exposure via food and drinking water would present no harm, based on the lack of mammalian toxicity and allergenicity potential, and the rapid digestibility demonstrated in SGF for the PIP.

Non-occupational dermal and inhalation exposure is not expected, since the PIP is expressed and contained within soybean plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. The use sites of this PIP are all agricultural, for insect control, so there would be no exposure to infants and children from residential, school or lawn use. The amino acid sequence homology of known aeroallergens was included in the amino acid comparison of Cry1F protein with known food allergens, and the results indicated that no respiratory allergenicity would be expected if Cry1F protein were inhaled. The amino acid sequence results are discussed in Section II.B., above. Additionally, soybean flowers in a self-pollinating fashion wherein the anthers generally release pollen within a closed or covered floral tube. Pollen movement from soybean is minimal and highly localized when it happens (i.e., the pollen does not move readily on wind currents).

Taking all these data and information into consideration, EPA concludes that even if negligible aggregate exposure should occur it would present no harm to the U.S. human population due to the lack of mammalian toxicity and the rapid digestibility demonstrated for the Cry1F protein.

b. Cumulative Effects from Substances with a Common Mechanism of Toxicity

Section 408(b)(2)(D)(v) of FFDCA requires that, when considering whether to establish, modify, or revoke a tolerance, the Agency consider "available information" concerning the cumulative effects of a particular pesticide's residues and "other substances that have a common mechanism of toxicity."

Cry1F protein is not considered toxic. It does not share a common mechanism of toxicity with any other substances, nor does it appear to produce a toxic metabolite produced by other substances. For the purposes of this assessment, therefore, EPA has assumed that Cry1F protein does not have a common mechanism of toxicity with other substances. Thus, EPA concludes that there are no cumulative effects associated with Cry1F protein that need be considered. For information regarding EPA's efforts to determine which chemicals have a common mechanism of toxicity and to evaluate the cumulative effects of such chemicals, see EPA's website at *http://www.epa.gov/pesticides/cumulative*

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

To evaluate human risk, EPA considered the validity, completeness, and reliability of the available data from the studies cited in Unit III regarding potential health effects for Cry1F protein. This evaluation included the low levels of expression of Cry1F protein in soybean, as well as the lack of acute oral toxicity at high dose levels, heat stability, and *in vitro* digestibility of this protein. EPA also considered the minimal potential for allergenicity and the non-toxic source of the protein. Because of this lack of demonstrated mammalian toxicity, no protein residue chemistry data for Cry 1F were required for a human health effects assessment.

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

Based on its review and consideration of all the available information, as discussed in this BRAD, EPA concluded that there are no threshold effects of concern and, as a result, that an additional margin of safety for infants and children is unnecessary in this instance.

4. International Residue Limits

In making its tolerance decisions, EPA seeks to harmonize U.S. tolerances with international standards whenever possible, consistent with U.S. food safety standards and agricultural practices. In this context, EPA considers the international maximum residue limits (MRLs) established by the *Codex Alimentarius* Commission (Codex), as required by FFDCA section 408(b)(4). The *Codex Alimentarius* is a joint U.N. Food and Agriculture Organization/World Health Organization food standards program, and it is recognized as an international food safety standards-setting organization in trade agreements to which the United States is a party. EPA may establish a tolerance that is different from a Codex MRL; however, FFDCA section

408(b)(4) requires that EPA explain the reasons for departing from the Codex level. No Codex maximum residue level exists for *Bacillus thuringiensis* Cry1Ac and Cry1F proteins as expressed in Event DAS-81419-2 Soybean.

C. Environmental Effects Assessment

Ecological Effects Data for Cry1F and Cry1Ac Insecticidal Proteins Expressed in Event DAS-81419-2 Soybean

All ecological and environmental effects data requirements for the *Bt* soybean PIPs containing Cry1Ac and Cry1F proteins expressed in DAS-81419-2 Soybean have been *satisfied*. The submission of results of a confirmatory soil degradation study, however, is needed to evaluate the degradation rate of the Cry1Ac and Cry1F proteins sampled specifically from representative soybean soil systems. The completed soil degradation study must be received by the EPA within 18 months of the DAS-81419-2 Soybean Notice of Pesticide Registration date. For a more comprehensive discussion of the Agency's assessment of the data and information submitted concerning ecological and environmental risks for the registration of these PIPs (Cry1Ac, Cry1F, and their combination in DAS-81419-2 Soybean) refer to Appendix B. The tiered approach EPA uses to evaluate ecological and environmental effects, and summaries of the data evaluated, are included.

In the absence of PIP-specific risk assessment guidance, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins. These requirements include testing on birds, mammals, nontarget insects, honey bee, plants, and aquatic species, and information has been submitted to address these requirements. Limit dose testing on representative organisms from several taxa was performed in support of Section 3 FIFRA registration of event DAS-81419-2 expressing *Bt*-derived Cry1F and Cry1Ac insecticidal proteins. As stated above, BPPD's risk assessments focus more greatly on beneficial nontarget invertebrates, since they are most closely related to organisms susceptible to the insecticidal action of *Bt* toxins. The Cry1F and Cry1Ac proteins are meant to target species within the order Lepidoptera (e.g. moths). *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 nontarget insect evaluations should include several invertebrate species that are not related to the target pests. Earthworm studies are also recommended.

The applicant has requested to bridge to the Agency database of previously reviewed toxicity studies of select nontarget organisms (NTOs) that supported the currently registered WideStrike® PIP cotton product (EPA Reg. No. 68467-3). The WideStrike® PIP cotton line was produced by conventional cross breeding of cotton lines DAS-21023-5 (also described as 3006-210-23) expressing Cry1F protein and DAS-24236-5 (also described as 281-24-236) expressing Cry1Ac protein. The environmental risk assessment for the insecticidal proteins (Cry1Ac and Cry1F) expressed in WideStrike® cotton was previously evaluated for adverse impacts on nontarget organisms and have been shown to pose negligible risk (US EPA 2005). Since exposure may also occur to other nontarget organisms, EPA has received a number of

studies and data waiver rationales to comply with the Agency's published data requirements on other nontarget organisms.

The toxicity of Cry1F and Cry1Ac proteins has been previously evaluated on several species of invertebrates including: lady beetle (*Hippodamia convergens*), green lacewing (*Chrysoperla rufilabris*), honey bee (*Apis mellifera*), collembola (*Folsomia candida*), parasitic wasp (*Nasonia vitripennis*) and earthworm (*Eisenia fetida*). Two new dietary toxicity studies were submitted in support for event DAS-81419-2 soybean, which includes Northern Bobwhite (*Colinus viginianus*) and Rainbow Trout, (*Oncorhynchus Mykiss*). The individual results for the cited and newly submitted nontarget organism studies in support for Cry1F and Cry1Ac are summarized in Table 1. The studies are described in more detail below, and full reviews of each study can be found in the individual Data Evaluation Records.

To support data bridging from the previously conducted environmental risk assessment for Cry1F and Cry1Ac insecticidal proteins co-expressed in WideStrike® PIP cotton line, an environmental risk assessment was conducted using a "weight-of-evidence" approach based on the following lines of evidence to support the registration of event DAS-81419-2 soybean:

1) Confirmation of biological equivalency of Cry1F and Cry1Ac proteins expressed in event DAS-81419-2 soybean plant and previously characterized microbial-derived Cry1F and Cry1Ac protein test substances;
2) No significant increase in exposure to NTOs based on calculated margins of exposure for protein concentrations in event DAS-81419-2 plant tissue matrices relative to the dose concentrations used in the NTO laboratory toxicity tests conducted on Cry1Ac and Cry1F; and
3) No adverse effects on nontarget arthropods on a population and community level observed from a field monitoring study and other supporting supplemental information (e.g. meta-analysis, target feeding studies, and biological specificity to the target pest).

Exposure estimates were compared with endpoints reported from Tier I toxicity studies previously conducted on various representative nontarget test species to determine a Margin of Exposure (MOE), the ratio of the endpoint value to the EEC. The MOEs for representative NTOs were recalculated based on high-end exposure estimates (HEEEs) (US EPA, 2009) derived from Cry1Ac and Cry1F protein expression levels quantified from various plant tissues of event DAS-81419-2 soybean in a field study. A MOE of 50% mortality at 10X the estimated environmental exposure is regarded as sufficient to demonstrate negligible risk and was also the threshold for consideration of uncertainties in the risk assessment (US EPA 1998; US EPA 2007). If subsequent expression characterization in the commercial event leads to a safety margin of <10X, then the consequences are considered in terms of uncertainties in the ecological risk assessment below (US EPA 2007).

Endangered Species

Because of the selectivity of Cry1F and Cry1Ac insecticidal crystal proteins 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 PIP event DAS-81419-2 soybean plants or consumption of DAS-81419-2 pollen that falls on non-soybean plants within the soybean field and its immediate margins. However, oral exposure to significant amounts of Cry1F and Cry1Ac via pollen consumption is not likely. Little pollen is expected to be released from soybean flowers, since soybean plants are predominantly 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). 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 DAS-81419-2 soybean plants on the field or via tritrophic effects, in which predatory beneficial arthropod species consume soybean pest species feeding on PIP event DAS-81419-2 soybean plant tissues.

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, 2004b and 2010a and b) and in the endangered species assessment section in the BRAD for event MON87701 soybean expressing Cry1Ac protein (see US EPA 2010c). As previously discussed, soybean pollen is not expected to be deposited on plants in or around soybean fields in amounts sufficient to cause effects in sensitive lepidopterans. Ecology and life history information demonstrated that habitat requirements for larvae and adults of two of the identified species (Mitchell's Satyr butterfly and Saint Francis' Satyr butterfly) do not overlap with commercial soybean acreage, and are therefore not expected to be impacted by PIP event DAS-81419-2 cultivation (Bartel and Sexton 2009; Barton and Bach 2005; US FWS 1997). There is a possible overlap between the geographic range for Karner blue butterfly and soybean use sites. 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) (US EPA 2001b). Karner blue larvae feed only on lupines (*Lupinus* spp.) and soybean is neither identified as a larval food source, nor is soybean open-pollinated. Thus, pollen from event DAS-81419-2 soybean is highly unlikely to be deposited on lupine leaves (NatureServe 2013). Furthermore, soybean is not identified as a nectaring source for Karner blue adults. Therefore, the risk posed to the Karner blue butterfly was considered to be negligible.

Based on the above analysis, EPA determines that there will be no direct effect to listed lepidopteran species as a result of the cultivation of DAS-81419-2 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 DAS-81419-2 plants, are not currently known. Since the Cry1F and Cry1Ac in DAS-81419-2 soybeans targets only lepidopteran insects, loss of the pest insects as a result of DAS-81419-2 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 Cry1F and Cry1Ac. Effects on species other than insects have also been determined to be very unlikely because of the specificity of Cry1F and Cry1Ac.

Lastly, since the Agency previously determined Cry1F and Cry1Ac proteins are not expected to have adverse effects on mammals, birds, plants, freshwater and estuarine/marine fish and invertebrates, nontarget insects and other invertebrate species at the EEC, a "No Effect" determination is made for direct and indirect effects to Federally listed threatened and endangered species and their designated Critical Habitats. In addition, EPA does not expect that any threatened or endangered plant species will be affected by outcrossing to wild relatives or by competition with such entities. Hybrid soybean does not exist in the wild in the United States, nor do any wild plants that can interbreed with soybean in the United States.

D. Insect Resistance Management

The goal of insect resistance management (IRM) is to mitigate the potential risk of resistance in *Bt* crops, including *Bt* soybean. IRM typically involves two phases: 1) data submitted to assess the risk of resistance for a certain *Bt* toxin and crop including information and data on pest biology, dose expression of the toxins in the PIP, simulation modeling, cross resistance potential and baseline susceptibility of the target pests to the toxin(s); and 2) a post-registration stewardship program to implement the resistance mitigation measures which includes resistance monitoring, remedial action (should resistance occur), grower compliance with IRM requirements, and grower education.

Dow submitted IRM considerations with their application for registration of DAS-81419-2 Soybean (MRID No. 48828415 and its replacement, MRID No. 49024110). BPPD concluded that Dow's IRM submissions *satisfied* the IRM requirements for DAS-81419-2 Soybean when used for breeding/seed increase.

BPPD's major IRM conclusions from its assessment of DAS-81419-2 Soybean are detailed below.

Conclusions and Requirements

1) Because DAS-81419-2 Soybean is intended for breeding/seed increase, Dow did not propose a species-specific IRM plan for the product. Rather, the company proposed to mitigate resistance risk by acreage limitations on a county (1,000, 10,000, or 25,000 acres) and national (250,000 acres) scale. BPPD agrees that given the low acreage projected by Dow, it is unlikely that there will be a significant risk of resistance to the main soybean lepidopteran pests in the United States. These insects include soybean looper, velvetbean caterpillar, beet armyworm, cotton bollworm, green cloverworm, tobacco budworm, and cutworms (*Agrotis* spp.). This conclusion is further supported by the biology of the target insects, which are highly polyphagous (feeding on a number of wild hosts and cultivated crops) and (for some species) have limited overwintering in non-tropical areas.

2) Should a full commercial registration or expanded acreage be sought in the future, Dow must submit a complete, species-specific IRM assessment for DAS-81419-2 Soybean. Data needed to support such a review should include additional information on pest biology, dose, simulation modeling, cross resistance, resistance monitoring, and potential impacts on the natural refuge strategy for *Bt* cotton.

3) The use of DAS-81419-2 Soybean on a limited basis for seed increase purposes should not significantly impact the natural refuge strategy in place for Bt cotton PIPs. Soybean is one of the non-cotton crops that have been considered as part of natural refuge for tobacco budworm and cotton bollworm. While it can be expected that plantings of DAS-81419-2 soybean will remove some of the currently-available natural refuge for Bt cotton, this potential reduction will likely be small relative to the total amount of natural refuge present in southern cotton regions. Dow has proposed to further mitigate the impact on natural refuge by limiting DAS-81419-2 to 10,000 acres in counties with > 25,000 total acres of soybean and 1,000 acres in counties with < 25,000 total acres of soybean.

4) As a term of registration, Dow must submit a resistance monitoring and remedial action plan for DAS-81419-2 Soybean. A resistance monitoring plan limited to investigations of unexpected pest damage is appropriate for a capped acreage registration. BPPD notes that monitoring of two potential target insects, tobacco budworm and cotton bollworm, is already being conducted as part of the WideStrike *Bt* cotton monitoring program. Dow must also compile annual sales and acreage data for each state and provide a DAS-81419-2 Soybean-specific report to EPA if requested. Since DAS-81419-2 Soybean will be deployed without a structured refuge requirement, a compliance assurance plan and grower education program is not necessary.

Overall, BPPD concluded that Dow has submitted adequate resistance management data and information to support breeding/seed increase use of the product. There are no data gaps, though Dow must submit additional information, described above, to support any future request for either expanded acreage or commercial registration.

A full discussion of BPPD's IRM review of the supporting data for the Cry1Ac and Cry1F proteins expressed as DAS-81419-2 Soybean PIP, including technical references, is available as Appendix C of this BRAD.

IV. ENVIRONMENTAL JUSTICE

EPA seeks to achieve environmental justice, the fair treatment and meaningful involvement of all people, regardless of race, color, national origin, or income, in the development, implementation, and enforcement of environmental laws, regulations, and policies. To help address potential environmental justice issues, the Agency seeks information on any groups or segments of the population who, as a result of their location, cultural practices, or other factors, may have atypical, unusually high exposure to the *Bt* proteins Cry1Ac and Cry1F as expressed in PIP Event DAS-81419-2 Soybean compared to the general population.

For additional information regarding environmental justice issues, please visit EPA's web site at http://www.epa.gov/compliance/environmentaljustice/index.html.

V. RISK MANAGEMENT AND PROPOSED REGISTRATION DECISION

Section 3(c)(5) of FIFRA provides for the registration of a new active ingredient if it is determined that (A) its composition is such as to warrant the proposed claims for it; (B) its labeling and other materials required to be submitted comply with the requirements of FIFRA; (C) it will perform its intended function without unreasonable adverse effects on the environment; and (D) when used in accordance with widespread and commonly recognized practice, it will not generally cause unreasonable adverse effects on the environment.

The four criteria of the Eligibility Determination for Pesticidal Active Ingredients are satisfied by the science assessments supporting the PIPs containing *Bacillus thuringiensis* proteins Cry1Ac and Cry1F as expressed in Event DAS-81419-2 Soybean. These PIPs are not expected to cause unreasonable adverse effects and are likely to provide protection as claimed when used according to label instructions. The EPA believes, therefore, that DAS-81419-2 Soybean is eligible for registration for the proposed uses.

VI. TERMS AND CONDITIONS OF THE REGISTRATION(S)

As a term of registration, the applicant must submit or provide the following:

Expression in a Terrestrial Environment: OCSPP 885.5200 (40 CFR § 158.2150)
Although the registrant submitted a sufficient rationale of rapid degradation of Cry1F and Cry1Ac proteins in other crop soil systems, a soil degradation study has not been evaluated in soybean for this specific PIP event. A previous EPA FIFRA SAP noted that in soil degradation and field persistence studies, toxin degradation rates may vary with the crop, the toxin produced by the transformation event, the microbial community in the recipient soil microcosm, the statistical characterization of toxin persistence, and/or the initial dose of toxin in the experiment (US EPA 2001a). These differences in Cry toxin concentration may be due to the fact that different genetic constructs were used and/or inserted at different locations in the plant genome (Yu *et al*. 2012). Plant genotype and parental backgrounds have also been recognized as important factors influencing protein expression (Kranthi *et al.* 2005; Adamczyk and Sumerford 2001; and Torres *et al.* 2006). Other important factors that affect degradation of Cry toxins include environmental conditions such as temperature, light, drought, and soil properties (e.g. pH, texture, clay content) (Adamczyk *et al.* 2004; Chen *et al.* 2005; Jiang *et al.* 2006; Rochester 2006; Dong *et al.* 2008; Hallikeri *et al.* 2009; Addison and Rogers 2010; Chen *et al.* 2011; Ranjithkumar *et al.* 2011). To reduce this uncertainty, a soil fate study to determine the degradation rate (DT_{50}) of Cry1F and Cry1Ac proteins sampled from representative soils of soybean growing regions is to be conducted as confirmatory data. The completed soil degradation study must be received by the EPA within 18 months of the DAS-81419-2 Soybean Notice of Pesticide Registration date.

