C. ENVIRONMENTAL ASSESSMENT

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

1. Gene Flow and Weediness

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

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

The FIFRA EPA Scientific Advisory Panel meeting held on October 18-20, 2000 further discussed the matter of gene flow and offered some issues for consideration in this matter. The

panel agreed that the potential for gene transfer between corn (maize) and any receptive plants within the U.S., its possessions and territories was of limited probability and nearly risk free. Similarly, potatoes were seen as nearly risk free with regards to gene flow in that proximity of compatible wild relatives to this crop is insufficient to allow for cross pollination. Some concern was expressed, however, with respect to *B.t.* cotton grown in areas where wild relatives or feral populations of the crop are known to exist.

a. *B.t.* Corn Plant-Incorporated Protectants

1) Gene Transfer - Gene Flow

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

2) *Zea mays* ssp. *mays* - Maize - General Biology

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

A recent study has indicated that cross-pollination of commercial maize cultivars at 100 ft downwind from the source of genetically modified maize was 1 % and this proportion declined exponentially to 0.1 % at 130 ft and further declined to 0.03 % at 160 ft. At 1000 ft, the farthest distance measured, no cross-pollination was detected (Jemison and Vayda, 2000). For production of Foundation Seed, a distance of 660 ft has been generally required to ensure separation of pollen types. The relatively large size of corn pollen and its short viability period under most conditions preclude long distance transfer for purposes of outcrossing (Schoper, personal communication,1999). Under conditions of high temperature or low humidity, corn

pollen may only survive for a matter of minutes. Under more favorable conditions in the field or with controlled handling in the laboratory, pollen life may be extended to several hours.

3) *Tripsacum* species - Gama Grass - General Biology

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

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

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

Even though some *Tripsacum* species occur in areas where maize is cultivated, gene introgression from maize under natural conditions is highly unlikely, if not impossible (Beadle, 1980). Hybrids of *Tripsacum* species with *Zea mays* are difficult to obtain outside of the

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

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

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

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

4) *Zea* species - Teosintes - General Biology

Teosintes, specifically *Z. mays* ssp. *mexicana* (Schrader) Iltis, *Z. mays* ssp. *parviglumis* Iltis and Doebley, *Z. mays* ssp. *huehuetenangensis* (Iltis and Doebley) Doebley, *Z. luxurians* (Durieu and Ascherson) Bird, *Z. perennis* (Hitchc.) Reeves and Mangelsdorf and *Z. diploperennis* Iltis, Doebley and Guzman, have co-existed and co-evolved in close proximity to maize in the

Americas over thousands of years; however, maize and teosinte maintain distinct genetic constitutions despite sporadic introgression (Doebley, 1990).

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

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

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

Consultation with botanists and agronomists familiar with Texas flora suggested that no teosinte populations exist in the state (Benz, personal communication, 2000; Read, personal communication, 2000; Orzell, personal communication, 2000; Wilson, personal communication, 2000). Further, given the day length characteristics of *Z. diploperennis*, it is highly unlikely a sustaining population would result from introduction of this species. *Z. mays* ssp. *mexicana, Z.*

mays ssp. *parviglumis, Z. luxurians* and *Z. diploperennis* may cross with maize to produce fertile hybrids in many instances (Wilkes, 1967). None of these teosinte species have, however, been shown to be aggressive weeds in their native or introduced habitats (John Schoper, personal communication, 1999). Except for special plantings as noted above, teosinte is not present in the U.S. or its territories. Its natural distribution is limited to Mexico, Honduras, Nicaragua, El Salvador and Guatemala.

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

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

5) Conclusions

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

Many of the *Zea* species loosely referred to as “teosintes” will produce viable offspring when crossed with *Zea mays* ssp. *mays*. None of these plants are known to harbor weedy characteristics and none of the native teosinte species, subspecies or races are considered to be

aggressive weeds in their native or introduced habitats. In fact, many are on the brink of extinction where they are indigenous and will be lost without human intervention (*i.e*., conservation measures). Further, none of the landraces or cultivated lines of *Zea mays* are considered to have weedy potential and are generally considered to be incapable of survival in the wild as a result of breeding practices (*i.e*., selection) during domestication of the crop.

b. *B.t.* Cotton Plant-Incorporated Protectants

EPA has reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories. EPA has concluded that there is a possibility for gene transfer in limited geographic locations where wild or feral cotton relatives exist.

1) *Gossypium* - The Genus

Approximately 50 species of cotton are typically considered as within the genus *Gossypium* L., including both diploid and tetraploid species, although some authors offer a more conservative estimate of 32 species (Hortus Third, 1976; see also Seelanan *et al*., 1997). Species are indigenous to Africa, Central and South America, Asia, Australia, the Galapagos Islands, and Hawaii (Reinisch *et al.*, 1994). Plants include annuals, shrubs and small trees with generally palmately lobed, simple leaves, 1 to several axillary flowers, and 5 lobed flower petals. The fruits are loculocidal capsules, 3 to 5 celled and produce seeds typically covered in a close tomentum (fuzz) or a loose, wooly tomentum (lint). Flowers are ephemeral in that they generally open in the morning and fade the same day. *G. tomentosum* is an exception in that flowering is nocturnal and stigmas are typically not receptive during the day.

Domesticated *Gossypium* species often exist as feral populations that are self-sustaining in their native or introduced habitats in the tropics and subtropics. Although capable of persisting in disturbed areas, such as beaches or adjacent areas along the coast, this group does not contain any species considered to be noxious or problematic weeds in the U.S., its possessions or territories (Wendel, 2000[B]). Cotton and related congenerics do not withstand cold temperatures and would not overwinter in the temperate areas of the United States. A review of the weeds of the world list for *Gossypium*, notes only *G. tomentosum* of Hawaii as a weed (Holm *et al*. 1979). This species is considered as on the decline, however (Meredith, 2000).

Seven different genome types have been designated within the genus based upon chromosomal pairing and other cytogenetic characteristics. These are designated as A, B, C, D, E, F, and G. Of the Old World cottons, *G. arboreum* and *G. herbaceum* are the species grown for fiber in Africa and Asia, but not in the U.S. or its territories or possessions. Both are diploid species (2n = 26) and share a considerable complement of their A genomes with *G. hirsutum*, or Upland Cotton, a New World species. They are considered to have shared a common ancestor with *G. hirsutum.*

There are five species of New World allotetraploids which share the A-D genome complement: *G. barbadense*, *G. darwinii*, *G. hirsutum*, *G. mustellinum*, *G. tomentosum*. Of these, *G. barbadense*, *G. tomentosum*, and, of course, *G. hirsutum* are found in the United States or its possessions and territories. All are interfertile to some degree.

2) *Gossypium hirsutum* / *G. barbadense* - General Biology

G. hirsutum, or Upland Cotton, grows as an annual or perennial herb or shrub, typically 5 ft in height, but occasionally taller in its perennial habit. Seeds are produced in an ovoid, beaked capsule, 3 to 5 celled, which splits in a loculocidal manner and contains copious lint (Hortus Third, 1976). Upland Cotton is grown as an annual across much of the southern U.S. and has been the subject of numerous agronomic and genetic studies aimed at varietal improvement. Fibers of Upland Cotton are well suited to textile applications and the species is the most widely grown crop for fiber and is also an important source of food oils and seed meal / hulls.

Seeds of Upland Cotton and Pima Cotton, *G. barbadense*, typically require some form of treatment to ensure adequate germination. This may take the form of heat treatment, particularly in hard-seeded Pima types, and a sulfuric acid de-linting treatment to remove fuzz or linters from the seed coat. De-linting can also be done mechanically, but is most often performed chemically; failure to remove residual lint or fuzz can complicate the mechanics of planting as seed will aggregate. Additionally, those seeds that may escape from cultivation in the U.S., during transport of cotton at harvest for example, do not give rise to persistent populations due to the seed treatment requirements and the competition of multiple plants from seed that is heavy and not commonly dispersed by animals or wind. The requirement for significant moisture also prohibits growth of escapes in many locations and those that do survive set few or no seeds (Bassett, 2000). Even in areas of significant rainfall (*e.g.*, Mississippi), escaped Upland Cotton has not been able to establish itself due to its poor colonizing ability (Percy, 2000).

Upland Cotton, *G. hirsutum*, is an allotetraploid New World species with a chromosome complement of 2n = 4x = 52 in euploid accessions. This species is a product of two genomes, A and D, designated as AA-DD, which derive from Old World and New World ancestry, respectively. Likely progenitors of this species are *G. arboreum* and *G. herbaceum*, both Old World diploids, although it is clear they were not the direct ancestors of Upland Cotton. A single polyploidization event involving contribution of A genome from the ancestral maternal parent and the D genome from the ancestral paternal parent is apparently responsible for the evolution of *G. hirsutum* (Cronn *et al.*, 1999). Data from phylogenetic and sequence divergence analysis indicate that the American A-D genome (of the New World allotetraploids) last shared a common ancestor with the African-Asian A genome approximately 5 to 10 million years ago (Wendel, 2000[B]).

3) *Gossypium* spp. in the United States, Territories, and Possessions

There are four species of cotton, *Gossypium*, in the United States. Two of them, *Gossypium hirsutum* (upland cotton) and *Gossypium barbadense* (sea island cotton, pulpulu haole, Pima), are used commercially and escaped plants can be found growing in the wild in climates where they can survive the winter (*e.g.*, Mexico, Caribbean basin) and have access to adequate water supply (*e.g.*, in or near creek beds). In addition, two native wild species of *Gossypium* occur in the United States: *G. thurberi* Todaro and *G. tomentosum* Nuttall ex Seeman.

Upland cotton, *G. hirsutum*, is cultivated throughout the world and is present in many southern U.S. locales from Virginia across the Gulf states as far north as Missouri and west to California. Feral populations of *G. hirsutum* exist in southern Florida, U.S. Virgin Islands, and possibly Puerto Rico, but are not known to persist elsewhere in the U.S. or its possessions and territories. Pima cotton, *G. barbadense*, is also found in the Carribean, including the Virgin Islands. The semi-wild cotton of the Virgin Islands may constitute an introgression of genetic components from *G. hirsutum* and *G. barbadense* (Wendel, 2000[A]). Upland Cotton is genetically compatible with *G. barbadense* or Pima Cotton, also a tetraploid, and will produce viable, fertile progeny when crossed. Alleles specific to *G. barbadense* were found at a low frequency in feral *G. hirsutum* populations in the tropics and subtropics in areas where they are sympatric (Ellstrand *et al.*, 1999).