Insect Resistance Management
Dow must submit a resistance monitoring and remedial action plan for DAS-81419-2 Soybean. Although a full resistance monitoring plan with pest sampling and detection bioassays is not

warranted for this seed increase registration, an approach based on investigations of unexpected pest damage reports is appropriate. Monitoring for the subject proteins is conducted as part of the WideStrike cotton (EPA Registration Number 68467-3) monitoring program, and a remedial action plan (should resistance be documented) aligned with the plans in place for *Bt* corn or cotton (e.g., a "stop sale" if resistance develops) can be adapted for DAS-81419-2 Soybean. Additionally, annual sales and total acreage data reports for each state must be compiled by Dow for submission to the EPA (upon request).

The subject registration is limited to seed increase and to a total of 250,000 acres per year with no more than 20,000 acres per county (in non-cotton growing regions); 10,000 acres per county (in cotton-growing counties with at least 25,000 acres of soybean); or 1,000 acres (in cotton-growing counties with less than 25,000 acres of soybean) per year

A. Adverse Effects

Reports of all incidents of adverse effects to the environment must be submitted to the EPA under the provisions stated in FIFRA Section 6(a)(2).

B. Hypersensitivity Incidents

All incidents of hypersensitivity (including both suspected and confirmed incidents) must be reported to the Agency under the provisions of 40 CFR §158.2140(d).

APPENDIX A: Product characterization and human health effects – *Bacillus thuringiensis* Cry1Ac and Cry1F Proteins expressed in Event DAS-81419-2 Soybean plant-incorporated protectant.

A.1. BACKGROUND

Event DAS-81419-2 expresses the δ-endotoxins Cry1Ac and Cry1F derived from *Bacillus thuringiensis*. Product characterization and human health data submitted for seed increase registration of DAS-81419-2 are summarized below.

A.2. PRODUCT CHARACTERIZATION

Event DAS-81419-2 was developed by *Agrobacterium*-mediated transformation of soybean using the 2T-DNA plasmid vector pDAB9582 and produces *Bacillus thuringiensis* protein δ-endotoxins, Cry1Ac and Cry1F. A single T-DNA insert containing each of the intact plant transcription units (PTUs) for the *cry1Fv3, cry1Ac(synpro),* and *pat* genes was integrated into soybean to create DAS-81419-2. This protein is intended to provide protection from feeding damage caused by a number of lepidopteran pests.

a. Transformation System and Genetic Elements

Molecular characterization of DAS-81419-2 soybean by Southern blot analysis confirmed that a single T-DNA insert containing each of the intact plant transcription units (PTUs) for the *cry1Fv3, cry1Ac(synpro),* and *pat* genes from plasmid pDAB9582, was integrated into DAS-81419-2 soybean. In addition, a minor (<100 bp) fragment of the *cry1Ac(synpro)* gene was identified on the 5′ end of the T-DNA insert. Data were generated to evaluate the integration and integrity of the *cry1Fv3, cry1Ac(synpro),* and *pat* genes inserted into the soybean genome. Characterization of the integration of non-coding regions (designed to regulate the coding regions), such as promoters and terminators was also performed. The inserted DNA was stably inherited across the five generations (T1, T2, T3, T4, and F2) evaluated. No transformation plasmid backbone sequence was found in DAS-81419-2 soybean as demonstrated by Southern blot analysis using probes covering the entire region of the plasmid flanking the T-DNA insert.

b. Protein Characterization

Protein characterization data demonstrate that the plant-produced Cry1Ac and Cry1F have biochemical and functional activities that are similar to those of the *Pseudomonas fluorescens*-produced proteins that were used in several toxicity studies. The following techniques were used to characterize and compare the plant-produced and the *Pseudomonas fluorescens*-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 *Pseudomonas fluorescens*-produced Cry1Ac and Cry1F proteins and justified the use of *Pseudomonas fluorescens*-produced protein in toxicity studies.

c. Analytical Detection Methods

An exemption from the requirement of a food tolerance is already in existence (since 1997) for Cry1Ac protein for all food commodities (40 CFR 174.510) and an appropriate analytical detection method was previously reviewed for Cry1Ac. An exemption from the requirement of a food tolerance exists for Cry1F, however, the exemption is limited to expression in maize (40 CFR 174.520) and cotton (40 CFR 174.504). A petition for an exemption from the requirement of a food tolerance for Cry1F in soybean was included with the application from Dow.

Dow AgroSciences LLC analytical method 110674, "Method Validation for the Determination Cry1Ac Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)", was demonstrated to be suitable for its intended purpose. The method was validated over the concentration range of 0.1 to 2 ng Cry1Ac/mg dry weight (DW) and has a validated limit of quantitation (LOQ) in soybean tissues of 0.2 ng/mg DW and a limit of detection (LOD) of 0.1 ng/mg DW. The Cry1Ac protein was recovered at acceptable levels from soybean tissues. The Cry1Ac protein was efficiently extracted from all tested soybean tissues. The assay was shown to have acceptable accuracy, precision and ruggedness, and no false positive or false negative results were seen below the target LOD. It is concluded that this Cry1Ac ELISA method has been demonstrated to be suitable for quantitative measurements of the Cry1Ac protein in soybean tissue.

Dow AgroSciences LLC analytical method 110675, "Determination of Cry1F Protein in Soybean Tissues Using an Enzyme-Linked Immunosorbent Assay (ELISA)", was demonstrated to be suitable for its intended purpose. The method was validated over the concentration range of from 0.05 ng/mg to 1 ng/mg DW for root R3, from 0.15 ng/mg to 1.5 ng/mg DW for forage R3, from 0.2 ng/mg to 2 ng/mg DW for grain, and from 0.25 ng/mg DW to 2.5 ng/mg DW for leaf V5 and leaf V10-12. The method has a validated limit of quantitation (LOQ) of 0.1 ng/mg DW for root R3, 0.3 ng/mg DW for forage R3, 0.4 ng/mg DW for grain, and 0.5 ng/mg DW for leaf V5 and leaf V10-12, and a limit of detection (LOD) of 0.05 ng/mg DW for root R3, 0.15 ng/mg DW for forage R3, 0.20 ng/mg DW for grain, and 0.25 ng/mg DW for leaf V5 and leaf V10-12. Cry1F protein was recovered at acceptable levels from soybean tissues. This Cry1F ELISA method has been demonstrated to be suitable for quantitative measurements of the Cry1F protein in soybean tissue.

d. Protein Expression

Expression level data were provided for Cry1Ac in different plant tissues and at different growth stages. Cry1Ac protein is expressed at relatively low levels in event DAS-81419-2 soybean. The data were produced using ELISA methods for this protein. Summary results are provided below in Table A.1. Table A.2. provides summaries of the product characterization studies and data provided.

Table A.1. Protein Expression in DAS-81419-2 soybean

Summary of Cry1Ac Protein Expression

	Test		Cry1Ac ng/mg Tissue Dry Weight				
Matrix	Entry	Description	Overall Mean	Std. Dev. (n=10)	Min/Max Range	STMR[a]	HAFT[b]
V5 Leaf	7	DAS-81419-2	25.44	6.61	12.10 - 40.20	26.90	35.25
V10-12 Leaf	7	DAS-81419-2	23.16	6.17	10.70 - 37.45	22.15	34.61
Forage	7	DAS-81419-2	5.54	2.54	1.38 - 11.83	5.44	10.28
Root	7	DAS-81419-2	0.39	0.24	[0.12] - 1.12	0.32	0.97
Grain	7	DAS-81419-2	1.04	0.10	0.79 - 1.40	1.04	1.20

[a] Supervised Trials Mean Residue [b] Highest Average Field Trial

Summary of Cry1F Protein Expression

	Test		Cry1F ng/mg Tissue Dry Weight				
Matrix	Entry	Description	Overall Mean	Std. Dev. (n=10)	Min/Max Range	STMR[a]	HAFT[b]
V5 Leaf	7	DAS-81419-2	56.75	15.03	24.60 - 99.50	56.30	76.05
V10-12 Leaf	7	DAS-81419-2	39.07	16.60	12.75 - 76.71	38.70	59.98
Forage	7	DAS-81419-2	20.28	11.29	5.34 - 44.62	20.64	40.23
Root	7	DAS-81419-2	5.23	3.74	1.09 - 16.08	4.12	14.21
Grain	7	DAS-81419-2	13.80	1.24	10.41 - 16.95	13.71	16.21

[a] Supervised Trials Mean Residue [b] Highest Average Field Trial

Summary of PAT Protein Expression

	Test		PAT ng/mg Tissue Dry Weight				
Matrix	Entry	Description	Overall Mean	Std. Dev. (n=10)	Min/Max Range	STMR[a]	HAFT[b]
V5 Leaf	7	DAS-81419-2	5.23	0.88	3.25 - 7.35	5.30	6.93
V10-12 Leaf	7	DAS-81419-2	5.60	1.14	2.55 - 7.56	5.76	7.32
Forage	7	DAS-81419-2	4.06	1.30	1.24 - 6.12	4.02	5.69
Root	7	DAS-81419-2	0.63	0.12	0.44 - 1.05	0.63	0.85
Grain	7	DAS-81419-2	0.86	0.13	0.63 - 1.12	0.83	1.06

[a] Supervised Trials Mean Residue [b] Highest Average Field Trial

Table A.2. Summary of Product Characterization Data for PIP Event DAS-81419-2 soybean (EPA Reg. No. 68467-EN)

Study Type/Title	Summary	MRID No.
Molecular Characterization of DAS-81419-2 Soybean	Data from multiple Southern blots probed with various fragments of the *cry* and *pat* genes, as well as promoter and 3' UTR sequences, indicate that a single insertion of T-DNA is present in DAS-81419-2 Soybean. Samples from five generations of DAS-81419-2 soybean yielded identical patterns of hybridization indicating that the insertion is stably introduced into the plant genome. Further, the absence of pDAB9582 backbone sequences was demonstrated with probing of Southern blots using 4 different sequences (i.e., $Spec^r$, Backbone, 1, 2 and 3) outside of the T-DNA region of the plasmid. All blots included genomic DNA from a non-transgenic soybean, Maverick, and a Maverick DNA sample spiked with pDAB9582 DNA as negative and positive controls, respectively. **Classification: ACCEPTABLE**	48828401
Protein Expression of a Transformed Soybean Cultivar Containing Cry1Ac, Cry1F, and Phosphinothricin Acetyltransferase (PAT) - Event DAS-81419-2	Measures of protein accumulation at different growth stages and in different organs of DAS-81419-2 Soybean plants were obtained using appropriate methodology. The authors of this study quantified Cry1Ac, Cry1F and PAT proteins in leaf, forage (aerial portions of plant without pods), root and seed of soybean cultivated in 10 different localities typical for this crop. The ELISA-based methods were typical for this technique and the findings were statistically acceptable (i.e., variance is within acceptable limits). Sample handling and processing were also performed appropriately. **Classification: ACCEPTABLE**	48828402
Biological Equivalency of (DAS-81419-2) and microbe-produced Cry1Ac and Cry1F Proteins	With respect to biological activity of the plant-produced and microbe-produced Cry1Ac and Cry1F proteins, LC_{50} data for the target pest *Chrysodeixis includens* (soybean looper) indicate that the plant- and microbe-derived Cry proteins retain similar activity against this insect. LC_{50} values vary by less than 15%, which is an acceptable level of variance for such a biological system. **Classification: ACCEPTABLE**	48828403
Method Validation for the Determination of Cry1Ac Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)	Analytical method 110674 was validated to perform robustly in the quantitation of Cry1Ac protein in soybean organs in the range of 0.2 ng/mg DW to 2.9 ng/mg DW using an ELISA-based technique with two monoclonal antibodies raised against Cry1Ac. The method performed with acceptable level of accuracy in the quantitation of Cry1Ac in soybean organ samples, including leaf, root, forage (aerial portions of plant without pods), and seed. Given some matrix effects, a 2X dilution of samples is recommended for soybean samples to more accurately quantify Cry1Ac. Variability in results was within	48828404

	acceptable limits as the Coefficient of Variation (CV) values were all less than 20%. No cross reactivity with a variety of nontarget proteins was detected and extraction efficiencies from soybean samples were at acceptable levels. **Classification: ACCEPTABLE**	
Method Validation for the Determination of Cry1F Protein in Soybean Tissues Using an Enzyme-Linked Immunosorbent Assay (ELISA)	The ELISA-based double antibody sandwich method employed by the study authors accurately quantified target protein Cry1F in soybean organs sampled (root, forage, leaves, seed). The analytical method 110675 was validated over a range of protein concentration values appropriate for the levels as found in cultivated soybean spiked with known quantities of Cry1F protein. The range is, however, on the lower end of most transgenic soy samples as evidenced in other volumes of this submission as some leaf samples contained concentrations of Cry proteins in the 25 to 56 ng/mg DW range. Matrix effects were adequately resolved and indicate that in practice, dilution of root, leaf, seed and forage samples will need to be performed at 1X, 5X, 4X, and 3X respectively to minimize these effects on quantification of Cry1F in soybean. The variance recorded among samples and between different analysts was less than 20%, an acceptable amount when working with complex plant organ extracts, with the exception of root samples spiked to yield quantities of Cry1F protein at or near the LOD (0.1 ng/mg DW). Higher variability at or near an LOD is not unexpected. The ELISA-based method 110675 was not confounded by false positives or negatives and only minimal cross reaction with Cry1Ac (0.114 %) was observed while other nontarget proteins were similarly non-cross reactive. The method appears robust enough for practical use in the detection of Cry1F in soybean. **Classification: ACCEPTABLE**	48828405
Characterization of the Cry1Ac Protein Derived from Transgenic Soybean Event DAS-81419-2	The study authors employed several techniques to adequately demonstrate that the Cry1Ac protein from soybean event DAS-81419-2 is biochemically equivalent to the Cry1Ac protein as expressed in *Pseudomonas fluorescens*. Lateral test flow strips (an ELISA-based method), immunoprecipitation, detection of glycosyl residues, Western blot, MALDI-TOF / MS, MALDI-TOF/TOF, MS/MS, and SDS-PAGE analyses all support the contention that the two proteins are biochemically equivalent. Samples were processed, stored and handled in an appropriate manner to maintain integrity of these protein samples. **Classification: ACCEPTABLE**	48828416
Characterization of the Cry1F Protein Derived from Transgenic Soybean Event DAS-81419-2	The study authors employed several techniques to adequately demonstrate that the Cry1F protein from soybean event DAS-81419-2 is biochemically equivalent to the Cry1F protein as expressed in *Pseudomonas fluorescens*. Lateral test flow strips (an ELISA-based method), immunoprecipitation, detection of glycosyl residues,	48828417

	Western blot, MALDI-TOF / MS, MALDI-TOF/TOF, MS/MS, and SDS-PAGE analyses all support the contention that the two proteins are biochemically equivalent. Samples were processed, stored and handled in an appropriate manner to maintain integrity of these protein samples. **Classification: ACCEPTABLE**	
Amended Response to EPA Comment on Characterization of the Cry1F Protein Derived from Transgenic Soybean Event DAS-81419-2 Study – Address amino acid sequence similarity between Dow's Cry1F microbial protein and soybean produced Cry1F: Related to MRID 488284-17	The synthetic Cry1F protein expressed in DAS-81419-02 Soybean and the Cry1F protein expressed in *Pseudomonas fluorescens* strain MR872 differ at 4 amino acid positions in the full length proteins. The variations or alterations in amino acid residues for these 4 positions (residues 604, 608, 624, 629) do not affect the biochemical or biological properties evaluated in this risk assessment. For the purposes of testing, as performed, the proteins are considered equivalent. **Classification: ACCEPTABLE**	49081501

A. 3. HUMAN HEALTH ASSESSMENT

Section 408(c)(2)(A)(i) of the Federal Food, Drug, and Cosmetic Act (FFDCA) allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of the FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Pursuant to section 408(c)(2)(B), in establishing or maintaining in effect an exemption from the requirement of a tolerance, EPA must take into account the factors set forth in section 408(b)(2)(C), which require EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue... ."

Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider "available information concerning the cumulative effects of a particular pesticide's residues" and "other substances that have a common mechanism of toxicity." EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

Dow submitted a pesticide tolerance petition (PP2F8066) under the FFDCA, requesting 40 CFR Part 174 be amended by establishing an exemption from the requirement of a tolerance for residues of *Bacillus thuringiensis* (*Bt*) Cry1F insect control protein and the genetic material necessary for its production in food commodities of soybean.

Consistent with section 408(b)(2)(D) of the FFDCA, 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 risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children.

a. 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. No indication of toxicity toward mammals was noted in the review of the two Cry proteins expressed in soybean.

It should be noted that the allergenicity of Cry1Ac protein was evaluated previously (Wozniak, 2010) as evidenced in MRID No. 47841709 (US EPA, 2010). Based upon sequence comparisons to known allergens and digestibility assays (MRID No. 47841708), Cry1Ac was considered to be of little potential to function as an allergen.