G. thurberi Todaro (*Thurberia thespesiodes* Gray) occurs in the mountains of Southern Arizona and northern Mexico at 2,500 to 5,000 feet (rarely at 7000 feet), and is rather common on rocky slopes and sides of canyons in late summer and autumn. The diploid species *G. thurberi* is not found in the areas where cotton is grown (*i.e.*, desert valleys) and the progeny would be sterile due to their triploid state if gene flow did occur with Upland or Pima Cottons. Attempts to cross the two deliberately (*G. hirsutum*) with *G. thurberi* as the female parent were unsuccessful (Stewart, 1992). Additionally, the flowering periods of the commercial cotton and *G. thurberi* are primarily incongruous. Any gene exchange between plants of *Gossypium hirsutum* and *Gossypium thurberi*, if it did occur, would result in triploid (3x = 39 chromosomes), sterile plants because *G. hirsutum* is an allotetraploid (4x = 52 chromosomes), and *G. thurberi* is a diploid (2x = 26 chromosomes). Such sterile hybrids have been produced under controlled conditions, but they would not persist in the wild; in addition, fertile allohexaploids (6x = 78 chromosomes) have not been reported in the wild.

The second wild native species, *Gossypium tomentosum*, occurs in Hawaii on the six islands of Kahoolawe, Lanai, Maui, Molokai, Nihau and Oahu (Stephens, 1964). Upland, Hawaiian and Pima cotton are all tetraploids (4x = 52) that can crossbreed. Introgression has been claimed for what one author considered hybrid swarms of *G. barbadense* x *G. tomentosum*, but conclusive proof of this is lacking. *G. tomentosum* is a tetraploid capable of forming fertile hybrids with *G. hirsutum* despite some fertility or compatibility factors (Stelly, 2000). Winter nursery seed increases on any of these islands could result in further exposure of wild *G. tomentosum* to cultivated species which will cross readily as all are tetraploids of the A-D genome type. It has been the policy of this Agency to preclude the culture of *B.t.*-cotton in Hawaii for this reason.

Unfortunately, the culture of non-modified cotton poses a threat to the biological diversity of this species and introgression of sequences from *G. barbadense* and *G. hirsutum* have likely occurred previously. As *G. tomentosum* may bloom at the same time as domestic cotton, there is no guarantee of either geographic or temporal isolation. For these reasons, EPA imposed stringent sales and distribution restrictions on the registration for cotton expressing the Cry1Ac delta-endotoxin grown in Hawaii. The Agency required the following labeling statement to mitigate the potential for the *cry1Ac* gene to move from cultivated cotton to *G. tomentosum*:

> "Not for commercial sale or use in Hawaii. Test plots or breeding nurseries established in Hawaii must be surrounded by either 12 border rows of non-*B.t.* cotton if the plot size is less than 10 acres or 24 border rows if the plot is over 10 acres and must not be planted within 1/4 mile of *Gossypium tomentosum*."

With respect to gene flow between varieties and species of *Gossypium*, four conditions need to exist: (1) sexual compatibility between the parents, (2) the periods of fecundity or style receptiveness / anthesis must coincide, (3) a vector capable of moving the pollen between the parents must be present, (4) the progeny of the cross must be fertile and viable in the environment in which they develop (Stewart, 1992). Although all species of *Gossypium* are self-fertile, they require an insect vector for cross-pollination as wind dissemination of pollen is not a factor.

There are only five areas in the United States, and its territories and possessions wherein cultivated cotton has the opportunity to outcross to wild or feral species which are genetically compatible: (1) southern Arizona, (2) Hawaiian islands, (3) southern Florida (Stewart, 2000), (4) U.S. Virgin Islands, and (5) Puerto Rico.

G. thurberi (Arizona Wild Cotton), is present in the elevated regions of Arizona and does not grow in areas of commercial cotton production. *G. thurberi* is a diploid and produces sterile, triploid progeny when crossed with the tetraploids *G. hirsutum* or *G. barbadense* (Percival, 2000).

In the very south of Florida, feral *G. hirsutum* exists in apparently self-sustaining populations (Percival, 2000; Wendel, 2000[A]). Since these would readily cross with cultivated cotton, sale of *B.t.*-Cotton is restricted south of Interstate 60. There is currently no commercial cotton production in the southern part of Florida.

Evidence from germplasm collections indicates that feral *G. barbadense* and possibly *G. hirsutum* exist in the Carribean, including Puerto Rico and the U.S. Virgin Islands (Meredith, 2000; Percy, 2000). There is presently no production of commercial cotton in either of these places, hence, outcrossing is not an issue at this time.

4) Pollination of Upland and Pima Cotton

The presence of these feral populations representing sexually compatible recipients of insect vectored pollen from commercial plantings of cotton may be mitigated by instituting isolation distance requirements in conjunction with border rows of non-*B.t.* cotton. Isolation distances applied to commercial seed production (*i.e.*, Foundation, Registered or Certified seed) vary with region and are designed to minimize cross-pollination between types, but not to absolutely exclude it.

G. hirsutum is self-pollinated in the absence of insects, but is readily cross-pollinated in the presence of appropriate insect vectors, such as the bumble bees of the genus *Bombus*, *Mellisodes* bees or the honey bee, *Apis mellifera* (McGregor, 1976). The potential for cross pollination between *G. hirsutum* and other relatives in the immediate vicinity is dependent on a variety of factors including ploidy, presence of insect vectors, use of broad-spectrum insecticides, temporal synchrony of anthesis, and distance between plants. Many species of *Gossypium* are interfertile, but some are predominantly inbreeding species by design and would not readily outcross with other species in a natural setting (Wendel, 2000[A]).

During the cultivation of cotton, *G. hirsutum* and *G. barbadense*, for commercial seed production, various states have instituted requirements and standards to preclude pollen-directed gene flow between species and varieties. For example, in Arizona the minimum distance mandated by the Arizona Crop Improvement Association (Simons, 2000) between fields containing different species (*i.e*., Pima vs. Upland), or between varieties differing substantially in leaf type, is 1320 feet (Simons, 2000). For varieties which differ in lint color (all classes), the distance increases to 5280 feet and requires a buffer zone of at least 100 feet of border rows or an intervening field.

In California, fields producing Foundation or Registered seed must be isolated at least 1320 feet from any other variety of similar type[1] or 2640 feet with an additional 20 buffer rows from other varieties of widely different types[2] (California Crop Improvement Association, 2000). Colored cotton shall be isolated from white cotton by a distance of at least 1 mile, as long as there is an intervening field at least 250 feet wide (100 rows) of cotton covering the full length of the colored cotton field. In the absence of an intervening field of this size, the required distance increases to 3 miles. For Foundation and Registered seed production, the field inspection must not reveal more than 1 in 45,000 off-types or the certification cannot be maintained. Seed

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[1] similar type - all upland cottons of the species *G. hirsutum* that are not naturally colored, including acala, delta, okra leaf, transgenic or non-transgenic, are considered as similar to one another, and widely different to Pima cottons *(G. barbadense*).

[2] widely different type - all Pima cottons of the species *G. barbadense* that are not naturally colored, transgenic or non-transgenic, shall be considered as similar types to one another, and widely different types to Upland cottons (*G. hirsutum*).

classified as Certified seed under the California guidelines may contain 1 in 9000 off-types.

Using a plot design as would be applied to certified seed production, Umbeck *et al.* (1991) measured the movement of pollen from a genetically modified Upland Cotton variety to border rows of *G. hirsutum* (non-modified) by assaying for a novel, introduced gene, neomycin phosphotransferase (*npt*II). A plot 136 x 30 m was surrounded by 25 m of commercial cotton on all sides to act as a recipient of transgenic pollen from the test plot. Progeny were tested for introgression of an active, intact *npt*II gene by evaluating F_1 seed germination in the presence of kanamycin and by the polymerase chain reaction. A consistent and significant reduction in pollen dissemination was noted as the distance from the test plot increased. Approximately 7 m away from the plot, outcrossing decreased from 5 % to < 1%. Within the remaining border rows, a small, but detectable (< 1%) number of fertilizations occurred out to a distance of 25 m. Containment was considered to have been achieved to a level appropriate for this transgenic field test. Since flowers were sampled with respect to height on the plant (*i.e*. age) and no correlations were noted with respect to position, it was concluded that there were no seasonal effects that were observed relative to pollen dispersal.

The inability of plants or seeds of either of *G. hirsutum* or *G. barbadense* to survive freezing temperatures restricts their persistence as perennials or recurrent annuals to tropical and sub-tropical areas. Feral *G. hirsutum* occurs in parts of southern Florida in the Everglades National Park and the Florida Keys. Cotton is not grown commercially in these areas at this time (*i.e.*, cultivated cottons are found in the northernmost portions of the state), but the containment provisions of the initial registration must continue for areas in Florida where feral cotton occurs. Wild cotton is a potential concern as it may increase the spread of resistance in Florida (with intensive vegetable production). EPA imposed sale and distribution restrictions on *B.t.* cotton in Florida, restricting its use to those sites North of Tampa (Route 60). The Agency is satisfied that the planting restrictions on *B.t.* cotton (*i.e*., no *B.t.* cotton south of Tampa) will mitigate concerns for gene transfer to wild cotton:

"In Florida do not plant south of Tampa, (Florida Route 60)."

Studies underway at the University of California Davis, Biotechnology Seed Center, indicate that outcrossing between cotton plots was detectable at 5,475 feet, the longest distance measured in this seed production area (Sundstrom, 2001). A continuation of this study with added design to simulate areas of low and high pollinator activity will provide greater information on pollen flow as this study progresses.

As noted in the FIFRA SAP report, the isolation distances typically required for seed production may not be adequate for mitigating pollen flow between crop types or between crops and their wild relatives. Additionally, the use of non-*B.t.* cotton as a trap crop surrounding *B.t.* cotton fields may not be sufficient in itself to preclude gene flow due to the insect vectors associated with pollination of this genus.

5) Potential Impacts of Gene Flow in Cotton

A wild relative of *Gossypium hirsutum* (Upland cotton) and *G. barbadense* (Pima cotton) exists in Hawaii on at least six of the islands (Stephens, 1964). This wild cotton, *G. tomentosum*, is pollinated in a nocturnal fashion, typically at dusk. The pollinators for this species are not known and may be the same or different from those insects pollinating cultivated *G. hirsutum* on the islands. Although it is presumed that the insect vectors for cultivated cotton are the same in the continental U.S. and in Hawaii, this has not been established (Wendel, 2001). Much of the basic biology of *G. tomentosum* remains to be discovered. Some efforts are underway to look at evidence of previous introgression of loci from cultivated cotton into *G. tomentosum* germplasm from collections made over 15 to 25 years ago.