An exemption from the requirement of a food tolerance was established previously for phosphinothricin acetyltransferase (PAT; 40 CFR 174.522) as expressed as part of a plant-incorporated protectant, so no further toxicological review of this protein is contained herein.

The human health studies submitted for Cry1Ac and Cry1F are summarized in Table A.3. below.

Table A.3. Summary of Human Health Data conducted for PIP Event DAS-81419-2 soybean (EPA Reg. No. 68467-EN)

Study Type/Title	Summary	MRID No.
Sequence Similarity Assessment of Cry1F Protein to Known Allergens by Bioinformatics Analysis	The study authors have utilized appropriate search methods in comparing the sequence of their Cry1F protein with the 'allergenonline' database of known allergens. The default parameters applied to the search are sufficient to identify sequences with > 35% homology over any 80 amino acid sequence and to detect any individual 8 amino acid sequences which may represent epitopes of allergens. Given the exhaustive analysis of Cry proteins, including Cry1F and closely related δ-endotoxins, over many years for the presence of sequences representing potential allergenic epitopes and the general lack of demonstrated IgE-mediated immune responses to those exposed to preparations of biopesticidal *Bacillus thuringiensis*,	48828406

	the findings are not surprising or unexpected. It should be noted that the allergenicity of Cry1F protein was evaluated previously (Wozniak, 2002; Matten 2005) as evidenced in MRID No. 44971701 and discussed in a Biopesticide Registration Action Document (US EPA, 2005; ibid 2010). Based upon sequence comparisons to known allergens and digestibility assays (MRID No. 45542312), Cry1F was considered to be of little potential to function as an allergen. **Classification: ACCEPTABLE**	
Sequence Similarity Assessment of Cry1F to Known Toxins by Bioinformatics Analysis	The study authors queried appropriate databases containing sequences of proteins, including those with potential toxicity to vertebrates. Parameters chosen for the search were selected to detect proteins with minimal homology in sequence such that even those with insufficient similarity to trigger a toxic reaction may be uncovered. Of the 672 proteins or hypothetical protein sequences returned from the search E-value < 1.0, including 646 with E-value <0.01, 618 were clearly related to δ-endotoxins from *Bacillus thuringiensis* and two were related to proteins from other *Bacillus* species (e.g, *B. cereus*). Of the remaining 28 protein hits from the databases, some of the sequences were from parasporal proteins of related species (e.g., *Lysinibacillus sphaericus*, *Paenibacillus lentimorbus*) and also from hypothetical proteins (of no known function) from eubacteria, slime molds / Mycetozoa and a fungal species (*Fusarium oxysporum*). These proteins and sequences of hypothetical proteins are not known to represent toxic moieties with respect to vertebrates. As is typical with previously characterized Cry proteins of *B. thuringiensis*, no toxicity to vertebrates, including man, is expected from consumption or contact with these proteins. **Classification: ACCEPTABLE**	48828407
Sequence Similarity Assessment of Cry1Ac Protein to Known Allergens by Bioinformatics Analysis	The study authors have utilized appropriate search methods in comparing the sequence of their Cry1Ac protein with the 'allergenonline' database of known allergens. The default parameters applied to the search are sufficient to identify sequences with > 35% homology over any 80 amino acid sequence and to detect any individual 8 amino acid sequences which may represent epitopes of allergens. Given the exhaustive analysis of Cry proteins, including Cry1Ac and closely related δ-endotoxins, over many years for the presence of sequences representing potential allergenic epitopes and the general lack of demonstrated IgE-mediated immune responses to those exposed to preparations of biopesticidal *Bacillus thuringiensis*, the findings are not surprising or unexpected. It should be noted that the allergenicity of Cry1Ac protein was evaluated previously as evidenced in MRID No. 45542319 (US EPA, 2005). Based upon sequence comparisons to known allergens and	48828408

	digestibility assays (MRID No. 47841708), Cry1Ac was considered to be of little potential to function as an allergen. **REFERENCES:** US Environmental Protection Agency (2010) 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] http://www.epa.gov/pesticides/biopesticides/pips/bt-cry1ac-protien.pdf **Classification: ACCEPTABLE**	
Similarity Assessment of Cry1Ac Protein to Known Toxins by Bioinformatics Analysis	The study authors queried appropriate databases containing sequences of proteins, including those with potential toxicity to vertebrates. Parameters chosen for the search were selected to detect proteins with minimal homology in sequence such that even those with insufficient similarity to trigger a toxic reaction may be uncovered. Of the 684 proteins or hypothetical protein sequences returned from the search, 662 hits were clearly related (E-value <0.01) to δ-endotoxins from *Bacillus thuringiensis* and two were related to proteins from other *Bacillus* species. Of the remaining 52 protein hits from the databases, some of the sequences were from parasporal proteins of related species (e.g., *Bacillus cereus*, *Paenibacillus lentimorbus*) and also from hypothetical proteins (of no known function) from eubacteria, slime molds / Amoebozoa and fungal species. These proteins and sequences of hypothetical proteins are not known to represent toxic moieties with respect to vertebrates. As is typical with previously characterized Cry proteins of *B. thuringiensis*, no toxicity to vertebrates, including man, is expected from consumption or contact with these proteins. **Classification: ACCEPTABLE**	48828409

b. Mammalian Toxicity and Allergenicity Assessment

Previously submitted acute oral toxicity data demonstrated a lack of mammalian toxicity at high levels of exposure (ingestion) to the pure Cry1Ac protein. These data demonstrate the safety of this protein 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 No. 45542313) indicated that Cry1Ac is non-toxic to humans and other mammals. 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 all food commodities precluded the need for a separate tolerance action in conjunction with this review of product characterization and human health assessment.

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. (MRID No. 45542319) The Cry1Ac protein was digested rapidly in simulated gastric fluid containing pepsin. Small peptides remaining following gastric simulated digestion were completely degraded to amino acid residues in SIF upon contact (US EPA 2005; 2010a).
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.

An acute oral toxicity study in mice (MRID No. 45542314) indicated that Cry1F is non-toxic to humans and other mammals. Cry1F protein is a δ-endotoxin from *B. thuringiensis* that has been used extensively in plant-incorporated protectants as a means of insect pest management. Existing exemptions from the requirement of a tolerance for Cry1F in cotton (CFR 40 Section 174.504; 72 FR 20434, April 25, 2007) and corn (CFR 40 Section 174.520; 72 FR 20435, April 25, 2007) precluded the need for a separate tolerance action in conjunction with this review of product characterization and human health assessment. Cry1F was previously granted an exemption from the requirement of a tolerance for expression in cotton (CFR 40 Section

174.504) and maize (CFR Section 174.520); a separate petition (PP2F8066) for an exemption from the requirement of a tolerance for Cry1F as expressed in soybean is included with the submission from Dow for registration of DAS-81419-2 Soybean.

Since Cry1F 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 Cry1F 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 Cry1F with known allergens showed no significant overall sequence similarity or identity at the level of eight contiguous amino acid residues.
3. Digestibility. (MRID No. 45542318) The Cry1F protein was digested rapidly (< 5 min) in simulated gastric fluid containing pepsin. Small peptides remaining following gastric simulated digestion were completely degraded to amino acid residues in SIF upon contact (US EPA 2005; 2010b).
4. Glycosylation. Cry1F expressed in soybean was shown not to be glycosylated.
5. Conclusion: Considering all of the available information, EPA has concluded that the potential for Cry1F to be a food allergen is minimal.

Cry1Ac and Cry1F were shown not to be glycosylated in extracts of DAS-81419-2 soybean; it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it needs to 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).

c. Overall Safety Conclusions

The continued use of Cry1Ac and Cry1F proteins 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-endotoxins Cry1Ac and Cry1F as an insect management mechanism is safe as proposed for use in soybean intended for cultivation, and human and animal consumption.

APPENDIX B: Ecological and environmental effects – *Bacillus thuringiensis* Cry1Ac and Cry1F proteins expressed in Event DAS-81419-2 Soybean plant-incorporated protectant

Dow submitted data in support for Sec. 3 Seed-Increase Registration of event DAS-81419-2 soybean line [EPA Reg. No. 68467-EN; OECD Unique Identifier: DAS-81419-2] for use as a Plant-Incorporated Protectant (PIP). The proposed PIP is a new transformation event that expresses lepidopteran-active Cry1F and Cry1Ac proteins in soybean. The insecticidal proteins Cry1F and Cry1Ac (encoded by the *cry1F* gene and *cry1Ac* genes, respectively) are co-expressed in WideStrike® cotton (OECD Unique Identifier: DAS-21Ø23-5 x DAS-24236-5), which is currently registered for use as a PIP cotton product (US EPA 2005). The cotton line WideStrike® was produced by crossing cotton lines 281-24-236 (OECD Unique Identifier: DAS-24236-5) with 3006-210-23 (OECD Unique Identifier: DAS-21Ø23-5) via conventional breeding methods. Since these proteins are identical to those expressed in the proposed PIP product, a "weight-of-evidence" approach was used to bridge data from WideStrike® cotton in support for the environmental risk assessment of event DAS-81419-2 soybean. Thus, the findings of the environmental risk assessment for event DAS-81419-2 soybean include the extrapolation of previously submitted nontarget organism (NTO) toxicity studies and results from field studies currently found in the Agency database for the registration of WideStrike® expressing Cry1F and Cry1Ac proteins for use as a PIP in cotton. The biological equivalency of Cry1F and Cry1Ac proteins expressed in event DAS-81419-2 soybean plant was confirmed with the previously characterized microbial-derived Cry1F and Cry1Ac protein test substances. No significant increase in exposure to NTOs was demonstrated based on calculated margins of exposure for protein concentrations in event DAS-81419-2 plant tissue matrices relative to the dose concentrations used in the NTO laboratory toxicity tests conducted on Cry1Ac and Cry1F. Lastly, a field monitoring study showing no differences on nontarget arthropods on a population and community level and other supporting supplemental information (e.g. meta-analysis, target feeding studies, and biological specificity to the target pest) provided additional certainty to support the "weight-of-evidence" that there is negligible risk to NTOs representing diverse taxonomic groups and exposure scenarios from the cultivation of DAS-81419-2 soybean.

Therefore, the submitted data supports the applicant's request and the findings from the environmental risk assessment conducted for WideStrike® cotton can be bridged to support the Sec. 3 Registration of PIP event DAS-81419-2 (US EPA 2005). Based on extrapolating from previous studies on *Bt* proteins, which tend to be very species specific in their activity, the Agency concludes that the cultivation of event DAS-81419-2 soybean will not result in adverse effects to nontarget organisms, are not likely to persist in the soil, and pose no risk of gene flow and development of weediness in wild relatives, and additionally will have no effect, direct or indirect, on Federally listed threatened and endangered species and their designated Critical Habitats.

BACKGROUND

Dow has developed event DAS-81419-2 soybean line [EPA Reg. No. 68467-EN; OECD Unique Identifier: DAS-81419-2] for use as a plant-incorporated protectant (PIP). Event DAS-81419-2

soybean was produced by *Agrobacterium*-mediated transformation with plasmid pDAB9582. The inserted T-DNA from this plasmid contains three transgenes: 1) synthetic truncated *cry1F* gene from *Bacillus thuringiensis* (*Bt*) var. *aizawai*; 2) *cry1Ac* gene isolated from *Bt* var. *kurstaki*; and 3) a version of the *phosphinothricin acetyl transferase* (*pat*) gene from *Streptomyces viridochromogenes* that has been optimized for expression in soybean. The Cry1F and Cry1Ac proteins, (encoded by the *cry1F* and *cry1Ac* genes, respectively) confer resistance to certain lepidopteran pests. The PAT enzyme encoded by the *pat* gene provides the plant with tolerance to herbicide applications containing glufosinate-ammonium by acetylating the herbicide glufosinate-ammonium and making it unable to bind glutamine synthase. The proposed PIP is intended to provide protection against several lepidopteran pests of soybean, including soybean looper (*Chrysodeixis includens,* formerly *Pseudoplusia includens*)*,* velvetbean caterpillar (*Anticarsia gemmatalis*)*,* fall armyworm (*Spodoptera frugiperda*) and tobacco budworm (*Heliothis virescens*). The Cry1F and Cry1Ac proteins are co-expressed in the currently registered PIP event WideStrike® cotton (EPA Reg. No. 68467-3). Dow has requested to bridge several environmental fate and effects studies previously reviewed for the registration of WideStrike® cotton (US EPA 2005) to support the registration of event DAS-81419-2 soybean PIP product.

A "weight-of-evidence" approach has been used to determine if the supporting data submitted by Dow is acceptable for bridging to the Agency's database on the Cry1F and Cry1Ac proteins expressed in WideStrike® cotton and whether extrapolation of those studies and other supplemental information support the environmental risk assessment of event DAS-81419-2 soybean.

I. Environmental Risk Assessment Process and Rationale for PIPs under EPA

The paragraphs below describe the process and rationale developed by the U.S. EPA's Biopesticides and Pollution Prevention Division (BPPD) within the Office of Pesticide Programs 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] Nontarget invertebrate hazard tests often are conducted at exposure concentrations several times higher than the maximum concentrations expected to occur under realistic exposure scenarios. This has customarily allowed an

Tiered tests are designed to first represent unrealistic worst case scenarios and ONLY progress to real world field scenarios if the earlier tiered tests fail to indicate adequate certainty of acceptable risk. Screening (Tier I) nontarget 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 nontarget 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 (nontarget) 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.

EPA's Office of Chemical Safety and Pollution Prevention (OCSPP) has published a series of test guidelines that contain testing methods and standards for conducting, evaluating, and reporting of data needed to support the registration of a pesticide under FIFRA. The testing methods, which utilize the tiered approach, are published in the OCSPP Harmonized Test Guidelines, Series 850 and 885.[2] These guidelines, as defined in 40 CFR § 158.70(c), apply to microbes and microbial toxins when used as biological 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. Further, most registered PIP products express insecticidal proteins derived from *Bacillus thuringiensis* (*Bt*), a common soil bacterium that is also found in several "reduced risk" microbial pesticides and currently available commercially to prevent the damage caused by insect pests. Microbial pesticides containing *Bt* proteins have a long history of safe use and undergone extensive toxicity testing showing no adverse effects to human or animal health (US EPA 2000 and 2001a; McClintock *et al.* 1995; Mendelsohn *et al.* 2003).

The Tier I screening maximum hazard dose (MHD) approach to environmental hazard assessment is based on some factor (whenever possible >10) times the Estimated Environmental

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

[2] OCSPP Harmonized Test Guidelines, Series 850 - Ecological Effects. Available at the EPA website: http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series850.htm
OCSPP Harmonized Test Guidelines, Series 885 - Microbial Pesticide. Available at the EPA website: http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series885.htm

Concentration[3] (EEC), which is the maximum amount of active ingredient expected to be available to terrestrial and aquatic nontarget organisms in the environment. Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels which increase the statistical power to test the hypotheses. Elevated test doses, therefore, add certainty to the assessment, and such tests can be well standardized. The Guidelines call for initial screening testing of a single group or several groups of test animals at the maximum hazard dose level. The Guidelines call for testing of one treatment group of at least 30 animals or three groups of 10 test animals at the screening test concentration. The Guidelines further state that the duration of all Tier I tests should be approximately 30 days. Some test species, notably nontarget 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. However, greater than 50% mortality does not necessarily indicate the existence of unacceptable risk in the field, but it does trigger the need to collect additional dose-response information and a refinement of the exposure estimation before deciding if the risk is acceptable or unacceptable. Where potential hazards are detected in Tier I testing (i.e. mortality is greater than 50%), additional information at lower test doses is required which can serve to confirm whether any effect might still be detected at more realistic field [1X EEC] concentrations and routes of exposure.[5]

When screening tests indicate a need for additional data, the OCSPP 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

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

4 It is notable that 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 nontarget organism may be ingesting in the field because most nontarget organisms do not ingest plant tissue.

replication, the number of test species, and/or repetition are needed to enhance statistical power in these circumstances.

Data that shows less than 50 % mortality at the maximum hazard dosage level – (i.e. LC_{50}, ED_{50}, or LD_{50} >10 X EEC) is sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. It is also notable that the recommended >10X EEC maximum hazard dose level is a highly conservative factor. The published EPA Level of Concern [LOC] is 50% mortality at 5X EEC [6] (US EPA 1998).

Validation: The tiered testing 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 nontarget species data requirements. In October 2000, another SAP also recommended that field testing should be used to evaluate population-level effects on nontarget organisms. The August 2002 SAP, and some public comments, generally agreed with this approach, with the additional recommendation that test surrogate organisms should be selected on the basis of potential for field exposure to the subject protein (US EPA 2000, 2001a, 2002 and 2004a). A White Paper developed by the U.S. States Department of Agriculture (USDA) and EPA discussed the tiered testing approach and provided additional guidance for conducting nontarget invertebrate ecological risk assessments specifically for crops expressing insecticidal proteins (US EPA 2007).

Chronic studies: Additional higher-tier (e.g. Tier II, III, and IV) testing for chronic effects on nontarget organisms are only conducted when they are triggered (i.e. toxicity studies that show 50% mortality when tested at least 5X EEC). Since delayed adverse effects and/or accumulation of toxins through the food chain are not expected to result from exposure to *Bt* PIP proteins, these protein toxins are not routinely tested for chronic effects on nontarget organisms. However, the 30 day test duration requirement does amount to subchronic testing when performed at field exposure test doses.