G. barbadense is found in the Carribean, including the Virgin Islands. The semi-wild cotton of the Virgin Islands may constitute an introgression of genetic components from *G. hirsutum* and *G. barbadense* (Wendel, 2000). Upland Cotton is genetically compatible with *G. barbadense* or Pima Cotton, also a tetraploid, and will produce viable, fertile progeny when crossed. Alleles specific to *G. barbadense* were found at a low frequency in feral *G. hirsutum* populations in the tropics and subtropics in areas where they are sympatric (Ellstrand *et al.*, 1999). Currently there does not appear to be any commercial cultivation of cotton on these islands. Puerto Rico is also known to contain feral populations of *G. hirsutum* and *G. barbadense*, or possibly hybrid swarms of the two species (Wendel, 2001). Although the precise distribution of these feral populations is not known with certainty, they are apparently common in this territory.

6) Gene Flow in Cotton - Mitigation

Current Agency restrictions in Hawaii preclude the sale and use of *B.t.* cotton for commercial planting in this state: "Not for commercial sale or use in Hawaii. Test plots or breeding nurseries established in Hawaii must be surrounded by either 12 border rows of non-*B.t.* cotton if the plot size is less than 10 acres or 24 border rows if the plot is over 10 acres and must not be planted within 1/4 mile of *Gossypium tomentosum*." The use of test plots for gathering field data following issuance of an experimental use permit or permission for a seed increase in the vicinity of *G. tomentosum* populations could have the same net effect (*i.e.*, gene flow). In light of the lack of basic biological data on *G. tomentosum* (*e.g.*, pollinator ecology, compatibility / sterility factors, potential impact of *B.t.* on herbivores, distribution of native populations), conservative measures may be needed to mitigate hybridization with cultivated cotton. If complete isolation and prevention of gene flow is desired, then test plot plantings of *B.t.* cotton in Hawaii may require a minimum 3 mile distance from *G. tomentosum* with 24 border rows of non-*B.t.* cotton surrounding the plots or exclusion from planting in this state. In situations where breeding nurseries and genetic purity are at stake, border rows surrounding the test plots consisting of a suitable floriferous malvaceous species which utilizes similar pollinators as *G. hirsutum* would lessen the possibility of cotton pollen moving out of these test plots and nurseries. These border plants would serve as a pollinator 'trap' to reduce long range pollen dissemination by insects and

must include a species genetically incompatible with upland cotton. Additionally, the flowering period of the border trap crop must by congruous with that of the breeding nursery in order to be effective. However, if these nurseries and test plots are frequently treated with chemical insecticides likely to eliminate all pollinators in the immediate area, the possibility of pollen movement would be negligible.

A restriction on the planting of *B.t.* cotton in Florida south of Route 60, near Tampa, precludes any chance of outcrossing with feral *Gossypium* spp. due to the extremely large distance between any commercial plantings and wild populations in the extreme south of the state. Presently there are no restrictions on planting of *B.t.* cotton in the Virgin Islands or Puerto Rico, both of which are known to have extant populations of wild *G. hirsutum* or *G. barbadense*. In the former case, no commercial plantings of cotton or test plots of *B.t.* cotton are planned for the U.S. Virgin Islands, hence, gene flow is not a concern there. Planting of *B.t.* cotton should be restricted, however, if prevention of gene flow is desired. Puerto Rico has seen use as a winter nursery for some commercial cotton breeding and the populations of wild *Gossypium* are apparently commonly distributed throughout the island. Similar restrictions, as imposed for Hawaii, would be appropriate in order to reduce the possibility of cotton pollen moving from the nurseries to wild cotton plants in this situation based upon the distribution of wild populations around the island and the propensity of insect pollinators to move substantial distances,. In both cases, monitoring of native populations of established *Gossypium* spp. may be necessary to assess the efficacy of this isolation procedure for *B.t.* cotton. This would entail monitoring of wild populations for evidence of gene introgression through PCR or similar sensitive methods. Alternatively, the absolute restriction of planting *B.t.* cotton in Puerto Rico and the U.S. Virgin Islands, would of course, alleviate any concerns over gene flow.

c. *B.t.* Potato Plant-Incorporated Protectants

EPA has reviewed the potential for gene capture and expression of the *B.t.* Cry3A plant-incorporated protectant by wild or weedy relatives of cultivated potato in the United States, its possessions and territories. Based on data submitted by the registrant and a review of the scientific literature, EPA concluded that there is no foreseeable risk of unplanned pesticide production through gene capture and expression of the *Bacillus thuringiensis* subsp. *tenebrionis (B.t.t)* Colorado potato beetle control protein gene (Cry3A) in wild relatives of the transformed plant, *Solanum tuberosum* L in the U.S. or its possessions or territories.

1) *Solanum* spp. in the United States, Territories and Possessions

Tuber-bearing *Solanum* species, including *S. tuberosum*, cannot hybridize naturally with the non-tuber bearing *Solanum* species in the U.S. Three species of tuber-bearing (section *Petota*) wild species of *Solanum* occur in the United States: *Solanum fendleri, Solanum jamesii,* and *Solanum pinnatisectum*. Successful gene introgression into these tuber-bearing *Solanum* species is virtually excluded due to constraints of geographical isolation and other biological barriers to

natural hybridization (USDA/APHIS, March 1995). These barriers include incompatible (unequal) endosperm balance numbers (EBN) that lead to endosperm failure and embryo abortion, multiple ploidy levels, and incompatibility mechanisms that do not express reciprocal genes to allow fertilization to proceed. No natural hybrids have been observed between these species and cultivated potatoes in the U.S.

In the U.S., *S. fendleri* and *S. jamesii* are restricted to high elevation habitats in the continental Southwest, far removed from the centers of commercial potato production. Their distribution has been described by Hawkes (1999):

1) *S. fendleri* subsp. *fendleri* Asa Gray. Arizona, Colorado, New Mexico and Texas at 1,600 to 2800 meters in dry oak-pine forest, but not under dense shade.

2) *S. fendleri* subsp. *arizonicum* Hawkes. Arizona in pine forest clearings and roadsides from about 2000-2550 meters.

3) *S. jamesii* Torr. Arizona, Colorado, New Mexico, Texas, and Utah.

S. pinnatisectum is reported to be found in Arizona, though it is considered primarily a Mexican species (USDA/NRCS, 1999). While somatic hybrids (protoplast fusion) can be made and some of these fusions produced plants that can be backcrossed with potato, it cannot naturally cross with *S. tuberosum* because of abortion of hybrid endosperm (Thieme *et al.*, 1997).

2) *Solanum* spp. - Gene Flow

If plants of *Solanum tuberosum* (commercial potato) and either of the three native tuber-bearing species were to grow contiguously, cytological differences in ploidy level and/or endosperm balance number between the wild and cultivated species would bar successful hybridization and gene introgression (Johnston *et al*., 1980). Controlled crosses between *S. fendleri* and *S. tuberosum*, for example, have been successful only with intermediate bridging crosses and have produced hybrids incapable of further sexual reproduction (Soest, 1986). This does not present a risk of spread because intermediate bridging crosses do not occur in nature.

All cultivated potatoes in the U.S. belong to the species, *S. tuberosum*. Although it is possible to produce potatoes sexually from true seed (Martin, 1987), commercial production of *S. tuberosum* in the United States is done asexually through the use of tubers. The production of fruits by the crop, when it occurs, is only incidental to plant growth necessary for tuber maturation. Therefore, even in cases where non-*B.t.* potato fields are in close proximity to *B.t.* potato fields, cross-pollination would not result in the tubers containing the *B.t.* gene since they are vegetatively propagated. Seed potato (*i.e*., cut tuber pieces) production from such tubers would also be *B.t.* gene free.

Many barriers exist for gene transfer from CPB-resistant potatoes to other potato cultivars or free-living relatives. The widely planted cultivar, Russet Burbank, is male sterile. Other cultivars range from Shepody with "almost nil" pollen shed (Young *et al*., 1983), and Atlantic, which is also largely male sterile (Schneider, 1995), to the self-fertile variety, Superior. Lack of floral nectaries and paucity of pollen production in many cultivars restrict insect-mediated (primarily bumblebees) cross pollination (Arndt *et al.*, 1990). Cross pollination drops to very low levels within a few meters of the pollen source (USDA/APHIS, 1995).

Berries produced by self- or cross-fertilization within potato fields have been reported to result in volunteer potato weeds in subsequent crops (Lawson and Wiseman, 1983). Factors reducing the probability of this event include: low self and / or cross fertility among many of the potato cultivars being grown in the United States, critical environmental conditions necessary for fruit set, even with fertile cultivars (Burton, 1989), and competitive disadvantage of seed-produced potatoes in tuber-produced fields. Therefore, CPB-resistant potatoes are unable to outcross to male-fertile potato cultivars, and the chances for successful cross-pollination of CPB-resistant potatoes by male-fertile potato cultivars and subsequent seed production will be minuscule. The potential for the CPB-resistant potatoes to become an aggressive weed in the U.S. is negligible.

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

EPA has evaluated the potential for horizontal gene transfer (hgt) from *Bt* crops and has considered possible risk implications if it occurred. As summarized below, several experiments published in the scientific literature have been conducted to assess the likelihood of hgt, and have been unable to detect gene transfer under typical conditions. Hgt has only been detected under conditions designed to favor transfer. In addition, as discussed below, the genes that have been engineered into the *Bt* crops are mostly found in, or have their origin in, soil inhabiting bacteria. Therefore, we conclude that hgt is at most an extremely rare event, and the traits engineered into the *Bt* crops are already present in soil bacteria or are unlikely to have selective value for soil microorganisms. In considering these data we further conclude that there is no significant risk from hgt from the transgenes found in the current *Bt* crops.

Horizontal gene transfer, regardless of the source of the gene, has been considered in the literature, and evidence presented for possible impact on the evolution of organisms over long periods of time (Smith *et al.* 1992). However, the likelihood of such transfer over a human time scale, and where the transgene has a positive impact on the fitness of the recipient, has not been demonstrated. Horizontal transfer of several traits might be of concern for the current *Bt* crops, for example the *cry* genes and antibiotic resistance genes.

Since bacteria are generally recognized as more promiscuous than other organisms concerning DNA transfer, exist in the soil in large numbers, and because the antibiotic resistance gene most often used in the transgenic crops, kanamycin resistance (*nptII*) as well as the *cry* genes, are of

bacterial origin, it is likely that bacteria would have the highest probability of integrating and expressing these genes. Horizontal gene transfer from transgenic plants to bacteria has not been demonstrated under field conditions (Schluter *et al*. 1995, Gebhard and Smalla 1999). There is one reported case of gene transfer from transgenic plants to the fungus *Aspergillus niger* (Hoffman *et al*. 1994). However, the presence of hygromycin resistant fungal colonies in the control (non-transgenic plant) treatments and the use of plant homogenates to effect the transfer cast doubt on the relevancy of these findings. The *hph* gene was recovered from *A. niger* colonies co-incubated with *Datura innoxia* plants / plant homogenate, however, the vast majority of hygromycin resistant colonies failed to indicate the presence of foreign (plant) DNA as detectable by Southern blotting. Further work is needed in this area to clarify the potential for transfer to soil fungi, although current evidence (*e.g.*, lack of plant sequences in fungal genomes) does not support this avenue of horizontal gene transfer.