Conclusion: The tiered testing approach to test guidelines ensures, to the greatest extent possible, that the Agency requires the minimum amount of data needed to make scientifically sound regulatory decisions. The EPA believes the Tier I screening maximum hazard dose (MHD) approach presents a reasonable approach for evaluating environmental hazards related to the use of biological pesticides and for identifying negative results with a high degree of confidence (US EPA 2007). The Agency expects that Tier I testing for short-term hazard assessment will be sufficient for most studies submitted in support of PIP registrations (US EPA 2007). However, if

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

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 nontarget organisms directly in the field. This approach, with an emphasis on testing invertebrates found in *Bt* cotton fields, was also recommended by the August 2002 SAP and was supported by several public comments. Based on these recommendations, the Agency has required large-scale, long term field studies as a condition of registration on: 1) monitoring nontarget invertebrate populations (e.g. community level effects); and 2) determining the bioavailability/persistence of Cry proteins remaining in soils representative of different growing regions of *Bt* crops. These studies were conducted as a precautionary measure 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 term 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 nontarget invertebrates are generally more abundant in fields of *Bt* crops 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* crops have not revealed any unexpected nontarget 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.

Proteins are not known to persist in the environment due to the ubiquitous nature of proteases in microbes. The biological nature of proteins makes them readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Sprayable microbial pesticide formulations containing *Bt* have a long history of safe use. Several laboratory studies have demonstrated rapid degradation of Cry proteins in various soils sampled from *Bt* crop growing regions. Long term field studies have been conducted showing no accumulation (i.e. persistence) of Cry proteins as a result of continuous planting of *Bt* crops (Head *et al.* 2002; Herman *et al.* 2002; Shan *et al.* 2008). Thus, the issue of long term effects of Cry proteins has also been addressed by several field studies showing a lack of persistence in various soil types sampled from representative *Bt* crop growing region. While there are reports that some proteins (Cry proteins) binding to soil particles, it has also been shown that these proteins are degraded rapidly by soil microbial flora upon elution from soil particles (Icoz and Stozky 2008).

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

As a result, the Agency has determined no additional long-term field studies are required for monitoring nontarget invertebrate populations or soil protein persistence for *Bt* crops that contain similar Cry proteins expressed in previously registered PIP events with the exception of Tier I laboratory studies that "trigger" additional testing or if the proposed registration is a novel PIP.

Overall, the Agency is in agreement with the conclusions of these studies and collectively, these results provide extensive data to support that *Bt* crops registered to date have not caused long term adverse effects on a population level to organisms not targeted by *Bt* proteins. Moreover, these field studies further substantiate the "tiered testing approach" by confirming that the absence of hazard in the Tier I toxicity studies is predictive of an absence of ecological harm in the field. These results are also consistent with earlier studies of *Bt* strains used as microbial insecticides, which demonstrated no significant detrimental impacts on populations of the nontarget organisms that were studied. Based on these considerations, the "tiered testing approach" has been demonstrated as an appropriate method for assessing environmental risk of *Bt* crops while ensuring, to the greatest extent possible, that the Agency requires the minimum amount of data needed to make scientifically sound regulatory decisions.

A. Environmental Exposure Assessment

Two separate FIFRA Scientific Advisory Panel reports (US EPA SAP 2000 and 2002) recommended that nontarget testing of *Bt* Cry proteins should focus on invertebrate species exposed to the crop in which the protein(s) will be expressed. Following SAP recommendations, the EPA determined that nontarget organisms with the greatest exposure potential to Cry protein in transgenic soybean fields are beneficial insects that feed on soybean leaf foliage and pods, particularly lepidopteran insects, and soil invertebrates. While EPA's risk assessments of *Bt* soybean 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 on adverse effects at the field exposure rates, which are typically based on protein expression levels within the plant for PIPs. Although it is recommended that nontarget 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 nontarget organism studies submitted in support of Cry1F and Cry1Ac expressed in event DAS-81419-2 soybean, the test material dose levels were based on an estimated concentration of Cry1F and Cry1Ac proteins expressed in the tissue(s) with the highest concentration calculated from a field study quantifying protein expression levels in several soybean plant tissue parts (MRID No. 48828402).

B. Ecological Effects Data for Cry1F and Cry1Ac Insecticidal Proteins Expressed in Event DAS-81419-2 Soybean

In the absence of PIP-specific risk assessment guidance, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins. These

requirements include testing on birds, mammals, nontarget insects, honey bee, plants, and aquatic species, and information has been submitted to address these requirements. Limit dose testing on representative organisms from several taxa was performed in support of Section 3 FIFRA registration of event DAS-81419-2 expressing *Bt*-derived Cry1F and Cry1Ac insecticidal proteins. As stated above, BPPD's risk assessments focus more greatly on beneficial nontarget invertebrates, since they are most closely related to organisms susceptible to the insecticidal action of *Bt* toxins. The Cry1F and Cry1Ac proteins are meant to target species within the order Lepidoptera (e.g. moths). *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 nontarget insect evaluations should include several invertebrate species that are not related to the target pests. Earthworm studies are also recommended.

The applicant has requested to bridge to the Agency database of previously reviewed toxicity studies of select nontarget organisms (NTOs) that supported the currently registered WideStrike® PIP cotton product (EPA Reg. No. 68467-3). The WideStrike® PIP cotton line was produced by conventional cross breeding of cotton lines DAS-21023-5 (also described as 3006-210-23) expressing Cry1F protein and DAS-24236-5 (also described as 281-24-236) expressing Cry1Ac protein. The environmental risk assessment for the insecticidal proteins (Cry1Ac and Cry1F) expressed in WideStrike® cotton was previously evaluated for adverse impacts on nontarget organisms and have been shown to pose negligible risk (US EPA 2005). Since exposure may also occur to other nontarget organisms, EPA has received a number of studies and data waiver rationales to comply with the Agency's published data requirements on other nontarget organisms.

The toxicity of Cry1F and Cry1Ac proteins has been previously evaluated on several species of invertebrates including: lady beetle (*Hippodamia convergens*), green lacewing (*Chrysoperla rufilabris*), honey bee (*Apis mellifera*), collembola (*Folsomia candida*), parasitic wasp (*Nasonia vitripennis*) and earthworm (*Eisenia fetida*). Two new dietary toxicity studies were submitted in support for event DAS-81419-2 soybean, which includes Northern Bobwhite (*Colinus viginianus*) and Rainbow Trout, (*Oncorhynchus Mykiss*). The individual results for the cited and newly submitted nontarget organism studies in support for Cry1F and Cry1Ac are summarized in Table 1. The studies are described in more detail below, and full reviews of each study can be found in the individual Data Evaluation Records.

To support data bridging from the previously conducted environmental risk assessment for Cry1F and Cry1Ac insecticidal proteins co-expressed in WideStrike® PIP cotton line, an environmental risk assessment was conducted using a "weight-of-evidence" approach based on the following lines of evidence to support the registration of event DAS-81419-2 soybean:

1) Confirmation of biological equivalency of Cry1F and Cry1Ac proteins expressed in event DAS-81419-2 soybean plant and previously characterized microbial-derived Cry1F and Cry1Ac protein test substances;
2) No significant increase in exposure to NTOs based on calculated margins of exposure for protein concentrations in event DAS-81419-2 plant tissue matrices relative to the

dose concentrations used in the NTO laboratory toxicity tests conducted on Cry1Ac and Cry1F; and

3) No adverse effects on nontarget arthropods on a population and community level observed from a field monitoring study and other supporting supplemental information (e.g. meta-analysis, target feeding studies, and biological specificity to the target pest).

Exposure estimates were compared with endpoints reported from Tier I toxicity studies previously conducted on various representative nontarget test species to determine a Margin of Exposure (MOE), the ratio of the endpoint value to the EEC. The MOEs for representative NTOs were recalculated based on high-end exposure estimates (HEEEs) (US EPA, 2009) derived from Cry1Ac and Cry1F protein expression levels quantified from various plant tissues of event DAS-81419-2 soybean in a field study. A MOE of 50% mortality at 10X the estimated environmental exposure is regarded as sufficient to demonstrate negligible risk and was also the threshold for consideration of uncertainties in the risk assessment (US EPA 1998; US EPA 2007). If subsequent expression characterization in the commercial event leads to a safety margin of <10X, then the consequences are considered in terms of uncertainties in the ecological risk assessment below (US EPA 2007).

Table B.1. Summary of Environmental Effects Studies conducted for PIP Event DAS-81419-2 soybean (EPA Reg. No. 68467-EN) and Scientific Rationales in support for bridging Cry1F and Cry1Ac data from the Agency database on WideStrike® PIP cotton (EPA Reg. No. 68467-3)

OCSPP Guideline	Study Type	SUMMARY OF RESULTS AND CLASSIFICATION	MRID No.
WideStrike® PIP cotton data (EPA Reg. No. 68467-3)			
885.4150	Wild Mammal Testing, Tier I	Not required; Mammalian wildlife exposure to Cry1F and Cry1Ac proteins is considered likely; however, the results from two acute oral toxicity studies indicated no adverse effects on mice from microbial-derived Cry1F and Cry1Ac proteins administered via oral gavage at the maximum hazard dose. The NOAEL exceeded 600 mg/kg body weight for Cry1F (MRID No. 45542312) and 700 mg/kg bw for Cry1Ac (MRID No. 45542313). 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 Cry1F and Cry1Ac proteins is not expected. **Classification: ACCEPTABLE for data bridging**	45542312 and 45542313
850.1010	Aquatic Invertebrate Acute Toxicity Test, *Daphnia magna*	In a 48-hour static test with freshwater daphnids (*Daphnia magna*) there were no observed adverse effects with Cry1F and Cry1Ac in combination at respective concentrations of 510 and 2,500 µg/L. No immobility or other adverse effects were seen during the study. The 24-hour and 48hour EC50s for daphnia exposed to the Cry1F + Cry1Ac mixture were >510 µg Cry1F/L and >2500 µg Cry1Ac/L (represents a worst-case exposure of one kg of transgenic cotton pollen per liter of pond water). This rate of fortification represents 298X and >23,000X the anticipated EEC for Cry1F and Cry1Ac protein in surface water. Therefore, no hazard to aquatic invertebrates is expected from incidental exposure to WideStrike cotton pollen.	45808412

OCSPP Guideline	Study Type	SUMMARY OF RESULTS AND CLASSIFICATION	MRID No.
		Classification: ACCEPTABLE for WideStrike Cotton; SUPPLEMENTAL for DAS-81419-2 soybean.	
885.4280	Estuarine and Marine Animal testing, Tier I	Not required. Exposure in marine and/or estuarine environments is not expected to be significant.	N/A
885.4300	Nontarget Plant Studies, Tier I	Not required. *Bt* and its proteins are not known plant pathogens or toxins, and adverse effects in plants are not anticipated. Exposure to nontarget plants is expected to be minimal.	N/A
885.4380	Honey Bee Larva Testing, Tier I (*Apis mellifera*)	At 1.98 µg Cry1F + 11.94 µg Cry1Ac per mL sugar water no effect on survival of larvae to adult emergence was seen. The LC_{50} is >4X pollen expression. Therefore no hazard to honey bee larvae and adult bee emergence is anticipated. **Classification: ACCEPTABLE for data bridging**	45542316
885.4340	Nontarget Insect Testing, Tier I (*Nasonia vitripennis*)	At 5.2 µg Cry1F + 46.8 µg Cry1Ac per mL sugar water at 10 days, no effect of limit dose with LC_{50} > 13X pollen expression was seen on parasitic hymenoptera larva. Minimal exposure and no hazard to parasitic Hymenoptera from Cry1F and Cry1Ac proteins are expected. Testing of a species more common to cotton fields is recommended. **Classification: ACCEPTABLE for data bridging**	45808411
885.4340	Nontarget Insect Testing, Tier I (*Chryosperla camea*)	A dietary toxicity study on green lacewing (*Chrysoperla rufilabris*) larvae was conducted by presenting the green lacewings with a moth egg (*Sitotroga* sp.) diet incorporated with *Bt* Cry1F and Cry1Ac proteins. The dietary LC_{50} was >48.2 µg/g diet for Cry1Ac and >5.2 µg/g diet for Cry1F, which represented at least at 14X the concentration found in pollen. No effect was noted at the Cry1F and Cry1Ac protein levels expressed in pollen that would be encountered by green lacewings in the field. Because of questionable ingestion of the test material another species (e.g, minute pirate bug), which is more likely to be exposed, should be tested. **Classification: ACCEPTABLE for data bridging**	45808410
885.4340	Adult Lady Beetle Testing, Tier I (*Hippodamia convergens*)	At 300 µg Cry1F + 22.5 µg Cry1Ac per mL sugar water, no effect of limit dose with LC_{50}> 780X Cry1F pollen expression and > 8X Cry1Ac pollen expression on adult lady beetles. Based on these results, no hazard to *H. convergens* is expected when feeding on WideStrike® cotton pollen in the field. **Classification: ACCEPTABLE for data bridging**	45542315
885.4340	Collembola Chronic Dietary Toxicity Study, Tier I (*Folsomia candida*)	The combination of 709 µg Cry1F + 22.6 µg Cry1Ac per g diet and cotton leaf tissue showed no effect on adult survival and reproduction at up to 10X the anticipated field level of expression. Therefore, no hazard to decomposers represented by collembola is expected from exposure to WideStrike® cotton in the field. **Classification: ACCEPTABLE for data bridging**	45808409

OCSPP Guideline	Study Type	SUMMARY OF RESULTS AND CLASSIFICATION	MRID No.
OECD Guideline 207	Earthworm Toxicity Study, (*Eisenia foetida*)	A 14-day study on earthworms exposed to soils treated with microbial-produced Cry1Ac and Cry1F, individually and in combination, was performed. The 14-day LC50s were >247 mg a.i./kg for Cry1F; >107 mg a.i./kg for Cry1Ac, and > 247 mg a.i./kg Cry1F + >107 mg a.i./kg Cry1Ac in the test with the two proteins combined. There were no overt signs of toxicity to earthworms exposed to soils containing nominal concentrations of Cry1F and Cry1Ac at 50X the expected worst case EEC [this represents concentrations which are 792X and 5479X higher than the expected EEC for incorporation of defoliated cotton plants into the top 15 cm of soil]. **Classification: SUPPLEMENTAL**	45580701
885.5200	Expression in a terrestrial Environment Tier II	The soil half-life of the plant expressing Cry1F and Cry1Ac was estimated as 1.3 days in a laboratory study with a representative soil from a cotton growing region. The Cry proteins were not detectable after 14 days. These results verify that the Cry1F and Cry1Ac proteins degrade rapidly in cotton soil. **Classification: ACCEPTABLE for WideStrike Cotton; SUPPLEMENTAL for DAS-81419-2 Soybean.**	45556801
DAS-81419-2 Soybean data (EPA Reg. No. 68467-EN)			
885.4050	Avian Dietary Toxicity Study, Tier I (*Colinus viginianus*)	In a 14-day acute dietary toxicity study, Northern bobwhites (*Colinus virginianus*) were exposed to avian diet fortified with a 7.9:1.0 mixture of Cry1F and Cry1Ac proteins with a test substance concentration of 128 mg /kg body weight (24.52 mg a.i./kg body weight based on percent a.i.) by oral gavage. Results showed no apparent effects on mortality, sublethal observations, body weight, and food consumption at this treatment level. The 14 day acute oral LD_{50} for the test substance was > 128 mg /kg bw (24.52 mg a.i./kg body weight based on percent a.i.) and the 14 day NOEL for the test substance was > 128 mg /kg bw (24.52 mg a.i./kg body weight based on percent a.i.). Monogastric animals like birds cannot safely consume raw soybean seed since it contains endogenous antinutrients (Friedman *et al*., 1991). The estimated feed consumption per bird was 33 grams/day. Therefore, in relation to the daily diet, Cry1F was dosed at 0.660 mg AI/gm (660 ppm) and Cry1Ac was dosed at 0.083 mg AI/gm (83 ppm). The HEEE concentration of Cry1F in raw DAS-81419-2 soybean seed is 14.34 ng/mg dw (ppm) and 1.08 ng/mg dw (ppm) for Cry1Ac (MRID No. 48828413). The MOEs was calculated for Cry1F and Cry1Ac in the quail study relative to raw soybean seed consumed as 100% of the diet was 46X (660 ppm/14.34 ppm) and 77X (83 ppm/1.08 ppm), respectively, which provides a large safety margin even if bird could safely ingest raw soybean as their sole source of food. Thus, no adverse effects on avian wildlife are expected from incidental field exposure to DAS-81419-2 soybean expressing *Bt* Cry1F and Cry1Ac insecticidal proteins. **Classification: ACCEPTABLE**	48828410
885.4200	Freshwater Fish Testing, Tier I (*Onchorynchus mykiss*)	An acute oral dietary toxicity study on rainbow trout (*Onchorynchus mykiss*) was conducted to assess the effects of a basal fish diet fortified with a mixture of microbial-derived Cry1F and Cry1Ac proteins for eight days. No fish mortality and no sublethal effects were observed in any of the control or test treatment organisms during this study. Results show the 8-day LD_{50} value with rainbow trout was > 100 mg a.i./kg diet wet weight	48828411