DNA from crop plants has been shown to remain in soil for at least several months to several years (Gebhard and Smalla 1999, Paget *et al.* 1998), and there is evidence that some of this DNA is protected from soil nucleases by binding to clay or organic components of soil, similar to the case with Cry proteins, while remaining capable of transforming bacteria. The amount of this DNA, however, appears to be extremely small after several months to a year (Paget *et al.* 1998), so the probability of transformation diminishes considerably with time. In addition, even with much larger concentrations of DNA, transformation with plant transgenes has only been accomplished at low frequencies and under optimized conditions, i.e. where homology to existing DNA in the recipient bacteria occurs, as well as high selection pressure for the horizontal transfer event (Nielsen K.A. *et al.* 1998, Nielsen K.A. *et al.* 2000). Under conditions where homology does not occur, horizontal transfer has not been observed, even at extremely low frequencies of less than about 10^{-9} to 10^{-17} (Nielsen K.A. *et al.* 1998, Paget *et al.* 1998). Therefore, DNA transfer occurs rarely if at all from plants to bacteria. Nielsen *et al.* (2000) point out that homologous sequences already existing in soil bacteria would not be a risk factor from horizontal transfer of the same sequences from plants, but caution that other non-homologous transgenes might be transferred using the homology of surrounding bacterial sequences in transgenic plants. Homology between genes in soil bacteria and transformation vector sequences would generally be limited, although sequences that are common to many species, e.g. from transposons, insertion sequences, or broad host range plasmids, do exist. Probably the most likely target for homology mediated transfer of foreign genes would be to the species that originally carried the homologous DNA.

In addition, for a transgene to have a possible deleterious impact, it is likely that the trait would have to be otherwise unavailable. In other words, the prevalence of the trait in the soil prior to possible introduction by the transgenic plant should be considered. In the case of the transgenes used in the *Bt* crops, occurrence in soil bacteria or fungi has been well established. For example, Smalla et al. (Gebhard and Smalla 1999) demonstrated that kanamycin resistant bacteria were common in the soil and sewage. In the soils tested, numbers ranged from 6.6×10^3 to 2.8×10^5 (0.01 to 0.56 percent of total bacteria). Bacteria containing *nptII* were not found in cultured

bacterial species from soil, but were common in cultured bacteria sewage (9.1 to 47.6 percent). This would not be surprising since only a small fraction of the bacteria found in soil can currently be cultured. Additionally, other bacterial sequences such as type III neomycin phosphotransferases can provide resistance to kanamycin, but would not be detected by PCR using primers for nptII.

However, two of three soils showed consistent *nptII* specific PCR amplifications from total soil extracted DNA (that is, from soil organisms or extracellular DNA)[3]. This could indicate fortuitous matching DNA or false priming, but may also indicate the presence of *nptII* genes in bacteria that were not culturable by the techniques used in the other reported experiments. In addition, others have found *nptII* hybridization from DNA extracted from uninoculated soils (Holben *et al.* 1988). The authors ... "conclude that deliberate releases of organisms carrying Tn*5* or *nptII* are not inherently more hazardous than the 'natural' releases discussed here" (Gebhard and Smalla 1999).

Similarly, *Bt* species are generally common in soil, if not always abundant, and therefore various *cry* genes have been available for long periods of time for horizontal transfer from *Bt* to other soil species (Martin and Travers 1989). Similarly, $-lactamases that confer ampicillin resistance used for bacterial selection and found on some of the vectors used in making the *Bt* plants have long been known to be found in soil microorganisms. As concluded by Nielsen and colleagues (Nielsen K.A. *et al.* 1998), the likelihood of horizontal gene transfer is typically extremely low. Coupled with the common occurrence of the transgenic traits from *Bt* crops already in the soil, this low probability event makes adverse impact by these traits through horizontal transfer in the soil unlikely.

There is no evidence that horizontal gene transfer occurs from plants to microbes or bacteria to bacteria. *Bt* genes which naturally occur in many soils have never demonstrated horizontal gene transfer. The October 2000 SAP concluded that the "horizontal gene transfer assessment appear to be adequate, and no additional data are probably necessary for the Agency to complete a risk assessment."

3. Ecological Exposure

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

[3]These soils were not indicated to have been previously exposed to transgenic plants.

tissue bioactivity values.

Cry Protein Tissue Expression

Active Ingredient	Leaf	Root	Pollen	Seed	Whole Plant
Cry1Ab-*Bt*11 (006444)	3.3 ng/mg	2.2-37.0 ng/mg protein	< 90 ng Cry1Ab/ g dry wt. of pollen	1.4 ng/mg (kernel)	_
Cry1Ab-MON810 (006430)*	10.34 ng / mg	_	< 90 ng Cry1Ab/ g dry wt. of pollen	0.19-0.39 ng / mg (grain)	4.65 ng / mg
Cry1F-TC1507 (006481)	56.6 - 148.9 ng / mg total protein	data required	113.4 - 168.2 ng / mg total protein or 31 to 33 ng / mg pollen	71.2 - 114.8 ng / mg total protein	803.2 - 1572.7 ng / mg total protein
Cry1Ac (006445)	2.04 ng / mg	_	11.5 ng / g	1.62 ng / mg	_
Cry3A (006432)	28.27 ng / mg	0.39 ng / mg (tuber)	_	_	3.3 ng / mg

* 1994 Field Data

** All values reflect fresh tissue weight unless otherwise noted.

Exposure of *Bt* 11 (006444)

Study	Status, Classification & Comments	MRID #
Environmental Fate of *Bacillus thuringiensis* var.*kurstaki* Protein in Corn - Potential for Outcrossing and Weediness of Genetically Modified Insect Protected Corn; Levels of *Btk* Protein in Plant Tissue and per Acre; and Degradation in Soil	*Leaf* tissue: 9.4-168 ng Cry1Ab/mg protein. *Stalk* tissue: 8.8-27 ng/mg for pith and 9.0-36 ng/mg for stalk epidermis. *Root* expression: 2.2-37 ng/mg for sub surface roots and 3.2-7.0 ng/mg for brace roots. *Tassel* tissue: 6.8-8.8 ng /mg. *Silk*: 2.4-6.6 ng /mg . *Pollen*: 1.25 ng /mg protein. *Kernel* tissue: 0.4-8.2 ng/mg n. *Husk*, *cob* and *ear shank* tissue: 2.6-27.2 ng mg. CLASSIFICATION: ACCEPTABLE. Clarification of several points in the data needs to be submitted to upgrade the classification to CORE.	436960-01

Btk protein in *Bt* 11 is most highly expressed in the leaf tissue, in the order of 3.3 microgram/g fresh weight at physiological maturity. Peak expression in the leaf occurs at around 25 days after planting at approximately 168 ng/mg plant protein (uncorrected for extraction efficiency). At 84 days after planting, levels in the leaf drop to 10.2 ng/mg. Levels in other tissues at maturity range from 0.4 ppm in the kernel to 16.2 ng/mg plant protein in the cob, with most tissues averaging approximately 6 ng/mg plant protein.

The highest expression levels for Cry1Ab protein in *Bt*11 corn is found in the leaf tissue with the most recently emerged leaf tissue having the highest values. The range of values for leaf tissue was from 9.4-168 ng Cry1Ab/mg protein. Stalk tissue ranged from 8.8-27 ng Cry1Ab/mg protein for the pith and from 9.0-36 ng Cry1Ab/mg protein for the stalk epidermis. Root expression values ranged from 2.2-37 ng Cry1Ab/mg protein for typical sub surface roots and from 3.2-7.0 ng Cry1Ab/mg protein for brace roots. Tassel tissue ranged from 6.8-8.8 ng Cry1Ab/mg protein and silk ranged from 2.4-6.6 ng Cry1Ab/mg protein. The pollen was stated as 1.25 ng Cry1Ab/mg protein. Kernel tissue ranged from 0.4-8.2 ng Cry1Ab/mg protein. Husk, cob and ear shank tissue values ranged from 2.6-27.2 ng Cry1Ab/mg protein. Nearly 90% of the *Btk* protein was in the leaf tissue according to these calculations. The values for a study conducted in Hawaii were 80.4 ng *Btk* protein/g total protein for leaf tissue and 15.3 ng *Btk* protein/g total protein for stalk tissue. These values declined by the first week (3.89 to 6.9 ng *Btk* protein/g total protein for leaves; and 8.2 to9.03 ng *Btk* protein/g total protein for stalks) then stayed fairly steady for the next 14 days. The range of *Btk* protein values/g protein at the final determination was 0.0 to 5.65 ng *Btk* protein/g total protein for leaf tissue and 9.6 to 11.4 ng *Btk* protein/g total protein for stalk tissue.

An acre of corn was assumed to contain 89,300 lbs. of fresh tissue and produce 0.57 lb. *Btk* protein/acre of corn. The European corn borer (ECB) bioassay data indicates that *Bt*11 leaf tissue subject to soil degradation decreased in its ability to kill ECB larvae or decreased larval weight gain between the initial sample and that obtained on week 3. Bioassays with *Bt*11 stalk tissue showed a similar trend but the decrease was not as marked. The bioassay values for purified *Btk* protein (*Bt*103) added to soil also decreased between week 0 and week 3 but again the decrease was not as marked. The bioassay results suggest that active *Btk* protein is degraded, but not eliminated, over the 3 week period when incorporated into soil. The decrease is apparently faster in leaf tissue compared to stalk tissue or pure toxin added to the soil. *Btk* Cry protein could not be quantitatively extracted from spiked soil for ELISA, possibly because *Btk* protein binds to soil particles and cannot be released by the standard extraction procedures. *Btk cry* protein could be detected in the initial soil spiking sample but not thereafter.

b. Exposure of MON 810 (006430)

As noted elsewhere in this document, some of the expression data for MON 810 is based on data

developed on MON 801 which used the same plasmid construct for the tranformation. EPA has determined that MON 801 data can be bridged to MON 810.

Study	Status, Classification & Comments	MRID #
Laboratory Degradation Study	Bioactivity in soil: DT_{50}) of 1.6 days and DT_{90} of 15 days. *Tissue without soil* : DT_{50} of 25.6 d., DT_{90} of 40.7 d. *Purified protein in soil*: DT_{50} of 8.3 d., DT_{90} of 32.5 days. CLASSIFICATION: ACCEPTABLE.	436960-01
Expression Levels	*Leaf* 1.3 /g; *seed* 0.57/g; *whole plant* 1.77/g; *pollen*: below detection limits. CLASSIFICATION: ACCEPTABLE.	436960-01
Evaluation of Insect Protected Corn Lines in 1994 U.S. Field Locations	*Whole plant* 4.15, *grain* 0.31, *leaf* 9.35, over season leaf 9.78, *pollen* 0.09. CLASSIFICATION: ACCEPTABLE.	436655-02

B.t.k. HD-1 protein levels measured in the MON801 leaf averaged 1.3 microrams/g protein fresh weight, with a range of 0.84-2.36 : g/g; seed averaged 0.57 : g/g, with a range of 0.23-0.95 : g/g; and whole plant averaged 1.77 : g/g fresh weight, with a range of 1.44-2.01 : g/g. In this study, B.t.k. toxin was below the limits of detectability in pollen. Cry1Ab protein levels in MON 810 measured in microrams/g protein fresh weight were: whole plant 4.15 : g/g, grain 0.31 : g/g, leaf 9.35 : g/g, over season leaf 9.78 : g/g, pollen 0.09 : g/g. 1994 MON 810 field data demonstrated expression levels of 0.18-0.39 micrograms/gram protein (fresh weight) in grain, 7.93 -10.34 : g/g in the leaf, 3.65-4.65 : g/g in the whole plant, and 0.09 : g/g in the pollen. MON 810 does not express detectable levels of the marker gene products and the Cry1Ab protein is more truncated than in MON 801. MON 810 was shown to be stable in expression between 1994 and 1995. 1995 U.S. field data showed 5.2-10.6 : g/g in the leaf, 2.3-4.5 : g/g in forage, and 0.4-0.9 : g/g in the grain. 1995 French field data showed 7.6-9.4 : g/g in the leaf, 4.1-5.6 : g/g in forage, and 0.4-0.7 : g/g in the kernel.

Cry1Ab protein bioactivity of corn line #754-10-1 tissue, added to the soil as a component, decreased with an estimated DT_{50} (Degradation Time) of 1.6 days and an estimated DT_{90} of 15 days. Cry1Ab protein bioactivity of corn line #754-10-1 tissue incubated without soil decreased with an estimated DT_{50} of 25.6 days, and a DT_{90} of 40.7 days. The bioactivity of purified Cry1Ab protein in soil decreased with an estimated DT_{50} of 8.3 days and a DT_{90} of 32.5 days.

c. Exposure of Cry1F (006481)

Guideline No.	**Study**	**Status, Classification & Comments**	**MRID #**

155-18	Environmental Fate of Cry1F Protein Incorporated Into Soil	DT_{50} of 3.13 days. Cry1F will degrade in the soil within 28 days (the duration of this test). CLASSIFICATION: SUPPLEMENTAL	450201-05

Test line grain samples contained an average Cry1F expression of 89.8 (71.2to 114.8) pg / µg total protein. Leaf sample expression from Cry1F maize lines was 110.9 (56.6 to 148.9) pg / µg total protein. Pollen and silk samples yielded 135.5 (113.4 to 168.2) pg/µg total protein for pollen and 50.3 (26.8 to 79.8) pg / µg total protein for silk. The Cry1F expression for stalk samples was 550.0 (355.9 to 737.4) pg / µg total protein. For whole plant samples, the expression level averaged 1063.8 (803.2 to 1572.7) pg / µg total protein. In senescent whole plant samples the expression of Cry1F was 714.3 (622.2 to 845.3) pg / µg total protein. Of the leaf samples tested for PAT expression, the test line samples ranged from below the LOD to 58.2 pg / µg total protein. All of the following tissues were below the LOD for PAT: pollen, silk, stalk and grain from both test and control lines. Both whole plant samples and senescent whole plant samples were negative or below the LOD for PAT.

d. Exposure of Cry1Ac (006445)

The levels of expression of the Cry1Ac delta endotoxin in cotton have been determined. The Cry protein is detectable in leaves, seeds, and whole plant assays. *Btk* protein was undetectable in cottonseed meal, and was present only at or near the level of detection in pollen and below the level of detection in nectar. Based upon estimates of 60,000 plants per acre, a total of 1.44 grams of Cry protein per acre would enter the soil.

Study	Status, Classification & Comments	MRID #
Gene Expression	Field grown plants: 1.10 to 2.04 : g/g of fresh leaf tissue; 0.49 to 1.62 : g/g per gram fresh seed tissue. Greenhouse studies: Bt expressed in pollen (11.5 ng/gram) at a level of detection of 8.0 ng/gram, and is below the level of detection in nectar (<1.6 ng/gram). CLASSIFICATION: ACCEPTABLE.	MRID is not availble
Soil Degradation	Purified endotoxin produced in *E. coli*: DT_{50} of 9.3-20.2 d. Ground, lyophilized Cry1A(c) cotton line 931tissue: DT_{50} of 41 d. CLASSIFICATION: ACCEPTABLE.	439995-09

The test substances were cotton lines 531 and 931. Six locations in Mississippi, Louisiana, Texas, Georgia, Arizona, and Alabama were used for field expression studies. Proteins in leaf, seed, whole plant, cottonseed meal and refined cotton seed oil were analyzed. Expression level ranges were identified by validated ELISA procedures. Reported mean *Btk* protein expression levels from field grown plants ranged from 1.10 to 2.04 : g/g of fresh leaf tissue and from 0.49 to 1.62 : g/g per gram fresh seed tissue. Greenhouse studies indicate that *Btk* protein is expressed in pollen (11.5 ng/gram) at a level of detection of 8.0 ng/gram, and is below the level of detection in nectar (<1.6 ng/gram). The Cry protein was reduced to undetectable levels in cottonseed meal after processing. No detectable levels were found in refined oil at a level of detection of 1.3 ppm.

Based upon planting rates of 60,000 plants per acre, a total of 1.44 grams of *Btk* protein would enter the soil per acre due to post harvest incorporation of the plants into the soil.
The submitted data demonstrated a loss, following soil incorporation, in activity of *Btk* endotoxin against a susceptible insect, the tobacco budworm *Heliothis virescens*. Purified endotoxin produced in *E. coli* shows a soil DT_{50} (Degradation Time) of 9.3-20.2 days. Ground, lyophilized Cry1A(c) cotton line 931 tissue has a soil DT_{50} of 41 days.

e. Exposure of Cry3A (006432)

Study	Status, Classification & Comments	MRID #
Expression levels in field grown potatoes	Cry3A delta-endotoxin expression levels for leaf samples: 5.39 to 28.27 micro g/g tissue or 0.03-0.2% of the total foliage protein. Tuber CryIIIA delta-endotoxin expression levels: 0.40 to 2.00 micro g/g or 0.002-0.01% of the total tuber protein. CLASSIFICATION: ACCEPTABLE.	429322-02

Monsanto submitted data for seven advanced CPB resistant Russet Burbank lines and a nontransformed Russet Burbank control grown at seven locations representative of potato growing regions in the United States. Tissues assayed included leaf, whole plant (minus tubers) and tuber, three harvest dates for leaves, two for whole plants and one for tubers were conducted for Cry3A.

Tuber samples were lyophilized before determining protein expression levels. The fresh weight equivalent of each tuber sample was obtained by determining the amount of water removed during the lyophilization process. Leaf and whole plant samples were processed from frozen, but not lyophilized, samples. Percent moisture was not reported for these tissues. Leaf or whole plant samples with low moisture content would be expected to yield higher expression levels than otherwise comparable tissue with relatively higher moisture content.

Cry3A levels expressed as a percentage of total protein were based on the assumption that total protein comprises 1.6 and 2.0% of the fresh weight of foliage and tubers, respectively. These levels are comparable to average values reported in the literature (Burton, 1989). The range expected will vary with genetic and cultural variables. Tuber protein, for example, has been reported to range from 0.7 to 4.6 % of tuber fresh weight (Kadam *et al*., 1991).

The relatively low expression levels reported for the Cry3A protein in tuber tissue reflect, in part, the high starch concentration in storage parenchyma, cells which comprise the bulk of the tuber. Reported Cry3A delta-endotoxin expression levels for leaf samples ranged from 5.39 to 28.27 micro g/g tissue or 0.03-0.2% of the total foliage protein. Tuber Cry3A delta-endotoxin expression levels ranged from 0.40 to 2.00 micro g/g or 0.002-0.01% of the total tuber protein.

f. Fate of *Bt* Proteins in Soil

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

Experiments addressing amounts and persistence of *-endotoxins in the soil have been submitted by registrants and reviewed for the current conditional registrations. In addition, a number of publications in the scientific literature have addressed the degradation of Cry proteins in the soil. These experiments consist of the incorporation of purified *-endotoxin or transgenic plant material in soil in a laboratory setting. Cry protein DT_{50} (time to 50% degradation) studies were submitted for registration for corn containing Cry1Ab and Cry1F, and published studies were available for Cry1Ac cotton. Cry1Ab produced an estimated DT_{50} of 1.6 days for Cry protein as expressed in transgenic corn tissue and 8.3 days for purified protein (Sims and Holden 1996). Based on a bioassay with the tobacco budworm (*Heliothis virescens*), a target species, purified Cry1F proteins incorporated into test soils biodegraded with a DT_{50} of approximately 3.13 days. Data produced by Monsanto for Cry1Ac protein and transgenic Cry1Ac in cotton give degradation rates (DT_{50}) of approximately 9-20 days for the purified protein, and 41 days for the protein in cotton tissue (MRID# 43999509). Published data for Cry1Ab or Cry1Ac in cotton tissue or as purified protein produced DT_{50}s of 2.2 to 46 days, where measurable (in 4 of 11 experiments), with DT_{50}s in transgenic tissue shorter than for purified protein in two of three experiments (Palm *et al.* 1994). DT_{50}s of purified Cry1Ac in two different non-sterile soils were

22 d and 40 d (Palm *et al.* 1994). None of the studies discussed above have been performed under field conditions, although most have used field soil in laboratory microcosms.

Several studies indicate that Cry proteins bind to clays and humic acids (Crecchio and Stotzky 1998, Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998, Stotzky 2000b). The results of these studies suggest that this binding slows the rate of microbial degradation of these toxins compared to when these soil components are not present (Stotzky 2000b). However, this protection is not absolute, since degradation does in fact occur under several experimental conditions. Several factors influence either the affinity of binding or the rate of degradation. In particular, pH near neutrality generally substantially increases degradation. At pH above 5.8 to near neutrality, degradation of Cry protein bound to clay minerals in soil was much faster than degradation at pH 4.9-5.0 (Tapp and Stotzky 1998). For example, it was found (Tapp and Stotzky 1998) that Cry toxin added to nonsterile soil containing kaolinite or montmorillonite showed little degradation even after around six months at lower pH (pH~5), while substantial degradation occurred over this time period at higher pH.