OCSPP Guideline	Study Type	SUMMARY OF RESULTS AND CLASSIFICATION	MRID No.
		(>200 mg a.i./kg diet dry weight). The NOEC was reported as > 100 mg a.i./kg diet weight (>200 mg a.i./kg diet dry weight) based on the lack of mortalities, sublethal effects, and apparent lack of effects on standard length and body weight. In addition, exposure to freshwater fish in aquatic environments is also considered to be limited . In view of the lack of toxicity and minimal aquatic exposure, no adverse effects to freshwater fish as a result of the cultivation of DAS-81419-2 soybeans are expected. **Classification: ACCEPTABLE**	
Non-guideline	Nontarget Arthropod Census Field Study	A field study was conducted to monitor nontarget arthropods in fields of DAS-81419-2 soybean in comparison to non-transgenic fields for any statistically significant differences in the types of taxa collected and their abundance for determining any adverse effects on a community level to nontarget arthropods. DAS-81419-2 soybean, a near-isogenic non-transgenic control (Maverick) and four non-transgenic reference lines (DSR 3590; IL 3505; Porter 75148; Williams 82) were monitored in replicated field trials at two locations in the USA, Richland, Iowa and York, Nebraska. Each trial site included the six soybean lines arranged in a randomized complete block design with four replicate blocks. Three sampling methods were used to monitor arthropod abundance: 1) pitfall trapping, 2) sticky card trapping and 3) vertical beat sheet sampling. A total of 74 taxonomic groupings were detected and assessed across field sites. To determine biological relevance of any statistical differences, results were interpreted in the context of the magnitude of differences, observations in reference plots, and trend consistency across sampling methods, sampling periods, and locations. Analysis of data found no statistically significant differences of biological relevance in the nontarget arthropod populations present in fields planted with DAS-81419-2 soybean and the non-transgenic control soybean lines. **Classification: SUPPLEMENTAL**	48828412
Non-guideline	Environmental Risk Assessment Summary on Nontarget Arthropods	An environmental risk assessment was separately prepared by the registrant to summarize the potential for adverse effects of cultivating PIP event DAS-81419-2 soybean on nontarget arthropods associated with soybean agroecosystems with a special emphasis on beneficial taxa and threatened or endangered species. Dow requested data bridging to the existing ecotoxicological studies on the effects of *Bt* Cry1F and Cry1Ac proteins expressed in WideStrike® PIP cotton on representative nontarget insect species, including beneficials, pollinators, detritivores, and incidentals. Exposure estimates for Cry1F and Cry1Ac protein concentrations in PIP event DAS-81419-2 soybean were compared with endpoints reported from toxicity studies conducted for WideStrike® cotton on select test species to determine the MOE, the ratio of the endpoint value to the EEC. Most MOEs exceeded 10X the estimated environmental concentrations. None of the estimated exposure values exceeded NOECs for any of the tested species, indicating that the most sensitive individual in a population is not likely to be adversely affected. Other supplemental information (i.e. meta-analyses, biological specificity to the pest species, targeted toxicity feeding studies), the biology of the crop and lack of exposure (due to feeding habits/food preferences) support the conclusion of no adverse effects to NTO species from PIP event DAS-81419-2 soybean. Other supporting data includes environmental fate data	48828413

OCSPP Guideline	Study Type	SUMMARY OF RESULTS AND CLASSIFICATION	MRID No.
		of the Cry1Ac and Cry1F proteins, results from a field survey showing no effects on the populations of nontarget arthropods associated with PIP event DAS-81419-2, and supplemental information from the FIFRA Endangered Species Task Force databases for identifying threatened and endangered species that may be exposed to PIP event DAS-81419-2 soybean. **Classification: SUPPLEMENTAL** *A soil fate study is needed to demonstrate rapid protein degradation of Cry1F and Cry1Ac in a variety of soil types that are common in soybean cultivation as confirmatory data.*	
Non-guideline	Supporting document	A response to EPA Comments on Cry1F(synpro) ICP and Cry1Ac(synpro) insecticidal crystal protein was submitted for the acute oral toxicity study with the northern bobwhite (MRID No. 488284-10). Protein identity and analytical assays confirmed the equivalence of and their respective certificates of analysis were submitted to demonstrate the equivalence of Dow's test material #101811 to plant-produced protein and provide a justification of the test dosage. **Classification: SUPPLEMENTAL** to MRID No. 48828410	49044807
Non-guideline	Supporting document	A response to EPA Comments on Cry1F(synpro) and Cry1Ac(synpro) insecticidal crystal proteins was submitted to demonstrate the equivalence of Dow's test material #101811 to the plant-produced protein used in the 8-day dietary study with the rainbow trout (*Oncorhynchus mykiss*) WALBAUM (MRID No. 488284-11). **Classification: SUPPLEMENTAL** to MRID No. 48828411	49044808
Non-guideline	Supporting document	A response to EPA Comments on Cry1F(synpro) ICP and Cry1Ac(synpro) insecticidal crystal protein was submitted to address several data gaps in the registrant's environmental risk summary (MRID No. 48828413) for PIP event DAS-81419-2 soybean. Additional data was needed to complete the risk assessment on the environmental fate of Cry1F and Cry1Ac proteins expressed in PIP event DAS-81419-2 soybean. Supporting literature and data waiver rationales were presented for evaluating soil fate, gene flow and the potential for weediness. **Classification: SUPPLEMENTAL** to MRID No. 48828413	49044809

Additional support for a complete environmental risk assessment is gained from an overview of the assessment conducted to support the WideStrike® cotton EPA Sec. 3 registration (EPA Reg. No. 68467-3). A brief summary of the EPA's environmental risk assessment is provided in the following section:

C. WideStrike® cotton (lepidopteran active) Environmental Risk Assessment Summary (EPA Reg. No. 68467-3)

Potential adverse effects to nontarget organisms by Cry1F and Cry1Ac proteins have been reviewed in *Bt* Cry1F/Cry1Ac WideStrike® cotton BRAD (US EPA 2005). The following is a

summary of the environmental risk assessment for Cry1F and Cry1Ac. EPA performed risk assessments on plants, wild mammals, birds, fish, aquatic invertebrates, earthworms, terrestrial nontarget insects (including honey bee, parasitic wasps, green lacewings, ladybird beetle, springtails [Collembola toxicity/reproduction], and monarch butterflies), as well as field evaluations of the effects of Cry1F and Cry1Ac exposure on nontarget insects in cotton fields, soil degradation/persistence studies, and an endangered species impact assessment, particularly for Lepidoptera. In addition, gene flow and weediness assessments via pollen and Cry protein DNA uptake by plants were also performed. Cry1F and Cry1Ac protein in soil has been shown to degrade rapidly to very low levels. EPA concluded that there is sufficient information to believe that there is no risk from the uses of Cry1F and Cry1Ac cotton to nontarget wildlife, aquatic, and soil organisms. These studies were individually reviewed by the Agency and reported in Data Evaluation Reports (DERs). For detailed information of each study, refer to the DER according to its respective assigned MRID number in the associated environmental effects data summary tables for WideStrike® cotton (reviewed in US EPA 2005).

At present, the Agency is aware of no identified significant adverse effects of Cry1F and Cry1Ac proteins on the abundance of nontarget organisms in any population in the aquatic or terrestrial field environment. Field testing and field census data submitted to the Agency show minimal to undetectable changes in the beneficial insect abundance or diversity. To date the available field test data show that compared to crops treated with conventional chemical pesticides, the transgenic crops have no detrimental effect on the abundance of nontarget invertebrate populations. In addition, no direct or indirect effects on Federally listed endangered and threatened species or effects on their critical habitat are expected. The EPA has reviewed the potential for gene capture and expression of Cry1F and Cry1Ac proteins by wild or weedy relatives of cotton in the United States, its possessions or territories and has found that there is no significant risk in the United States, its possessions or territories (US EPA 2005).

In conclusion, the risk assessment found no unreasonable adverse effects on the environment from cultivation of WideStrike® cotton expressing Cry1F and Cry1Ac proteins for use as a PIP.

D. Protein Equivalence

The October 2000 SAP recommended that while actual plant material is the preferred test material, microbial-derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume cotton plant tissue and where large amounts of Cry protein are needed for maximum hazard dose testing. In support of event DAS-81419-2 soybean registration, several of the nontarget toxicological studies used microbial-produced, purified Cry1F and Cry1Ac protein test substances that were originally developed for the Widestrike® PIP cotton registration; in addition to plant tissue (lypholized leaf tissue) from event DAS-81419-2 soybean.

In support of bridging data from previously reviewed NTO toxicological studies conducted for WideStrike® cotton (US EPA 2005), the biochemical properties the microbial-derived Cry1F and Cry1Ac protein test substances were compared with plant-produced Cry1F and Cry1Ac proteins expressed in event DAS-81419-2 soybean to determine that the proteins from the two host sources were biologically equivalent. The proteins were characterized and equivalence was

evaluated based on a panel of analytical tests and assays. The results of these evaluations provide a detailed characterization of the Cry1F and Cry1Ac proteins isolated from event DAS-81419-2 soybean and confirmed their equivalence to the microbial-derived Cry1F and Cry1Ac proteins (MRID Nos. 48828403 and 49044803; reviewed in US EPA 2013). Therefore, the results of the NTO toxicity studies that utilized the microbial-derived Cry1F and Cry1Ac test protein substances can be bridged to support the environmental risk assessment of event DAS-81419-2 soybean. Furthermore, development of additional nontarget organism toxicity testing can be waived for PIP event DAS-81419-2 soybean and the results and conclusions from the environmental risk assessment conducted for the WideStrike® cotton PIP line can be bridged to support event DAS-81419-2 soybean (US EPA 2005).

E. Nontarget Organism Effects Cry1F and Cry1Ac insecticidal proteins expressed in Event DAS-81419-2 Soybean (EPA Reg. No. 68467-EN)

1. Avian Wildlife

The primary routes of exposure of birds to Cry1F and Cry1Ac insecticidal proteins expressed in PIP event DAS-81419-2 soybean plants expected to occur through-consumption of event DAS-81419-2 plant material (e.g., leaf foliage and pods) and invertebrates that feed on event DAS-81419-2 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 Cry1F and Cry1Ac proteins are contained primarily within the plant.

In a 14-day acute dietary toxicity study, Northern bobwhites (*Colinus virginianus*) were exposed to avian diet fortified with a 7.9:1.0 mixture of Cry1F and Cry1Ac proteins by oral gavage (MRID No. 48828410). Results showed no apparent effects on mortality, sublethal observations, body weight, and food consumption at this treatment level. The 14 day acute oral LD_{50} for the test substance was > 128 mg/kg bw (24.52 mg a.i./kg body weight based on percent a.i.) and the 14 day NOEL for the test substance was > 128 mg /kg bw test substance (24.52 mg a.i./kg body weight based on percent a.i.).

BPPD estimates the exposure to birds in the field for soybeans is minimal. Monogastric animals like birds cannot safely consume raw soybean seed since it contains endogenous antinutrients (Friedman et al., 1991). The estimated feed consumption per bird was 33 grams/day. Therefore, in relation to the daily diet, Cry1F was dosed at 0.660 mg a.i./gm (660 ppm) and Cry1Ac was dosed at 0.083 mg a.i./gm (83 ppm). The HEEE concentration of Cry1F in raw DAS-81419-2 soybean seed is 14.34 ng/mg dw (ppm) and 1.08 ng/mg dw (ppm) for Cry1Ac (MRID No. 48828413). The MOE was calculated for Cry1F and Cry1Ac in the quail study relative to raw soybean seed consumed as 100% of the diet was 46X (660 ppm/14.34 ppm) and 77X (83 ppm/1.08 ppm), respectively, which provides a large safety margin even if birds could safely ingest raw soybean as their sole source of food.

Furthermore, the Cry1F and Cry1Ac proteins have been registered as PIPs in several crops with no identified concerns for birds, and there is 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 the lack of avian

exposure, no adverse effects to birds are anticipated as a result of the registration of DAS-81419-2 Soybean.

2. Wild Mammals

Wild mammals could be exposed to Cry1F and Cry1Ac proteins produced by event DAS-81419-2 soybean plants by consumption of plant material and invertebrates that feed on event DAS-81419-2 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 Cry1F and Cry1Ac proteins from DAS-81419-2 soybeans to wild mammals. The results from the acute oral toxicity studies showed no significant toxicity to mice from acute oral testing with the Cry1F and Cry1Ac proteins, with a NOAEL that exceeds 600 mg/kg body weight for Cry1F (MRID No. 45542312) and 700 mg/kg for Cry1Ac (MRID No. 45542313). Based on the estimated expression level in leaf (V5) tissue (63.3 ng/mg dw) converted to dose (0.00153 mg/kg bw), adverse effects would not be expected at concentrations up to a MOE of $>3.9 \times 10^5$X for Cry1F. Based on the estimated expression level in leaf (V5) tissue (28.3 ng/mg dw) converted to dose (0.000683 mg/kg bw), adverse effects would not be expected at concentrations up to a MOE of $>1.0 \times 10^6$X for Cry1Ac. Thus, the MOEs greatly exceed the required 10X EEC showing no adverse effects when tested at the maximum hazard dose. 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 Cry1F and Cry1Ac proteins expressed in DAS-81419-2 Soybean is not expected.

3. Freshwater Animals

The registrant submitted an acute oral dietary toxicity study on rainbow trout (*Onchorynchus mykiss*) to assess the effects of consuming a basal fish diet fortified with a mixture of microbial-derived Cry1F and Cry1Ac proteins for eight days (MRID No. 48828411). No fish mortality and no sublethal effects were observed in any of the control or test treatment organisms during this study. Results show the 8-day LD_{50} value with rainbow trout was > 100 mg a.i./kg diet wet weight (>200 mg a.i./kg diet dry weight). The NOEC was reported as > 100 mg a.i./kg diet weight (>200 mg a.i./kg diet dry weight) based on the lack of mortalities, sublethal effects, and apparent lack of effects on standard length and body weight. In addition, exposure to freshwater fish in aquatic environments is also considered to be minimal.

For freshwater invertebrates, a 48-hour static toxicity study on daphnids (*Daphnia magna*) showed no adverse effects after exposure to WideStrike® cotton pollen expressing Cry1F and Cry1Ac in combination at respective concentrations of 510 and 2,500 µg/L (MRID No. 45808412). Based on a worst-case exposure of one kg of transgenic cotton pollen per liter of pond water, the rate of fortification at these concentration are 298X and >23,000X the anticipated EEC for Cry1F and Cry1Ac protein in surface water. However, this study was determined of minimal value for evaluating the potential effects to nontartget freshwater invertebrates because the length of the study was not conducted at the Agency's recommendation of at least 7-14 days, since the mode of action for *Bt* would likely take effect

after several days of exposure. In addition, EPA determined that exposure to Cry proteins in the freshwater environment were expected to be low, based on the assumption that exposure in aquatic environments primarily resulted from pollen deposition (see US EPA 2010a) and that post-harvest crop residue is the most likely route of exposure to aquatic organisms (Carstens *et al.* 2011). However, in light of published studies showing reduced growth in caddis flies exposed to anti-lepidopteran Cry1A protein (Rosi-Marshall *et al.* 2007), concerns regarding the potential for exposure to shredder invertebrate species via consumption of *Bt* crop plant litter in aquatic environments. EPA determined exposure to shredder invertebrate species from *Bt* crops is likely to be low, since the concentrations of Cry proteins in post-harvest crop tissues are limited temporally and spatially (Swan *et al*. 2009; Jensen *et al.* 2010; Wolt and Peterson 2010; Carstens *et al*. 2011). Nevertheless, EPA has required aquatic invertebrate testing lasting 7-10 days with *Daphnia* spp. to reduce uncertainty in past PIP registrations of *Bt* crops. A study with shredder species is even more appropriate since it would directly test the invertebrate species expected to be affected by *Bt* plant litter that may enter streams.

Specifically for soybean, the Agency has determined that the exposure of aquatic systems to Cry1F and Cry1Ac proteins produced in PIP event DAS-81419-2 soybean are negligible. Movement of Cry proteins from off-field pollen or post-harvest crop residues to aquatic habitats is not expected given that soybeans are primarily self-pollinated and cultivated soybean plants release pollen prior to flower opening (Abud *et al*. 2007; Caviness 1966). The Agency previously concluded that plant litter from both *Bt* corn and *Bt* cotton crops are not deposited in amounts high enough to result in adverse effects (US EPA 2001b). Since soybean produces less above-ground residue under cultivation (Green and Blackmer 1995), this conclusion is also made for the cultivation of DAS-81419-2 Soybean.

Based on the acute oral toxicity study on rainbow trout, adverse effects to freshwater fish are not anticipated as a result of the registration of Cry1F and Cry1Ac as expressed in event DAS-81419-2 soybean. Risk posed to aquatic invertebrates (including the aquatic Lepidoptera) through DAS-81419-2 cultivation was concluded to be much reduced due to a low likelihood of exposure (Carstens *et al.* 2011) and no verified instances of sensitive species. In view of the lack of toxicity and minimal aquatic exposure, the rainbow trout study and the data waiver rationale for freshwater invertebrates are considered to be adequate to support the conclusion that deployment of event DAS-81419-2 for use as a PIP in soybean poses little risk to aquatic organisms.

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 Cry1F and Cry1Ac for the registration of DAS-81419-2 Soybean, 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 ingredients are insect toxins (*Bt* δ-endotoxin) that have never shown any toxicity to plants. Therefore, BPPD

has concluded that adverse effects of Cry1F and Cry1Ac insecticidal proteins expressed in DAS-81419-2 Soybean to terrestrial and aquatic plants are not anticipated.

The active ingredients Cry1Ac and Cry1F are part of a larger group of insect toxins produced a naturally-occurring *Bacillus thuringiensis* (*Bt* δ-endotoxins) that have not shown toxicity to plants. Effects to nontarget plants in the terrestrial and aquatic environments are not expected, and BPPD concludes that adverse effects to terrestrial and aquatic plants as a result of the registration and use of Cry1F and Cry1Ac insecticidal proteins expressed in event DAS-81419-2 Soybean are not anticipated.

6. Invertebrate Species

BPPD assumes that nontarget insects receive exposure primarily through consumption of DAS-81419-2 Soybean leaf foliage, pods, and occasionally other soybean plant tissues as well as indirect exposure through consumption of insects that feed on soybean plant tissue. The principal route of exposure to soil-dwelling invertebrates, such as collembola and earthworms, is assumed to be consumption of decomposing plant tissue, and also possibly plant exudates, in soil during feeding.

The registrant requested data bridging to the existing ecotoxicological studies on the effects of *Bt* Cry1F and Cry1Ac proteins expressed in WideStrike® PIP cotton on representative nontarget species, including nontarget beneficial insects, pollinators, detritivores, and incidentals (MRID No. 48828413). As previously discussed, the Cry1F and Cry1Ac proteins produced in DAS-81419-2 soybeans were determined biologically equivalent with the microbial-derived Cry1F and Cry1Ac protein test substances used for producing toxicological data to support WideStrike® cotton (MRID No. 48828403 and 49044803; reviewed in US EPA 2013). The high-end exposure estimates (HEEEs) were derived from protein expression levels of Cry1F and Cry1Ac via Enzyme-linked immunosorbent assay (ELISA) analyses on various soybean plant tissue parts of PIP event DAS-81419-2. Exposure estimates for Cry1F and Cry1Ac protein concentrations in PIP event DAS-81419-2 soybean were compared with endpoints reported from toxicity studies previously conducted for WideStrike® cotton on various representative nontarget test species to determine a Margin of Exposure (MOE). The results of the MOE calculations are presented in Tables B.2. and B.3.

A MOE of 50% mortality at 10X the estimated environmental exposure is regarded as sufficient to demonstrate negligible risk and was also the threshold for consideration of uncertainties in the risk assessment (US EPA 1998; US EPA 2007). If subsequent expression characterization in the commercial event leads to a safety margin of <10X, then the consequences are considered in terms of uncertainties in the ecological risk assessment below (US EPA 2007). The ratio of the endpoint value to the EEC and are presented in Tables B.2. and B.3. for Cry1F and Cry1Ac, respectively. The published EPA level of concern is 50% mortality at 5× MEEC (US EPA 1998). It should also be noted that the dose margin can be less than 10× where uncertainty in the test system is low. 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 1× MEEC (US EPA 1998; US EPA 2007). The registrant note that the concentrations tested were much higher than those encountered under field conditions. Since most MOEs exceeded 10X the estimated

environmental concentrations (Tables B.2. and B.3.), the results of the MOE calculations indicates the most sensitive individual in a population is not likely to be adversely affected.

a. Beneficial Arthropods and Pollinators

Data from a dietary toxicity study on lady bird beetle previously conducted for WideStrike® cotton showed that adverse effects did not occur to the test insects at >300 µg/mL diet for Cry1F and >22.5 µg/mL diet for Cry1Ac assuming a maximum expected environmental concentration of 10.6 ng/mg fresh weight for Cry1F and 4.76 ng/mg fresh weight for Cry1Ac (see MRID No. 45542315). Dow did not analyze protein expression levels for pollen or soybean floral nectar, as Cry1 protein expression in this secretion in four cotton lines is negligible (Wolt 2002; MRID No. 45808420). Therefore, the MOE calculation for lady beetle relies on the highest level of protein expression found in the field expression study (V5 leaf). Based on the estimated expression level in leaf (V5) tissue, adverse effects would not be expected at concentrations up to 25.6X for Cry1F and 4.3X Cry1Ac.

The registrant requested data bridging to the dietary toxicity study on green lacewing (*Chrysoperla rufilabris*) larvae, which was conducted by presenting the green lacewings with a moth egg (*Sitotroga* sp.) diet incorporated with *Bt* Cry1F and Cry1Ac proteins (MRID No. 45808410). Results showed that adverse effects did not occur to the test insects at >48.2 µg/g diet for Cry1Ac and >5.2 µg/g diet for Cry1F. However, this study was considered supplemental. A previous EPA FIFRA Scientific Advisory Panel (SAP) determined that it is inappropriate to test the activity of insecticidal proteins by incorporating the *Bt* protein into a moth egg diet, since *Bt* protein may bind to the surface of the moth eggs, resulting in limited exposure to lacewings that feed with piercing sucking mouthparts (US EPA-SAP 2002). Furthermore, green lacewings do not consume much pollen in the field (US EPA-SAP 2002) and are not exposed to *Bt* proteins via consumption of aphids (Head *et al.* 2001). In the Agency's environmental risk assessment of WideStrike® cotton, this study was classified as supplemental and the Agency recommended other generalist predators (e.g. minute pirate bug) as a representative surrogate test organism for Tier I testing the effects of Cry1F and Cry1Ac proteins (US EPA 2005). Thus, the green lacewing study on the effects of Cry1Ac and Cry1F proteins expressed in DAS-81419-2 soybean can be bridged, but only offer supplemental information towards the environmental risk assessment of DAS-81419-2 soybean. Other information on the effects of Cry1Ac and Cry1F exist on green lacewing larvae concludes Cry1Ac and Cry1F toxins do not have a detrimental effect on the species when they are ingested either directly or through prey (Rodrigo-Simón 2006; Tian *et al.* 2013).

A dietary toxicity study that tested the effects of Cry1F and Cry1Ac expressed in DAS-81419-2 soybean in honey bee (*Apis mellifera*) was also cited for data bridging. Results showed no effect on survival of larvae to adult emergence at >1.98 µg/mL sugar water diet for Cry1F and >11.9 µg/mL sugar water diet for Cry1Ac (MRID No. 45542316). Pollen expression data for DAS-81419-2 soybean was not available, however, the MOE previously calculated for WideStrike® cotton showed the LC_{50} was >4X the levels of pollen expressed. These data can be inferred to represent the MOE for event DAS-81419-2 soybean, since the same genetic construct that was used to transform WideStrike® cotton was used for event DAS-81419-2 soybean and the constitutive expression in pollen should be similar. In addition, other information from a meta-analysis (Duan *et al.* 2008) of several laboratory acute oral toxicity studies on honeybee is

available, which consistently demonstrated no biological activity in Hymenoptera order of insects from Cry proteins that target lepidopteran pests. Six Cry1F and Cry1Ac studies were included in the meta-analysis, suggesting that regardless of expression level in event DAS-81419-2 soybean pollen the MOE will be acceptable.

An acute toxicity study on parasitic wasp species (*Nasonia vitripennis*) was cited for data bridging. Results showed adverse effects did not occur to the test insects at >5.2 µg/g diet for Cry1F, respectively, and >46.8 µg/g diet for Cry1Ac, which represented at least at 14X the concentration found in WideStrike® cotton pollen (see MRID No. 45808411). Similar to the green lacewing and a honey bee study, the MOE was not determined for the parasitic wasp species due to the lack of pollen and nectar expression data, and some uncertainty remains. . However, the HEEE of Cry1F and Cry1Ac from V5 leaf sample (as opposed to pollen) relative to the dose concentrations used on honeybees and nontarget arthropods (likely to be exposed via pollen ingestion) in toxicity studies is considered highly conservative and the margins of exposure (see Tables B.2. and B.3.) are well above dose levels of Cry1Ac and Cry1F protein toxicity testing showing no adverse effects.

The registrant noted that the concentrations tested were much higher than those encountered under field conditions. Since most MOEs exceeded 10X the estimated environmental concentrations (Tables B.2. and B.3.), the results of the MOE calculations indicates the most sensitive individual in a population is not likely to be adversely affected.

Further, in regards to exposure of Cry1F and Cry1Ac proteins expressed in event DAS-81419-2 soybean pollen, pollinators and other beneficial arthropods are anticipated to be less exposed based upon the cleistogamous nature of its flowers (in most cultivars). Soybean is predominantly self-pollinated and its pollen is essentially contained in the flower, since anthers usually dehisce and release pollen before flowers open (Caviness 1966; OECD 2000). Typical outcrossing rates are less than 1% (CPC 2004), so this suggests that contact of honeybees and other beneficial and predatory invertebrates to soybean pollen is minimal. So although the MOE to honeybees, green lacewings, and lady beetle were calculated from the HEEEs from Cry1F and Cry1Ac proteins in leaf tissue (V5) stage instead of pollen, the likelihood of actual field exposure to many nontarget invertebrates via pollen consumption or indirectly through consumption of pollen-feeding invertebrates is expected to be minimal. Since no pollen is expected to travel outside the field borders, off-field exposure to many nontarget invertebrates is further reduced.

In any case, no adverse effects were found on previously conducted toxicity testing of Cry1F and Cry1Ac proteins on representative nontarget beneficial arthropods and pollinators species (e.g. honeybees, lady beetles, green lacewings) based on the majority of the MOEs exceeded 10X the estimated environmental concentrations (Tables B.2. and B.3.). The results of the MOE calculations indicate the most sensitive individual in a population is not likely to be adversely affected. Risk to honeybees and the nontarget arthropods is further reduced due to minimal potential for exposure via pollen. Further, published meta-analysis of laboratory and field studies showed no significant differences were noted on populations of nontarget invertebrates on a community level for *Bt* crops expressing Cry1F and/or Cry1Ac vs. non-transgenic crops (Marvier, *et al* 2007; Duan *et al.* 2008). Lastly, an independent field census study showed no differences in the population of nontarget arthropods (MRID No. 48828412) in field sites containing event DAS-81419-2 soybean vs. non-transgenic conventional soybean lines. Among

the taxa showing no significant population impacts were two that were also tested in the laboratory: Coccinellidae (lady beetles) and Parasitica (parasitoid wasps). In view of the lack of toxicity and limited exposure via pollen consumption, no adverse effects are anticipated on nontarget beneficial arthropods and pollinators from the cultivation of PIP event DAS-81419-2 Soybean.

b. Soil Dwelling Invertebrates

An earthworm study conducted for WideStrike® cotton was also cited for data bridging. Earthworms (*Eisenia foetida*) were exposed to artificial soils treated with 247 mg/kg and 107 mg/kg of microbial-produced Cry1Ac and Cry1F proteins, respectively, for 14 days (MRID No. 45580701). The study showed no effects on mortality or burrowing time resulting from exposure to Cry1F and Cry1Ac present in artificial soil at approximately 716X for Cry1F and 1176X for Cry1Ac at the maximum EEC. The maximum EEC of the soil was 0.345 mg/kg for Cry1F and 0.091 mg/kg for Cry1Ac based on soybean plant residue in the top 15cm of soil.

Data from a toxicity study on Collembolas (*Folsomia candida*) previously conducted for WideStrike® cotton showed that adverse effects did not occur to the test insects at >702 mg/kg soil for Cry1F and >22.6 mg/kg soil for Cry1Ac (MRID No. 45808409). The MOE for Cry1Ac was calculated as >2035X and >248X for Cry1F, assuming a maximum EEC of 0.345 mg/kg for Cry1F and 0.091 mg/kg for Cry1Ac based on soybean plant residue in the top 15cm of soil. A lack of adverse effects following exposure to Cry1Ac protein has also been shown for collembolans (*Folsomia candida*) (Sims and Martin, 1997).

Based on the studies submitted for DAS-81419-2, as well as other information for Cry1F and Cry1Ac, risk to nontarget soil invertebrates resulting from the cultivation of PIP event DAS-81419-2 Soybean is not expected.

Table B.2. Margins of Exposure calculated from Cry1F Protein Concentrations in PIP Event DAS-81419-2 soybean plant tissues relative to Dose Concentrations used in the Nontarget Arthropod Ecotoxicological Studies previously conducted on WideStrike® PIP cotton plant- and microbial- derived Cry1F protein [a]

MRID No.	Study Type	Species	Tissue sample	HEEE[b]	Tox. Endpoint	MOE	Comment
45542316	Honey Bee Larva Testing, Tier I (885.4380)	Honeybee (*Apis mellifera*)	Pollen	ND	>1.98 µg/mL diet NOEC	ND	No effects found for honey bee larvae and adults in published meta-analysis with Cry1F and/or Cry1Ac expressing *Bt* crops (Duan *et al.* 2008)
45808410	Nontarget Insect Testing, Tier I (885.4340)	Green lacewing (*Chryosperla camea*)	Nectar, pollen	ND	>5.2 µg/g diet acute oral LC50	ND	No effects found in Cry1Ac and Cry1F protein lab study (Rodrigo-Simón *et al.* 2006) and tritrophic feeding study on green lacewings exposed to a diet of Cry1F or Cry1Ac resistant prey (Tian *et al.* 2013)
45542315	Nontarget Insect Testing, Tier I (885.4340)	Lady beetle (*Hippodamia convergens*)	Pollen (used V5 Leaf)	10.6 ng/mg fw	>300 µg/mL diet acute oral LC50	>25.6	Pollen expression data is not available; Highest expression in leaf converted to fw for MOE calculation.
45808411	Nontarget Insect Testing, Tier I (885.4340)	Parasitic wasp (*Nasonia vitripennis*)	Nectar	ND	>5.2 µg/mL diet acute oral LC50	ND	No feeding in narrow soybean flowers
45808409	Nontarget Insect Testing, Tier I (885.4340)	Collembola (*Folsomia candida*)	R3 Forage soil residue	0.345 mg/kg soil	>702 mg/kg soil NOEC	>2035	Plant residue in top 15 cm soil
45580701	Earthworm Toxicity Study	Earthworm (*Eisenia foetida*)	R3 Forage soil residue	0.345 mg/kg soil	247 mg/kg soil NOEC	716	Plant residue in top 15 cm soil

[a] Microbe-derived Cry1F protein used in laboratory studies was demonstrated to be equivalent with the Cry1F protein expressed in DAS-81419-2
[b] HEEE = mean expression level + ($t_{0.1,\ \text{upper tail},\ n-1}$ x std. dev.) / $n^{1/2}$. HEEE values based on Cry1F expression level determined for DAS-81419-2 plant tissue relevant to the potential exposure route for the organism of interest.
ND = Not Determined (data not available).
Table B.2 reproduced from Table 3, page 26 from MRID No. 48828413

Table B. 3. Margins of Exposure calculated from Cry1Ac protein concentrations in DAS-81419-2 Soybean plant tissues relative to dose concentrations used in the Nontarget Arthropod Ecotoxicological Studies previously conducted on WideStrike® PIP cotton plant- and microbial- derived Cry1Ac protein [a]

MRID No.	Study Type and OCSPP Guideline No.	Species	Tissue sample	HEEE[b]	Tox. Endpoint	MOE	Comment
45580701	Earthworm Toxicity Study	Earthworm (*Eisenia foetida*)	R3 Forage soil residue	0.091 mg/kg soil	107 mg/kg soil NOEC	1176	Plant residue in top 15 cm soil
45808409	Nontarget Insect Testing, Tier I (885.4340)	Collembola (*Folsomia candida*)	R3 Forage soil residue	0.091 mg/kg soil	>22.6 mg/kg soil NOEC	>248	Plant residue in top 15 cm soil
45542316	Honey Bee Larva Testing, Tier I (885.4380)	Honeybee (*Apis mellifera*)	Pollen	ND	>11.9µg/mL diet NOEC	ND	No effects found for honey bee larvae and adults in published meta-analysis with Cry1F and/or Cry1Ac expressing *Bt* crops (Duan *et al.* 2008)
45808410	Nontarget Insect Testing, Tier I (885.4340)	Green lacewing (*Chryosperla camea*)	Nectar, pollen	ND	>46.8 µg/g diet acute oral LC50	ND	No effects found in Cry protein lab study (Rodrigo-Simón *et al.* 2006) and tritrophic feeding studies on green lacewings exposed to a diet of Cry1F or Cry1Ac resistant prey (Tian *et al.* 2013)
45542315	Nontarget Insect Testing, Tier I (885.4340)	Lady beetle (*Hippodamia convergens*)	Pollen (used V5 Leaf)	4.76 ng/mg fw	>22.5 µg/mL diet acute oral LC50	>4.3	Highest expression in leaf converted to fw. No effect found in Cry1Ac feeding study in *Coleomegilla maculata* (Li *et al*. 2011)
45808411	Nontarget Insect Testing, Tier I (885.4340)	Parasitic wasp (*Nasonia vitripennis*)	Nectar	ND	>46.8 µg/mL diet acute oral LC50	ND	No feeding in narrow soybean flowers

[a] Microbe-derived Cry1Ac protein used in laboratory studies have been demonstrated to be equivalent with the Cry1Ac protein expressed in DAS-81419-2

[b] HEEE = mean expression level + ($t_{0.1,\ \text{upper tail},\ n-1}$ x std. dev.) / $n^{1/2}$. HEEE values based on Cry1Ac expression level determined for DAS-81419-2 plant tissue relevant to the potential exposure route for the organism of interest.

ND = Not Determined (data not available). Table B.3 reproduced from Table 3, page 26 from MRID No. 488284-13

7. Supplemental Information

Where a lack of expression data prevented calculation of high-end exposure values, other supplemental information (i.e. meta-analyses, biological specificity to the pest species, targeted toxicity studies, or food preferences) was used as a supporting line of evidence to support the conclusion of no adverse effects on NTOs from Cry1F and Cry1Ac proteins expressed in DAS-81419-2 soybean. To address any uncertainties in the risk assessment focused on taxonomic groups that were most likely to be exposed to Cry1F and Cry1Ac proteins expressed in PIP event DAS-81419-2 soybean in field settings, the potential for adverse effects of DAS-81419-2 on nontarget arthropod populations was assessed in a US field study (MRID No. 48828412). A nontarget arthropod field study with PIP event DAS-81419-2 soybean, a near-isogenic non-transgenic control (Maverick) and four non-transgenic reference lines (DSR 3590; IL 3505; Porter 75148; Williams 82) was conducted in 2011 at two field sites in the U.S. located in Richland, Iowa and York, Nebraska. Each trial site included the six soybean lines arranged in a randomized complete block design with four replicate blocks.