Corn does not grow well below ~pH 5.6 (Aldrich *et al.* 1975), and therefore most corn growing soils are expected to be at a higher pH. Potato prefers acid soils (Smith 1977), and the optimum range is pH 5.0-6.5 (Ware and McCollum 1980). The optimal range for cotton is pH 6.0-6.5 (Donahue *et al.* 1990). Therefore, under most production conditions, cotton and corn would not be grown on soils that would inhibit the rate of degradation compared to what is seen at near neutral pH. On the other hand, potato may be grown at soil pH levels that approach those in which a substantial reduction in degradation rates has been shown to occur. However, effects of pH on degradation rates in the range of pH 5.0-5.8, which overlaps with potato growing conditions, has not been explored.

Tapp and Stotzky (1998) proposed that the reason for more rapid degradation of Cry proteins at near neutral pH is a greater amount of microbial activity near pH 7. The authors point out that even at pH near neutrality, protein toxin activity (lethal concentrations against a sensitive bioassay) remained after six months, and they interpret these data as evidence of prolonged persistence of Cry protein in the soil. In these experiments substantial degradation (loss of biological activity) typically occurred rapidly in the first several weeks, with much slower subsequent breakdown (Tapp and Stotzky 1998). A similar pattern was observed in some experiments performed by other workers over a range of Cry1Ab and Cry1Ac protein concentrations from around 2-700 ng toxin/g soil (Palm *et al.* 1994, Palm *et al.* 1996). These experiments suggest that testing for persistence in the field should be determined over sufficiently long periods to assure an accurate assessment of degradation.

These results must be interpreted with caution regarding implications for persistence in the field. Field deposition of Cry protein would be associated with plant material (pollen or crop residue) or plant root exudates (e.g. carbohydrates and amino acids) which typically stimulate microbial activity and reproduction (Cheng and Coleman 1990, Griffiths *et al.* 1999, Jensen and Soerensen

1994, Meharg 1994). Many of the experiments examining persistence of Cry proteins reported in the published literature have apparently been conducted in bulk soils or soil components. Bulk soil generally does not support populations of microorganisms as high as in the rhizosphere or where plant residues are incorporated into the soil. Other work suggests typical ratios of 5-20 for rhizosphere to bulk soil microbes, with rhizosphere populations commonly 100 fold higher than in bulk soil (Atlas and Bartha 1993). Therefore, degradation rates under field conditions may be higher than those shown in bulk soil experiments.

This conclusion is supported by data submitted by Sims (Sims and Holden 1996) where the DT_{50} of free Cry protein alone (in bulk soil) is about 5 fold higher than in ground corn tissue added to soil (although proteases from the corn tissue might also contribute to degradation). In addition, Donegan *et al.* (1995) found that microbial populations increased 100-1000 fold with the addition of plant material (cotton) with or without Cry1Ab or Cry1Ac proteins. Palm *et al.* (1996) found more rapid degradation of truncated Cry1Ab or Cry1Ac proteins when incorporated with cotton crop residues more often than when purified protein was used. In these microcosm experiments, toxin DT_{50}s, where possible to determine, varied greatly from 2.2 to 46 days (microbial populations in these previously dried, rehydrated, soils were not determined). These experiments relied on ELISA of extracted Cry proteins to quantify residual Cry protein in the soil, so it is not clear what fraction of the extracted protein may have retained biological activity. The extraction efficiency was reported to be about 27-60%, with lower efficiency corresponding to higher clay and organic content, and it is unclear whether unextracted protein degraded at a similar rate as the extracted protein and retained biological activity. However, other work (Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998) suggests that at least some of the clay-bound fraction of Cry protein is more resistant to extraction and degradation while retaining biological activity.

Some experiments that show relatively long persistence of Cry proteins in the soil do not consider rates of degradation, reporting instead only on the duration of protein activity or presence of protein. These experiments sometimes begin with very high concentrations of the protein compared to the amounts found in the plant. For example, Tapp and Stotzky (1995) and Koskella and Stotzky (1997) use approximately 100 µg Cry protein/g soil for the former and approximately 100-300 : g toxin/g soil for the latter experiments, while *Bt* plants typically produce less than 10 : g/g plant tissue and the concentration in soil from incorporation is typically estimated to be at the PPB to low PPM levels. In addition, the bioassays or immunoassays used to detect the protein in the soil are very sensitive, able to detect the protein at concentrations of around 5-10 PPB (ng/g). Depending on the experiments, reductions of 10^3 to10^5 may be required to reduce the amounts of Cry protein added to soil below detectable levels. Therefore, it is not necessarily surprising that relatively long term persistence can be detected under these conditions. In order to predict persistence under field conditions, it is important to know starting concentrations as well as degradation rates. Other experiments discussed above used amounts of Cry protein more representative of many plant tissues in *Bt* plants.

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

It is important to consider that a number of factors are expected to influence persistence under actual field conditions, including: humic acid and clay content of the soil, clay type, pH, moisture, soluble ion content and type, and temperature, all of which affect microbial activity, composition, and population levels. These factors may also affect binding affinity of Cry proteins for soil components. Since these factors may vary considerably in the field, persistence of Cry proteins could likewise vary considerably. However, the conditions examined by the registrants generally replicate common field soil conditions, although performed in a laboratory setting. Field tests of Cry protein degradation under a range of conditions typical of *Bt* crop cultivation would yield relevant data on persistence and natural variation.

g. Secretion of Cry Proteins by Plant Roots

Saxena *et al*. (1999) reported that Cry protein was exuded by the roots of transgenic corn plants in laboratory studies. In this study, *Bt*11 and nontransgenic corn plants were grown in Hoagland's solution or in sterile or nonsterile soil (sandy loam with 6% montmorillonite clay added, pH 6.0-6.5) (Saxena *et al.* 1999, Stotzky 2000d). Results of this study showed that the amount of total protein found in the medium averaged 105 ：g per plant, as determined by the Lowry method. Although it is possible that the observed Cry1Ab protein was the result of small pieces of plant tissue (e.g. root cells or root hairs), it is unlikely since the Hoagland's solution was centrifuged, which would remove cellular debris (Stotzky 2000a). In addition, the simple protein band pattern reported (66kDa) by Saxena *et al.* (1999) was the same as the Cry1Ab protein and found in *Bt* corn exudates only after 7 and 15 days. However, the procedure used to isolate the Cry protein from soil, including vortexing in extraction buffer prior to centrifugation, could have ruptured or lysed cellular material.[4] In addition, small root material is difficult to

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

separate from the rhizosphere soil used in the subsequent assays. The SDS-PAGE protein profile from non-sterile soil was reported to contain many more bands than from the SDS-PAGE from the Hoagland's solution (Stotzky 2000d). This is consistent either with extraction of plant cellular proteins and/or microbial proteins, possibly in addition to exuded or secreted proteins. SDS-PAGE gels of extracted proteins from the sterile soil treatment were not performed (Stotzky 2000d) so such data may have been expected to result in a protein profile similar to that from the Hoagland's solution if plant cellular material was not included.

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

Relatively long persistence of Cry proteins is not surprising when the starting amounts are high and the assays for the protein (bioassay or immunoassay) are very sensitive. Saxena *et al.* (1999) reported that the 66KDa band disappeared after 25 days when the soil was no longer sterile. With a single incorporation at the concentrations estimated by Saxena *et al.* (1999), and DT_{50} estimates submitted by the registrant, it is possible that Cry protein would not be detectable after 25 days of exposure in the soil, even with sensitive bioassays or immunoassays (Sims and Holden 1996). The presence of Cry protein activity after 25 days in nonsterile soil appears to support either the persistence or continuous exudation of Cry protein, or both.

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

Proteins secreted into the soil by plant roots are limited in number and specialized for that purpose, containing specific endoplasmic reticulum (ER) export signals in the form of short amino terminal amino acid sequences that target the protein to the lumen of the ER, and other

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

Available data for other secreted proteins suggest that the amount of a secreted protein found in culture medium may be as high or higher than the amount associated with plant tissue after several days growth (Borisjuk *et al.* 1999). In *Bt*11 corn roots, Cry1Ab is expressed at an average of 20.2 : g/g total root protein. It is difficult to predict whether this level of root expression of Cry1Ab is consistent with active secretion, based on the roughly estimated amounts of Cry1Ab found in the media (soil or Hoagland's solution) reported by Saxena *et al.* (1999).

The corn plants used in the exudation experiments were identified as NK4640*Bt*, which correspond to Syngenta *Bt*11 corn lines. This variety of corn contains a modified *cry1Ab* gene. Previous soil fate data supplied by the registrant was from experiments performed with nonsterile soil in the laboratory and consisted of replicated single incorporations of transgenic plant material or purified Cry protein. All other studies of Cry protein stability in the soil performed for registration or other purposes also used a single incorporation of purified protein or protein in the transgenic plant, with subsequent monitoring of residual activity using sensitive bioassays or other means, such as immunoassays. Single incorporation studies were performed because it was believed that most of the exposure of soil organisms to the Cry proteins would be through the incorporation of plant residues after harvest, and to a lesser extent due to pollen shed.

Degradation studies were performed by Monsanto, registrant of Mon 810 corn which also contains the *cry1Ab* gene. In these studies, Cry1Ab protein degraded in non-sterile soil with a 1.6 d DT_{50} when ground plant material is used, and 8.3 d for the purified Cry1Ab protein in soil. The ground tissue used in this study would be expected to have a significantly increased surface area compared to crop residue incorporated at the end of a growing season. This increase in surface area might reduce degradation times. Use of ground tissue has been criticized (NAS/NRC 2000). Cry1Ab protein bioactivity of corn tissue incubated without soil decreased with an estimated DT_{50} of 25.6 days, and a DT_{90} of 40.7 days. After one week in soil, approximately 1% and 10% of the original levels of B.t.k. protein remained in leaf and stalk tissue, respectively. After three weeks, B.t.k. protein was still detected in the stalk tissue, but the level in transgenic leaves was similar to the background levels seen in control leaf tissue. B.t.k.

protein apparently binds to soil particles, making quantitative extraction difficult. Biological activity, assessed by European corn borer bioassay, is reduced to control levels after three weeks of incubation in soil (MRID# 43696001).