Arthropod abundance was sampled during the vegetative and reproductive soybean stages and monitored using three sampling methods: pitfall trapping, sticky card trapping, and vertical beat sheet sampling. Arthropods collected during the study included representatives from multiple orders and diverse ecological roles, including phytophagous, predatory, parasitic, and saprophagous modes of feeding. The analyses encompassed several taxonomic groups, including: Oribatida (soil mites), Araneae (foraging and web spiders), Collembola (springtails), Hemiptera (e.g. *Orius* spp.), Coleoptera (e.g. ground beetles, lady beetles), Neuroptera (e.g. *Chrysopa* spp.), Lepidoptera, Diptera, and Hymenoptera (e.g. parasitoid wasps). Consistent across locations and sampling methods, results showed arthropod populations associated with DAS-81419-2 soybean were similar to those of non-transgenic soybean. Therefore, the lack of differences in between populations of nontarget arthropod taxa and their abundance from the field monitoring suggests no adverse effects on nontarget arthropod communities from deployment of event DAS-81419-2 soybean cultivation.

F. Soil Fate

Since proteins are not known to persist in the environment due to the ubiquitous nature of proteases in microbes, the registrant submitted a rationale for demonstrating rapid degradation of Cry1F and Cry1Ac proteins in various crop soils. Data from a previously submitted laboratory study showed that Cry1F and Cry1Ac expressed in cotton plants degrades quickly and does not accumulate in soil for WideStrike® PIP cotton (MRID No. 45556801). The soil half-life of the plant expressing Cry1F and Cry1Ac was estimated as 1.3 days and the Cry proteins were not detectable after 14 days. These results verify that the Cry1F and Cry1Ac proteins degrade rapidly in soils sampled from cotton growing regions.

The registrant also provided a data waiver rationale to support rapid degradation of *Bt* Cry1F and Cry1Ac proteins in soil based on the history of safe use in sprayable *Bt* formulations. Cry1Ac is present in both WideStrike® cotton event DAS-21023-5 (also described as 3006-210-23) and Bollgard® cotton (event MON 531, as well as a discontinued corn event: DBT418Cry1Ac corn) (Mendelsohn *et al.* 2003; Sanahuja *et al.* 2011). Cry1F is also present in WideStrike® cotton

event DAS-24236-5 (also described as 281-24-236), as well as, in Herculex® and SmartStax® corn (event TC1507) (Mendelsohn *et al.* 2003; Sanahuja *et al.* 2011). Other supporting information included previously conducted field studies showing no accumulation (i.e. persistence) of the proteins as a result of continuous planting of other *Bt* crops containing these proteins (Head *et al.* 2002; Herman *et al.* 2002; Shan *et al.* 2008). As a result, the Agency has determined that no additional long-term field studies are required for PIP event DAS-81419-2 soybeans because currently registered PIPs have not been shown to persist in soil.

Although the registrant submitted a sufficient rationale of rapid degradation of Cry1F and Cry1Ac proteins in other crop soil systems, a soil degradation study has not been evaluated in soybean for this specific PIP event. A previous EPA FIFRA SAP noted that in soil degradation and field persistence studies, toxin degradation rates may vary with the crop, the toxin produced by the transformation event, the microbial community in the recipient soil microcosm, the statistical characterization of toxin persistence, and/or the initial dose of toxin in the experiment (US EPA 2001a). These differences in Cry toxin concentration may be due to the fact that different genetic constructs were used and/or inserted at different locations in the plant genome (Yu *et al*. 2012). Plant genotype and parental backgrounds have also been recognized as important factors influencing protein expression (Kranthi *et al.* 2005; Adamczyk and Sumerford 2001; and Torres *et al.* 2006). Other important factors that affect degradation of Cry toxins include environmental conditions such as temperature, light, drought, and soil properties (e.g. pH, texture, clay content) (Adamczyk *et al.* 2004; Chen *et al.* 2005; Jiang *et al.* 2006; Rochester 2006; Dong *et al.* 2008; Hallikeri *et al.* 2009; Addison and Rogers 2010; Chen *et al.* 2011; Ranjithkumar *et al.* 2011). To reduce this uncertainty, a soil fate study to determine the degradation rate (DT_{50}) of Cry1F and Cry1Ac proteins sampled from representative soils of soybean growing regions is to be conducted as confirmatory data.

G. 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 studies discussed in the WideStrike® cotton BRAD show Cry1F and Cry1Ac proteins degrade quickly in soil (US EPA 2005).

With regard to the impact of genetically engineered crops on soil, EPA has previously noted (US EPA 2010a) that agricultural practices themselves cause large changes in soil and soil microbial composition. Furthermore, factors such as variations in seasons and weather, plant growth stage, and plant varieties, independent of being genetically engineered, are also responsible for significant shifts in soil microbial communities. 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.

H. 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, or have their origin, in soil-inhabiting bacteria. Soil is also the habitat of other toxin-producing bacteria, and transfer of these genes and/or toxins to other microorganisms or plants has not been detected. There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Conner *et al.* 2003). 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 event DAS-81419-2 are derived from soil-inhabiting bacteria, the EPA has concluded that the risk of HGT of transgenes found in event DAS-81419-2 soybean expressing Cry1F and Cry1Ac proteins are low.

I. Gene Flow and Weediness Potential

The movement of transgenes from the host plant into weeds has been a significant concern for EPA due to the possibility of novel exposures to the pesticidal substance. This concern has been considered for each of the *Bt* plant-incorporated protectants currently registered, and was extensively reexamined in 2010 (see US EPA 2010a), and EPA believes that these concerns have been satisfactorily addressed.

The FIFRA EPA Scientific Advisory Panel meeting held on October 18-20, 2000 discussed the matter of gene flow and offered some issues for consideration in this matter. The panel agreed that the potential for gene transfer between *Gossypium hirsutum* (cotton) and any receptive plants within the U.S., its possessions and territories, was of limited probability and nearly risks free (US EPA 2000). Since soybean is also predominantly a self-pollinating species, the dispersal of pollen is limited because the anthers mature in the bud and directly pollinate the stigma of the same flower (OECD 2000). Thus, the risk of potential gene transfer between soybean and any receptive plants is further negligible. Lastly, there are no known wild or weedy relatives exist in the U.S. with which *Glycine max* (L.) Merr. can form viable hybrids in nature, and soybean is not weedy in character or invasive (US EPA 2007). Therefore, 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.

J. Endangered Species Considerations

Because of the selectivity of Cry1F and Cry1Ac insecticidal crystal proteins 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 PIP event DAS-81419-2 soybean plants or consumption of DAS-81419-2 pollen that falls on non-soybean plants within

the soybean field and its immediate margins. However, oral exposure to significant amounts of Cry1F and Cry1Ac via pollen consumption is not likely. Little pollen is expected to be released from soybean flowers, since soybean plants are predominantly 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). 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 DAS-81419-2 soybean plants on the field or via tritrophic effects, in which predatory beneficial arthropod species consume soybean pest species feeding on PIP event DAS-81419-2 soybean plant tissues.

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, 2004b and 2010a and b) and in the endangered species assessment section in the BRAD for event MON87701 soybean expressing Cry1Ac protein (see US EPA 2010c). As previously discussed, soybean pollen is not expected to be deposited on plants in or around soybean fields in amounts sufficient to cause effects in sensitive lepidopterans. Ecology and life history information demonstrated that habitat requirements for larvae and adults of two of the identified species (Mitchell's Satyr butterfly and Saint Francis' Satyr butterfly) do not overlap with commercial soybean acreage, and are therefore not expected to be impacted by PIP event DAS-81419-2 cultivation (Bartel and Sexton 2009; Barton and Bach 2005; US FWS 1997). There is a possible overlap between the geographic range for Karner blue butterfly and soybean use sites. 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) (US EPA 2001b). Karner blue larvae feed only on lupines (*Lupinus* spp.) and soybean is neither identified as a larval food source, nor is soybean open-pollinated. Thus, pollen from event DAS-81419-2 soybean is highly unlikely to be deposited on lupine leaves (NatureServe 2013). Furthermore, soybean is not identified as a nectaring source for Karner blue adults. Therefore, the risk posed to the Karner blue butterfly was considered to be negligible.

Based on the above analysis, the EPA determines that there will be no direct effect to listed lepidopteran species as a result of the cultivation of DAS-81419-2 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 DAS-81419-2 plants, are not currently known. Since the Cry1F and Cry1Ac in DAS-81419-2 soybeans targets only lepidopteran insects, loss of the pest insects as a result of DAS-81419-2 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 Cry1F and Cry1Ac. Effects on species other than insects have also been determined to be very unlikely because of the specificity of Cry1F and Cry1Ac.

Lastly, since the Agency previously determined Cry1F and Cry1Ac proteins are not expected to have adverse effects on mammals, birds, plants, freshwater and estuarine/marine fish and invertebrates, nontarget insects and other invertebrate species at the EEC, a "No Effect" determination is made for direct and indirect effects to Federally listed threatened and endangered species and their designated Critical Habitats. In addition, EPA does not expect that any threatened or endangered plant species will be affected by outcrossing to wild relatives or by competition with such entities. Hybrid soybean does not exist in the wild in the United States, nor do any wild plants that can interbreed with soybean in the United States.

K. Data Needed to Confirm DAS-81419-2 Soybean Environmental Fate Assessment

Although the registrant submitted a sufficient rationale of rapid degradation of Cry1F and Cry1Ac proteins in other crop soil systems, the degradation rate (DT_{50}) of Cry1F and Cry1Ac proteins sampled from representative soils of soybean growing regions has not been evaluated and must be conducted as confirmatory data. The soil degradation study is to be submitted to the EPA within 18 months of the issuance date of the DAS-81419-2 Soybean Notice of Pesticide Registration.

II. Conclusion

The biological equivalency of Cry1F and Cry1Ac proteins expressed in event DAS-81419-2 soybean plant was confirmed with the previously characterized microbial-derived Cry1F and Cry1Ac protein test substances. No significant increase in exposure to NTOs was demonstrated based on calculated margins of exposure for protein concentrations in event DAS-81419-2 plant tissue matrices relative to the dose concentrations used in the NTO laboratory toxicity tests conducted on Cry1Ac and Cry1F. Lastly, a field monitoring study showing no differences on nontarget arthropods on a population and community level and other supporting supplemental information (e.g. meta-analysis, target feeding studies, and biological specificity to the target pest) provided additional certainty to support the "weight-of-evidence" that there is negligible risk to NTOs representing diverse taxonomic groups and exposure scenarios from the cultivation of DAS-81419-2 soybean.

Therefore, the submitted data supports the applicant's request and the findings from the environmental risk assessment conducted for WideStrike® cotton can be bridged to support a breeding/seed increase registration for PIP event DAS-81419-2 (US EPA 2005). Based on extrapolating from previous studies on *Bt* proteins, which tend to be very species specific in their activity, the Agency concludes that the cultivation of event DAS-81419-2 soybean will not result in adverse effects to nontarget organisms, are not likely to persist in the soil, and pose no risk of gene flow and development of weediness in wild relatives, and additionally will have no effect, direct or indirect, on Federally listed threatened and endangered species and their designated Critical Habitats.

APPENDIX C: Insect Resistance Management

Cry1Ac and Cry1F proteins as DAS-81419-2 Soybean

Discussions of EPA's risk assessments for Insect Resistance Management (IRM) are presented in this Appendix. Immediately below is the risk assessment completed by EPA's A. Reynolds on November 7, 2013.

I. Insect Resistance Management Review

Below is the BPPD review of Dow's Insect Resistance Management (IRM) plan and supporting data for the application to register DAS-81419-2 Soybean PIP for seed production and research purposes, as well as supporting documentation concerning natural refuge for *Bt* cotton (U.S. EPA memorandum 11/7/13; EPA File Symbol 68467-EN, MRID 49024110 replaced MRID 48828415).

CONCLUSION AND REQUIREMENTS

1) Because DAS-81419-2 soybean is intended for breeding/seed increase, Dow did not propose a species-specific IRM plan for the product. Rather, the company proposed to mitigate resistance risk by acreage limitations on a county (1,000, 10,000, or 25,000 acres) and national (250,000 acres) scale. BPPD agrees that given the low acreage projected by Dow, it is unlikely that there will be a significant risk of resistance to the main soybean lepidopteran pests in the United States. These insects include soybean looper, velvetbean caterpillar, beet armyworm, cotton bollworm, green cloverworm, tobacco budworm, and cutworms (*Agrotis* spp.). This conclusion is further supported by the biology of the target insects, which are highly polyphagous (feeding on a number of wild hosts and cultivated crops) and (for some species) have limited overwintering in non-tropical areas.

2) Should a full commercial registration or expanded acreage be sought in the future, a complete, species-specific IRM assessment for DAS-81419-2 will be required. Data needed to support such a review should include additional information on pest biology, dose, simulation modeling, cross resistance, resistance monitoring, and potential impacts on the natural refuge strategy for *Bt* cotton.

3) The use of DAS-81419-2 on a limited basis for seed increase purposes should not significantly impact the natural refuge strategy in place for *Bt* cotton PIPs. Soybean is one of the non-cotton crops that have been considered as part of natural refuge for tobacco budworm and cotton bollworm. While it can be expected that plantings of DAS-81419-2 soybean will remove some of the currently-available natural refuge for *Bt* cotton, this potential reduction will likely be small relative to the total amount of natural refuge present in southern cotton regions. Dow has proposed to further mitigate the impact on natural refuge by limiting DAS-81419-2 to 10,000 acres in counties with > 25,000 total acres of soybean and 1,000 acres in counties with < 25,000 total acres of soybean.

4) Dow must institute a stewardship program for DAS-81419-2 including resistance monitoring and remedial action (in the event of resistance). A resistance monitoring plan limited to investigations of unexpected pest damage is appropriate for a capped acreage registration. BPPD notes that monitoring of two potential target insects, tobacco budworm and cotton bollworm, is already being conducted as part of the WideStrike *Bt* cotton monitoring program. Dow must also compile annual sales and acreage data for each state and provide a report to EPA if requested. Since DAS-81419-2 will be deployed without a structured refuge requirement, a compliance assurance plan and grower education program should not be necessary.

IRM Rationale and Proposed Plan for DAS-81419-2 *Bt* Soybean (MRID No. 49024110)

Dow's proposed DAS-81419-2 soybean is intended for breeding, seed production, and research in the United States. Therefore, acreage of the product grown in the United States will be limited to a total of 250,000 per year. Individual counties would also be annually limited to either 20,000 (in non-cotton growing regions), 10,000 (in cotton growing counties with at least 25,000 acres of soybean), or 1,000 acres (in cotton-growing counties with < 25,000 soybean acres).

Dow's IRM rationale for DAS-81419-2 is based on the limited commercialization of the product in the United States. The company reasons that selection pressure should be limited by the acreage caps and notes that EPA has not mandated refuges for similarly-capped *Bt* corn breeding registrations. Further, the product provides two modes of action (Cry1F and Cry1Ac) for control of lepidopteran pests.

Soybean is one of the lepidopteran crop hosts that have been considered as natural refuge for *Bt* cotton. Dow's acreage proposal for DAS-81419-2 could therefore reduce the amount of soybean available for cotton refuge in areas where both crops are planted. To estimate the potential effects of DAS-81419-2 on natural refuge, Dow calculated the refuge contribution of soybean relative to the amount of cotton as such: [(acres of soybean – proposed DAS-81419-2 acres)/(acres of soybean + cotton) * 100]. DAS acreage was set at either 10,000 or 1,000 per county as described above. Acreage data for county cotton and soybean were obtained from USDA-NASS (http://quickstats.nass.usda.gov/) for the 2010 season. Calculations were conducted for all counties with plantings of both soybean and cotton in the states of Alabama, Arkansas, Georgia, Kansas, Louisiana, Mississippi, Missouri, North Carolina, Oklahoma, South Carolina, Tennessee, and Georgia.

A total of 183 counties were surveyed by Dow (data contained in Tables 1-7 in MRID No. 49024110). Of these, 84 had at least 25,000 acres of soybean planted during the 2010 season. In these counties up to 10,000 acres of DAS-81419-2 has been proposed. Even with this potential reduction in refuge, the amount of available soybean refuge averaged 62% among the 84 counties. Dow noted that this level of refuge is well above the 20% structured refuge that was required for *Bt* cotton prior to approval of natural refuge. In addition, commercial soybean fields are rarely treated for cotton bollworm which should further enhance production of susceptible insects.

The remaining 99 counties analyzed by Dow, with less than 25,000 total soybean acres, would have a limit of 1,000 DAS-81419-2 acres. Some of these counties grow little soybean (< 2000 acres) such that the relative contribution to natural refuge from the crop is likely low, regardless of the adoption of DAS-81419-2. Other counties grow larger amounts of soybean; however, in these counties full adoption of DAS-81419-2 (1,000 acres) would still have a small impact on natural refuge.

Dow indicated a number of other factors add to the conservatism of the IRM rationale:

- Seed production for soybean (the primary intended purpose for DAS-81419-2 in the U.S.) occurs in areas where the crop is favored – i.e., in counties with large amounts of soybean (>25,000 acres).
- Counties with not as much soybean (<25,000 acres) are less likely to be used for seed production. Nonetheless, a "regional" natural refuge may provide susceptible insects from other counties since *H. zea* is known to be highly mobile (Jackson et al. 2008).
- Soybean for seed production (non-commercial production) is frequently treated with insecticides for pod-feeding insects. Therefore, seed production soybean is likely to be limited in value as natural refuge regardless of the adoption of DAS-81419-2.
- In addition to soybean, *H. zea* is known to utilize numerous other crop and plant hosts including corn, peanuts, sorghum, and weed species. Accordingly, natural refuge for the insect is not limited solely to soybean acres and the county refuge estimates provided by Dow will be underestimates of the actual amounts.

BPPD Review

Dow's proposal to limit the overall acreage and distribution of DAS-81419-2 in the U.S. should be sufficient to mitigate potential resistance development for a seed increase registration. It should be noted, however, that BPPD does not have sufficient information in the submitted materials to completely assess the risk of resistance for a full commercial registration of DAS-81419-2.

Target Pests

Dow's submission did not indicate the primary insect pests targeted by DAS-81419-2 soybean. BPPD presumes this is because the product is intended for commercialization in South America; U.S. plantings will be solely for seed production. Nonetheless, DAS-81419-2 can be expected to confer protection against susceptible soybean-feeding lepidoptera while grown for seed production in the U.S.

Soybeans can be affected by a number of lepidopteran insects, including (among others) soybean looper (*Pseudoplusia includens*), velvetbean caterpillar (*Anticarsia gemmatalis*), beet armyworm (*Spodoptera exigua*), cotton bollworm/corn earworm (*Helicoverpa zea*), green cloverworm (*Plathypena scabra*), and cutworms (*Agrotis* spp.). Though not common on soybean, tobacco budworm (*Heliothis virescens*) has also been known to infest the crop (Sheck and Gould 1993). Other occasional lepidopteran pests include fall armyworm (*Spodoptera frugiperda*) and saltmarsh caterpillar (*Estigmene acrea*).

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. Soybean looper is known to feed on a wide range of vegetables, field crops (including corn and cotton), and weeds in addition to soybean (Capinera 2005).

Velvetbean caterpillar, similar to soybean looper, 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). Velvetbean caterpillar is polyphagous and feeds on a number of field crops (soybean, alfalfa, cotton, peanut, other legumes) and weeds (Barbara 2008).

Green cloverworm also follows a southern overwintering/migration pattern from Gulf Coast areas. The insect feeds on a variety of legumes, including soybean, and has up to four generations per season (http://ipm.ncsu.edu/AG271/soybeans/green_cloverworm.html).

Cotton bollworm and tobacco budworm are polyphagous Noctuid species that are primary target pests of *Bt* cotton registrations (see descriptions in BPPD 2001 and 2006). Fall armyworm and cutworms are secondary pests targeted by some *Bt* corn registrations (see BPPD 2010).

IRM Risk Considerations

No data were provided in the submission to evaluate the efficacy of DAS-81419-2 against any of the potential U.S. target pests. Published data for another Bt soybean cultivar (TIC107 with a *cry1A* gene) showed high activity against velvetbean caterpillar and soybean looper (McRae et al., 2005). Cry1Ac and Cry1F are also known to have toxicity to cotton bollworm and tobacco budworm (BPPD 2005), while fall armyworm is affected by Cry1F (BPPD 2010). However, without specific data for DAS-81419-2, it is not possible to evaluate dose considerations for the product or determine the resistance risk for targeted insect pests.

Rather than formulate an insect-specific IRM approach for DAS-81419-2, Dow has proposed acreage limitations to lower selection pressure for resistance development. A number of other factors should further reduce the risk of resistance such that a formalized refuge strategy is unnecessary. These considerations include biological aspects of soybean lepidopteran pests, the abundance of alternative hosts (natural refuge) for these insects, the pyramiding of two toxins in DAS-81419-2, and anticipated limited plantings of the product for seed increase purposes.

Most of the lepidopteran insects that infest soybean 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. Further, soybean looper, velvetbean caterpillar, and fall armyworm overwinter only in tropical areas (e.g., Florida in the U. S.); population migrate annually from the Caribbean, Mexico and Central America northward through the U.S. Other key pests, cotton bollworm and green cloverworm, overwinter in southern Gulf Coast states. Because of this, it is less likely that any resistant individuals that evolve during the growing season would be able to successfully overwinter and propagate the resistant trait to future generations.

As a pyramid, DAS-81419-2 expresses two toxins (Cry1Ac and Cry1F) for lepidopteran control. Pyramided PIPs are thought to increase the respective durability of each toxin relative to mosaics of single toxin PIPs (Roush 1998, Caprio 1998, Zhao et al. 2003, others). BPPD notes that the potential for some cross resistance exists between Cry1Ac and Cry1F in cotton bollworm and tobacco budworm (Gould et al. 1995; BPPD 2005, 2010), which could limit the effectiveness of the pyramid for those insects. Nonetheless, pyramiding two toxins in DAS-81419-2 should further decrease the likelihood of resistance development.

Acreage limitations have been employed for seed/breeding registrations issued for Bt corn PIPs as a means to lower overall selection pressure for resistance. These restrictions have been implemented on county (up to 20,000 acres) and national level (250,000 acres total). Dow's proposed acreage for DAS-81419-2 generally follows this standard, with up to 25,000 acres per county (250,000 nationally) except in counties where soybean is currently planted (see discussion below).

Based on the proposed acreage-limited (breeding) registration and in consideration of the biological aspects of the soybean pests discussed above, BPPD concludes the risk of resistance to Cry1Ac and Cry1F in DAS-81419-2 should be low. Therefore, a refuge strategy is not recommended at this time for DAS-81419-2. However, should any request be made to expand the scope of DAS-81419-2 to a commercial registration (with large or unlimited acreage), BPPD recommends 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.

Considerations for *Bt* Cotton Natural Refuge

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 highly polyphagous and are known to exploit numerous crop and wild plant hosts. Cry1Ac and Cry1F are expressed in both DAS-81419-2 and WideStrike *Bt* cotton (Bollgard II also expresses Cry1Ac).

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.

To evaluate potential impacts to cotton natural refuge, Dow conducted an analysis of counties in southern states growing soybean. The company tabulated soybean acreage in 2010 (USDA NASS data) and projected the impact DAS-81419-2 would have on the natural refuge contribution of soybean. In the surveyed counties with large soybean acreage (>25,000), full adoption of DAS-81419-2 would still leave a sizable (average 62%) contribution to natural

refuge from non-Bt soybean. As could be expected, the potential reduction in natural refuge was greater in counties with less than 25,000, particularly those with less than 5,000 acres. Soybean contribution in 19 counties (of 99 total) would be reduced to less than 10% with the full addition of DAS-81419-2. In counties with less than 1,000 soybean acres (3 total), refuge contribution from soybean could potentially be eliminated. Generally, soybean refuge was lower in Georgia and Texas than the other surveyed states. This was largely because these states each have a number of counties with low overall soybean acreage but relatively high (> 20,000) cotton planting.

Dow's analysis was limited by considering only soybean as a contributor to cotton natural refuge. BPPD agrees with the company that numerous other crops (e.g., tobacco, peanut, corn, sorghum, alfalfa) and wild or weedy hosts that support TBW and CBW are present in cotton-growing regions. As such, Dow's assessment of the impact of DAS-81419-2 on natural refuge was likely highly conservative and underestimated the amount of refuge relative to cotton.

Nonetheless, it can be expected that plantings of DAS-81419-2 soybean will remove some of the currently-available natural refuge for *Bt* cotton. But this potential reduction will likely be small relative to the total amount of natural refuge available in southern cotton regions. As part of the natural refuge assessment in 2006-2007, BPPD reviewed extensive host utilization data developed by Monsanto (see BPPD 2006, 2007; Jackson et al. 2008). TBW and CBW were sampled for host preference by testing insect bodies for the presence of bound gossypol (TBW) or insect wings for the ratio of C3 to C4 plant isotopes (CBW). These data showed that TBW and CBW in cotton-growing areas fed much more frequently on non-cotton hosts than on cotton. In many of the sampled states, 50% or more of sampled TBW developed on crops other than cotton. While soybean is a component of these non-cotton hosts, it is unlikely that it is the sole (or even a majority) source of natural refuge, given the relatively low county acreages (< 25,000 soybean acres in most counties). This is the case in Georgia, where low soybean acreage (relative to cotton) in Dow's analysis appeared to indicate a small natural refuge contribution from soybean (< 10% in many counties). However, Monsanto's host utilization data clearly showed a high proportion of natural refuge in the state, as very few sampled TBW (0 to 10%) developed on cotton (MRID No. 46717201, reviewed in BPPD 2006, 2007).

BPPD's assessment of the impact of DAS-81419-2 on *Bt* cotton natural refuge is contingent upon the acreage projections provided by Dow (as discussed in the previous section). A full commercial registration of DAS-81419-2 without acreage limitations would require a more detailed assessment potentially including revised natural refuge calculations and simulation modeling.

Stewardship Activities

BPPD's review indicated that other aspects of IRM including resistance monitoring and remedial action plan are needed for DAS-81419-2. A full resistance monitoring plan with pest sampling and detection bioassays should not be warranted for a seed increase registration; rather an approach based on investigations of unexpected pest damage reports should be appropriate. In addition, both TBW and CBW are monitored for Cry1Ac and Cry1F susceptibility as part of the WideStrike cotton monitoring program (see BPPD 2005). A remedial action plan, in the event of

documented resistance, could mimic those in place for *Bt* corn or cotton or could rely on a "stop sale" approach if resistance develops. It was also noted that Dow will need to compile DAS-81419-2–specific annual sales and total acreage data for each state and provide a report to EPA if requested. Since DAS-81419-2 will be deployed without a structured refuge requirement, a compliance assurance plan and grower education program should not be necessary.

APPENDIX D: References

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Appendix E. Bibliography of data submitted for registration of *Bacillus thuringiensis* Cry1Ac and Cry1F Proteins expressed in Event DAS-81419-2 Soybean.

Table E. 1. Data Submitted for Cry 1Ac and Cry1F proteins expressed in DAS-81419-2 Soybean

MRID No.	Citation	Receipt Date
49024100	Dow AgroSciences LLC (2012) Submission of Pesticide Use, Product Chemistry and Toxicity Data in Support of the Application for Registration of DAS-81419-2 Soybean. Transmittal of 10 Studies.	18-Dec-2012
49024101	Unknown (2012) Supplement to MRID 488284-01: Molecular Characterization of DAS/81419-2 Soybean. Unpublished study prepared by Dow AgroSciences. 5p.	18-Dec-2012
49024102	Embrey, S. (2012) Characterization for Soy Leaf Tissue Samples (TSN303074, TSN303075). Project Number: 120416. Unpublished study prepared by Dow AgroSciences LLC. 2p.	18-Dec-2012
49024103	Unknown (2012) Supplement to MRID 48828409: Similarity Assessment of Cry1Ac Protein to Known Toxins by Bioinformatics Analysis: (DAS-81419-2 Soybean). Project Number: 120416. Unpublished study prepared by Dow AgroSciences LLC. 1p.	18-Dec-2012
49024104	Unknown (2012) Supplement to MRID 48848216: Characterization of the Cry1Ac Protein Derived from Transgenic Soybean Event DAS-81419-2. Project Number: 110840. Unpublished study prepared by Dow AgroSciences LLC. 3p.	18-Dec-2012
49024105	Unknown (2012) Supplement to MRID 48828417: Characterization of the Cry1F Protein Derived from Transgenic Soybean Event DAS-81419-2. Unpublished study prepared by Dow AgroSciences LLC. 3p.	18-Dec-2012
49024106	Unknown (2002) Certificate of Analysis for Test/Reference/Control Substances (TSN103748): (DAS-81419-2 Soybean). Project Number: BIOT023159. Unpublished study prepared by Dow AgroSciences LLC. 7p.	18-Dec-2012
49024107	Unknown (2012) Supplement to MRID 488284-10: An Acute Oral Toxicity Study with the Northern Bobwhite: (DAS-81419-2 Soybean). Unpublished study prepared by Dow AgroSciences LLC. 1p.	18-Dec-2012
49024108	Unknown (2002) Certificate of Analysis for Test/Reference/Control Substances (TSN103748): (DAS-81419-2 Soybean). Project Number: BIOT023159. Unpublished study prepared by Dow AgroSciences LLC. 7p.	18-Dec-2012
49024109	Unknown (2012) Supplement to MRID 48828413: Potential Impact of Cry1F and Cry1Ac in DAS-81419-2 Soybean on Non-Target Organisms and Threatened and Endangered Species. Unpublished study prepared by Dow AgroSciences LLC. 2p.	18-Dec-2012
49024110	Storer, N. (2012) Insect Resistance Management Implications of Seed-Production and Research Registration for DAS-81419-2 Soybean - Revised. Project Number: IRM/2012/08. Unpublished study prepared by Dow AgroSciences LLC. 19p.	18-Dec-2012
49044800	Dow AgroSciences, LLC (2013) Submission of Product Chemistry, Efficacy, Toxicity and Exposure/Risk Data in Support of the Application for Registration of DAS-81419-2 Soybean. Transmittal of 9 Studies.	25-Jan-2013

49044801	Storer, N. (2012) Insect Resistance Management Implications of Seed-Production and Research Registration for DAS-81419-2 Soybean - Revised. Project Number: IRM/2012/08. Unpublished study prepared by Dow AgroSciences, LLC. 19p.	25-Jan-2013
49044802	Guttikonda, S. (2013) Response to EPA Comment on Molecular Characterization of DAS-81419-2 Soybean Study - Provide Information Concerning the Source of the Promoters and Terminators; Provide the Actual construction of the Cry Proteins; Related to MRID 488284-01. Project Number: IRSOY/2013/01. Unpublished study prepared by Dow AgroSciences, LLC. 8p.	25-Jan-2013
49044803	Han, L. (2013) Response to EPA Comment on Biological Equivalency of (DAS-81419-2) and Microbe-Produced Cry1Ac and Cry1F Proteins Study - Provide Explanation to Establish the Equivalence of Dow"s Cry1Ac and Cry1F Proteins to those Expressed in the Soybean; Identify the Leaf Powder Diluent Used in the Insect Bioassays: Related to MRID 488284-03. Project Number: IRSOY/2013/02. Unpublished study prepared by Dow AgroSciences, LLC. 8p.	25-Jan-2013
49044804	Han, L. (2013) Response to EPA Comment on Similarity Assessment of Cry1Ac Protein to Known Toxins Bioinformatics Analysis Study - Identify the Protein Toxin Source; Explain the Error on Page 11 of 16: Related to MRID 488284-09. Project Number: IRSOY/2013/03. Unpublished study prepared by Dow AgroSciences, LLC. 5p.	25-Jan-2013
49044805	Han, L. (2013) Response to EPA Comment on Characterization of the Cry1Ac Protein Derived from Transgenic Soybean Event DAS-81419-2 Study - Address Amino Acid Sequence Similarity between Dow"s Cry1Ac Microbial Protein and Soybean-Produced Cry1Ac; Provide a Replacement for the Illegible Figure on Page 55: Related to MRID 488284-16. Project Number: IRSOY/2013/04. Unpublished study prepared by Dow AgroSciences, LLC. 8p.	25-Jan-2013
49044806	Han, L. (2013) Response to EPA Comment on Characterization of the Cry1F Protein Derived from Transgenic Soybean Event DAS-81419-2 Study - Address Amino Acid Sequence Similarity between Dow"s Cry1F Microbial Protein and Soybean-Produced Cry1F: Related to MRID 488284-14. Project Number: IRSOY/2013/05. Unpublished study prepared by Dow AgroSciences, LLC. 7p.	25-Jan-2013
49044807	Herman, R. (2013) Response to EPA Comment on Cry1F(Synpro) ICP and Cry1Ac(Synpro) ICP: An Acute Oral Toxicity Study with the Northern Bobwhite - Provide Data Demonstrating the Equivalence of Dow""s Test Material #101811 to Plant-produced Protein; Provide a Justification of the Test Dosage: Related to MRID 488284-10. Project Number: IRSOY/2013/06. Unpublished study prepared by Dow AgroSciences, LLC. 14p.	25-Jan-2013
49044808	Han, L. (2013) Response to EPA Comment on Cry1F(Synpro) and Cry1Ac(Synpro) Insecticidal Crystal Proteins: An 8-Day Dietary Study with the Rainbow Trout Oncorhynchus mykiss, WALBAUM - Provide Data Demonstrating the Equivalence of Dow"s Test Material #101811 to Plant-produced Protein: Related to MRID 488284-11. Project Number: IRSOY/2013/07. Unpublished study prepared by Dow AgroSciences, LLC. 13p.	25-Jan-2013
49044809	Han, L. (2013) Response to EPA Comment on Potential Impact of Cry1F and Cry1Ac in DAS-81419-2 Soybean on Non-target Organisms and Threatened and Endangered Species Study - Provide Gene Flow and Weediness Information to Address Environmental Fate Concerns; Address Soil Degradation: Related to MRID 488284-13. Project Number: IRSOY/2013/08. Unpublished study prepared by Dow AgroSciences, LLC. 6p.	25-Jan-2013
49081500	Dow AgroSciences, LLC (2013) Submission of Pesticide Use Data in Support of the Application for Registration of DAS-81419-2 Soybean. Transmittal of 1 Study.	14-Mar-2013
49081501	Han, L. (2013) Amended Response to the EPA Comment on Characterization of the Cry1F	14-Mar-

	Protein Derived from Transgenic Soybean Event DAS-81419-2 Study - Address Amino Acid Sequence Similarity Between Dow's Cry1F Microbial Protein and Soybean-Produced Cry1F. Project Number: IRSOY/2013/05. Unpublished study prepared by Dow AgroSciences, LLC. 7p.	2013