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

h. Exposure of Soil Organisms to *Cry* Proteins from *In Situ* Roots or Incorporated Plant Tissue

In addition to possible concerns about exposure of soil organisms to Cry proteins by root exudation or secretion, exposure to soil organisms from the roots themselves during and after the growing season must be considered. Primary exposure to soil organisms has been considered to be from incorporation of crop residues at the end of the growing season, or to a lesser extent from deposition of pollen onto the soil. Therefore, degradation and possible accumulation of Cry proteins has been examined by determining degradation rates of Cry proteins, either in isolation or as expressed in the plant tissue, incorporated at a single point in time. In addition, determination of degradation rates of Cry proteins in soil is more feasible using a discreet starting point. Estimates of total Cry protein incorporated into the soil have been based upon the biomass of total plant tissue, although it is not clear whether root biomass has been included in these calculations.

In contrast, the roots of a crop may be comparable in biomass to the above ground portions of the crop at the end of the growing season. During the growing season, soil organisms are exposed to roots and their contents. In particular, organisms that feed on living roots will ingest expressed Cry protein directly from this source. Data for expression levels of Cry proteins in the roots are not available for all registered transgenic crops. Cry3A is expressed in potato tubers at 1.01 ：g/g tissue (NAS/NRC 2000). An average of 20.2 ：g/g total root protein (seasonal average for actively growing corn) for *Bt*11 corn containing Cry1Ab is expressed (Rusch and Kendall 1995). Because of the variability of expression levels between transformation events, the levels of root

expression in the other transgenic *Bt* crops cannot be predicted from these numbers or from expression in other tissues in those plants.

Similar to the case with above ground plant tissue, organisms that feed on root feeding organisms may be exposed indirectly and over an extended period of time. In addition, a significant amount of root tissue has been estimated to be lost during plant growth. Estimates of loss of root tissue range from about 11-72 percent of total root tissue and about 4-20 percent for rhizodeposition of insoluble root material into soil (Newman 1985). This dead tissue may consist of exuded high molecular weight materials such as root cap mucilage, root cortical cells, or whole root tissue (Newman 1985). The composition of this material is generally believed to consist largely of higher molecular weight materials, such as the structural components of the roots. It is therefore difficult to estimate whether the proportion of Cry protein in this material differs from that of living roots. Deposition of some Cry protein in the soil will likely occur during degradation of root tissue, in addition to Cry protein incorporated into the soil from the above ground parts of the crop at the end of the growing season.

As with the above ground portions of the plant, the root biomass increases during the growing season. Therefore, assuming that other factors are comparable, exposure of soil and soil organisms will be minimal early in the growing season and will increase with root volume. Therefore, the assumptions underlying a discrete, relatively short duration of exposure of both soil and soil organisms is likely to be inaccurate.

Exposure to Cry proteins of more species and higher numbers of beneficial soil coleopterans, compared to lepidopterans, is likely due the greater species diversity of non-pest (e.g. predatory) Coleoptera that inhabit the soil. In addition, soil inhabiting Lepidoptera that feed on crop roots would generally be considered to be pests in that setting, while coleopterans and other taxa that feed on these lepidopteran species would more likely be considered to be beneficial organisms. Therefore, it may be more important to perform further toxicity testing of soil inhabiting Coleoptera using Cry3A potatoes than testing with the other *Bt* crops containing Lepidoptera specific *cry* genes. However, some larval Lepidoptera found in some *Bt* crop soils likely cause minimal damage and are not considered to have economic impact (Dively 2000). These species could be considered to be non-target organisms and could be considered in non-target organism assessments.

It is also important to consider the amount of exposure based on the available active protein in the soil. As noted elsewhere, calculated DT_{50}s or persistence of several Cry proteins in the soil typically suggest an initial rapid degradation rate over a period of several weeks, commonly followed by small (nanograms/gram soil amounts) residual amounts that are more persistent. Therefore, exposure after deposition is likely to fall to levels that are not expected to adversely affect organisms other than sensitive species. On the other hand, root feeding organisms, their predators or parasites, or possibly detritus feeders encountering root tissues soon after death, might be exposed to levels similar to those found in the plant root.

Available studies on the impact of transgenic Cry producing plants indicates that adverse effects on soil microorganisms are unlikely. No effects have been seen due to the protein itself, and only a minimal, transient increase observed in soil microbes attributed to the transgenic cotton plant tissue rather than the Cry protein expressed in that tissue (Donegan *et al.* 1995). No adverse effects have been observed in a similar season long field study with Cry3A potato (Donegan *et al.* 1996).

In summary, sufficient evidence exists to suggest that adverse impacts of currently commercialized *Bt* Cry1Ab, Cry1F and Cry1Ac proteins in the soil are not likely, although the levels of expression in the root should be determined to assure that unexpectedly high levels of root expression are not found. It is somewhat less predictable what the effect of Cry3A might be given the limited testing of non-target coleopterans that has been performed and considering the relatively larger number of soil Coleoptera that might be affected. Levels of expression on Cry3A in potato tubers (1.01 : g/g tuber) are high enough that sensitive non-target Coleoptera (if any) exposed to these concentrations might be adversely effected. Several non-target Coleoptera in addition to lady bird beetles (*H. convergens*) have been previously tested with no adverse effects (MacIntosh *et al.* 1990).

4. Ecological Effects Testing

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

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

summarized in the individual Fact Sheets for each of the registered Cry proteins, and are described in supporting assessment documents.

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

EPA assesses the toxicity of a *Bt* Cry protein to potentially exposed non-target organisms by single species laboratory testing. If toxicity to a particular species is observed, the amount of exposure is quantified and a risk assessment is performed to determine if adverse effects would be expected at the concentrations used under field conditions. Based upon EPA's risk assessment methodology for determining adverse effects to non-target organisms, if detrimental effects to an individual species are observed under laboratory conditions, field studies (a census) are required to assess the actual abundance of non-target species (in the field, insects, for example, are usually exposed to smaller amounts of toxin than the laboratory test dose because in the field there is a greater choice in diet and because other environmental factors play a role in the field setting.)

The non-target test organisms are chosen as representative indicators of potential adverse effects. The choice of appropriate indicator organisms for testing is based on the potential field exposure as deduced from data on Cry protein expression in the plant. Although *Bt* Cry proteins are very specific in their activity to only certain insect species, for *Bt* Cry protein in plants, EPA has examined the toxicity of the Cry proteins to birds, fish, honeybees and certain other beneficial insects (a recent FIFRA SAP [USEPA, 2001] recommended against testing of non-targets species not related to those susceptible to the specific activity of *Bt* Cry proteins). Because of the extensive scientific literature on the susceptibility of lepidopteran species to *Bt* Cry1 proteins, EPA assumes that Cry1 proteins would be toxic to butterflies if they were exposed to high levels of the protein and therefore, did not require lepidopteran toxicity data. EPA nevertheless required data on *Collembola* (springtail) and earthworm species to ascertain effects on beneficial soil invertebrates because soil exposure to *Bt* Cry proteins was a possibility. In the honeybee study, effects studies on brood as well as adults were required when exposure to the *Bt* Cry protein in pollen is expected. Evaluations of risk to other non-targets which may be affected by the *Bt* pollen, specifically the monarch butterfly, were conducted in 1999 and 2000 by the USDA, ABSTC and university scientists in the USA and Canada (ABSTC, 2001). The reports from these studies are summarized below and in the supporting DCI review document (Rose, 2001).

Bt delta endotoxins are proteins and, unlike inorganic chemicals, do not have the potential to bioaccumulate causing delayed effects. An accumulation through the food chain is therefore not expected to take place, and there are no data to support this possibility for protein substances. The basic biological properties of proteins also make *Bt* Cry proteins readily susceptible to

metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports of soil binding under certain circumstances, the bound Cry proteins are also reported to be rapidly degraded by microbes upon elution from soil. The same sources report that *Bt* proteins in the soil of *Bt* corn fields have no detectable effect on soil invertebrates or culturable microbial flora. The *Bt* Cry proteins do not have any characteristics in common with persistent, bioaccumulative chemicals that are transferred through the food chain. Therefore, chronic effects testing of protein substances is not routinely performed.

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

Study	Status, Classification & Comments	MRID #
Larval Honey Bee Testing	*Btk* HD-1 protein at 20 ppm showed no toxicity to larval honey bees. An LC_{50} was not possible to calculate since this was a single dose test. Therefore, the NOEL is greater than 20 ppm.	434392-02
Verification of Test Substance from Nontarget Insect and Honey Bee Testing	Test substance was stable for up to 7 days in 1:1 honey:sucrose solution. Test material was bioactive.	434680-02
Non-Target Insect Testing - Green Lacewing Larvae	*Btk* HD-1 protein at 16.7 ppm showed no toxicity to green lacewing larvae after 7 days. The NOEL is greater than 16.7 ppm.	434680-03
Non-Target Insect Testing - Ladybird Beetles (*Hippodamia convergens*)	*Btk* HD-1 protein at 20 ppm showed no toxicity to ladybird beetles (*Hippodamia convergens*). The NOEL is greater than 20 ppm.	434680-05
Non-Target Insect - Parasitic Hymenoptera	*Btk* HD-1 protein at 20 ppm showed no toxicity to *Brachymeria intermedia*. Since this is a single dose study, an LC_{50} cannot be calculated. The NOEL is greater than 20ppm.	434680-04
Non-Target Adult Honey Bee Testing	There were no statistically significant differences among the various groups. However, sizable mortality occurred in all treatments. *Btk* HD-1 protein at 20 ppm resulted in a mean mortality of 16.2%. Because mortality was observed at the single dose tested, a NOEL could not be determined from this study, but it was less than 20 ppm. 20 ppm was determined to be significantly higher than exposure conditions in the environment.	434392-03
Avian Oral Toxicity in Northern Bobwhite Quail	No treatment related mortality or differences in food consumption, body weight or behavior occurred in birds fed 50,000 or 100,000 ppm transgenic corn meal derived from Monsanto's MON 80187 corn line (which contains Cry1Ab protein) relative to birds fed corn meal made from parental corn lines which did not express *Bt* protein. Although this study utilized Monsanto's *Bt* corn for testing, the test material was considered sufficiently similar to the *Bt*11 corn grain to bridge the data.	435332-05

Study	Status, Classification & Comments	MRID #
Corn Pollen Containing the Cry1Ab Protein: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*).	Monsanto submitted this study to support their MON 810 corn. The study is scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. These results indicate that *Daphnia magna*, a sensitive aquatic invertebrate species, is not affected by a 48 hour exposure to 100 mg of Cry1Ab protein containing MON810 corn pollen/L. This study adequately address potential aquatic toxicity concerns for MON 810 corn pollen expressing Cry1Ab protein. Mon 810 pollen is preferred over *Bt*11 pollen as a test material in studies supporting *Bt*11 corn. However, given the equivalent Cry1Ab expression in *Bt*11 and MON 810 corn (< 90 ng Cry1Ab/g dry wt.pollen) and the lack of treatment related effects seen in any *Bt* corn pollen *Daphnia magna* studies, the data requirement is satisfied for *Bt*11. The data suggest that at the expected environmental concentration the proposed use of Cry1Ab protein in corn is not likely to have any measurable effects on aquatic invertebrates.	442715-02
Daphnia magna Study and Bridging Rationale	Novartis cited the Ciba Seeds (now Novartis Seeds) Event 176 acute 48 hr. study (MRID No. 433236-10) to support *Bt*11. This 48-hour static renewal toxicity study of Event 176 maize pollen containing *Bt* Cry1Ab Cry protein was conducted using *Daphnia magna*. Test daphnids were dosed at five concentration levels, including a maximum hazard dose of 150 mg/L (nominal) of water. No mortalities were observed at any of the treatment levels tested. The 48-hour EC_{50} was determined to be greater than 150 mg/L. The NOEC was found to be >150 mg/L. These results indicate that *Bt* Cry1Ab protein expressed in corn showed not toxicity at 150 mg/L to Daphnia magna. In view of the above results, no freshwater aquatic invertebrate hazard is expected from the use of this product. *Bt*11 pollen is preferred over Event 176 pollen as a test material in studies supporting *Bt*11 corn. However, given the low level of expression of Cry1Ab in *Bt*11 pollen [(< 0.55 micro g Cry1Ab/ g protein) or (< 90 ng Cry1Ab/g dry wt.pollen)] compared to Event 176 pollen [80.63 micro g Cry1Ab/g protein) or (12.36 micro g Cry1Ab/g dry wt. pollen)] and the lack of effects seen in the cited *Daphnia magna* study using Event 176 pollen, the data requirement is satisfied for *Bt*11.	433236-10 442742-01

Study	Status, Classification & Comments	MRID #
Evaluation of the European Corn Borer Resistant corn Line MON 801 as a Feed Ingredient for Catfish.	Feed per fish, feed conversion ratios, final weight, percentage weight gain and survival were not significantly different between fish fed the control MON 800 diet when compared to those fed the diet containing transgenic corn from the test line MON 801. Body composition data exhibited no significant differences in percentage moisture, fat, or ash, with a higher protein content in the test fish on a dry weight basis. This difference in protein content disappears when one expresses the results on a wet weight basis. Data in this study are consistent with historical controls for catfish grown at the Delta Research and Extension Center. Although this study utilized Monsanto's *Bt* Cry1Ab corn for testing, the test material was considered sufficiently similar to the *Bt*11 Cry1Ab corn grain to bridge the data.	438879-01
Effect of Cry1Ab, on *Folsomia candida* and *Xenylla grisea* (Insecta: Collembola).	In the cited study, purified *Btk* insecticidal proteins derived from *E. coli* (200 ppm), including Cry1Ab protein, had no observable toxicological effect on two species of Collembola: *Folsomia candida* and *Xenylla grisea*. The Agency has required Novartis to submit a Collembola study using leaf material rather than bacterially-derived Cry1Ab.	439416-01
Chronic Exposure of *Folsomia candida* to Corn Tissue Expressing Cry1Ab Protein.	This study determined that the LD_{50} of lyophilized MON 810 corn leaf tissue containing the Cry1Ab protein to Collembola (*Folsomia candida*) over a 28-day exposure period is greater than 50% (by weight) of the diet. The no-effect-level for mortality was 50% of the diet. This same concentration in the diet had no effect on the reproduction of Collembola. According to the sponsor, the estimated concentration of Cry1Ab protein was 50.6 µg/g in lyophilized tissue and 6.27 µg/g in fresh tissue. The control substance was lyophilized leaf tissue from the non-transgenic corn line MON 823 which has a genetic background similar to the MON 810 line but does not carry the gene responsible for the Cry1Ab protein. Thiodicarb was used as a positive control or reference substance. While this study is useful in characterizing effects of Cry1Ab corn tissue on Collembola and satisfies the requirement for MON 810 corn, it does not adequately characterize the effect of *Bt*-11 corn tissue on Collembola since possible treatment related effects were observed in a *Bt* corn Collembola study.	MRID No. 442715-01

Study	Status, Classification & Comments	MRID #
Cry1Ab Insecticidal Protein: An Acute Toxicity Study with the Earthworm in Artificial Soil Substrate	The 14-Day LC_{50} value for earthworms exposed to Cry1Ab insecticidal protein derived from *E. coli* in an artificial soil substrate was determined to be greater than 200 mg/kg (ppm), which was the single concentration tested. There were no statistically significant effects at the single dose tested, therefore the NOEL is greater than 200 ppm. Although this study was graded supplemental, *Bt* Cry1Ab proteins expressed in the corn plant are not expected to generate a toxic effect in the earthworm, therefore, no additional follow-up of this study is required.	438879-02

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

Guideline No.	Study	Result (LC_{50})	Status, Classification & Comments	MRID #
OPPTS Series 885-4380	Evaluation of the Dietary Effect(s) on Honeybee (*Apis mellifera*) Development Using Bacterially Expressed Bt Cry1F Delta-Endotoxin and Pollen from Maize Expressing Bt Cry1F Delta Endotoxin.	LC_{50} > 64 ng Cry1F in 2 mg pollen /larva and 640 ng Cry1F protein /larva	The data show that at the expected environmental exposure the proposed use of Cry1F protein in corn is not likely to have any measurable deleterious effects on the honey bee (*Apis mellifera*). The study is scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. Cry1F protein as expressed in corn pollen showed no toxicity to honey bee larvae or their development into healthy adults. The test insects were exposed to a constant dose of pollen. This is more than the amount that the bees would be expected to consume under field use conditions. As a result, no discernible detrimental effects to honey bees are expected from the proposed uses of the Cry1F producing corn. This study adequately address potential toxicity concerns for honey bees exposed to Cry1F protein expressed in corn pollen in the field. Classification: Acceptable.	450415-03, 453078-05 (supplement)
71-2, 154-7	Transgenic Corn Expressing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F Delta-Endotoxin: A Dietary Toxicity Study with the Northern Bobwhite.	LC_{50} > 100,000 ppm (10% corn meal); NOEC > 100,000 ppm. (10% corn meal)	The dietary LC_{50} value for corn grain (meal) expressing *Bacillus thuringiensis* var. *aizawai* protein in corn grain when fed to juvenile northern bobwhite for 5 days was determined to be greater than 100,000 ppm (10% corn meal), the only concentration tested. The no-observed-effect concentration was also 100,000 ppm. These data show that there will be no adverse effects on avian wildlife from incidental field exposure to Cry1F corn. These data are, however, not sufficient to make a hazard assessment from repeated exposure(s) to higher doses of Bt corn. A six week study with 60 to 70% corn in the diet is necessary to assess hazards from chronic exposure of wild and domesticated fowl. Classification: Supplemental due to the concentration of corn meal tested.	450201-12

Guideline No.	Study	Result (LC_{50})	Status, Classification & Comments	MRID #
72-2, 154-9	A 48-Hr Static Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*) Using Bacterially-Expressed Bt Cry 1F Delta-Endotoxin and Pollen from Maize Expressing Bt Cry 1F.	The 48-hr EC50 was > 100 mg a.i./L. The NOEC was >100 mg a.i./L.	This study was conducted according to approved EPA guideline procedures. The 48-hr EC50 for *Daphnia magna* exposed to Bt Cry 1F delta-endotoxin was > 100 mg a.i./L. The no-mortality concentration and NOEC were estimated to be >100 mg a.i./L. There were no overt signs of toxicity to daphnids exposed to 100 mg *Bt*-pollen/L - (maize pollen containing the *Bt* Cry1F delta-endotoxin). These data show that there will be no adverse effects on daphnia from incidental field exposure to transgenic corn pollen containing Cry1F. Classification: Acceptable.	450201-08
OPPTS Series 885-4200	Waiver Request: Fish Toxicity Test with Transgenic Maize (Corn) Containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F Delta-Endotoxin.	N/A	The Agency previously has waived static renewal toxicity tests for freshwater fish due to the lack of substantial exposure to Cry protein in runoff and corn pollen. Therefore the registrant's request to waive rainbow trout and bluegill sunfish toxicity studies is granted. In addition, based on submitted results of a protein-specific ELISA analysis and bioasays, no Cry1F protein was detectable in catfish pellets processed from corn kernels containing Cry1F protein. The submitted data and results are sufficient to conclude that the Cry1F protein in aquafarm fish diets is unlikely to present hazardous exposures. Accordingly the registrant's request to waive farmed fish toxicity studies is granted.	450442-01

Guideline No.	Study	Result (LC_{50})	Status, Classification & Comments	MRID #
OPPTS Series 885-4340	Chronic Exposure of *Folsomia candida* to Bacterially Expressed Cry1F Protein.	LC50 and NOEL >12.5 mg Cry1F/kg soil	Three treatment levels (12.5, 3.1, 0.63 mg/kg) of Cry1F were replicated four times for a total of 12 replicates. Test or control diets and water were given to the Collembola every two or three days for 28 days. In addition to a test and control treatment, thiodicarb (carbamate) was used as a reference substance. The number of surviving Collembola in each treatment were counted every seven days and at the end of 28 days. It was estimated that the exposure rates in this study are 1560-, 388-, and 79-fold-higher than would be encountered in the field. Results of the study indicate that levels of Cry1F that might occur in the field are not expected to adversely effect the soil invertebrate Collembola species. This study adequately addresses the Collembola environmental hazard issue. Classification: Acceptable.	450201-07
OECD Guideline 207	Cry 1F Bacillus thuringiensis var. aizawai delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate.	LC50 and NOEL >2.26 mg Cry1F/kg dry soil	There were no apparent treatment-related effects on mortality or body weight of worms in study. The NOEC value was determined to be equal to 1.7 mg Cry1F/kg dry soil and LC_{50} value was determined to be greater than the test concentration of 1.7 mg Cry1F/kg dry soil. [Actually 2.26 mg (Reviewer's comment: 33% moisture content appears to have been subtracted twice to obtain the 1.7 mg figure)] The 14-day LC_{50} value for earthworms exposed to chloroacetamide, a positive control, was determined to be approximately 15.7 mg a.i./kg dry soil . The one limit test concentration of 2.26 mg Cry1F/kg dry soil represented up to 100X the estimated concentration present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants. This concentration is higher than any amount of Cry protein that may be present in the soil during any stage of the growing season (such as from root exudation). Based on the results of this study, it is not likely that Cry1F transgenic corn plantings will have adverse effects on earthworms. Classification: Supplemental since test material analysis did not meet GLP standards.	450201-06, 453078-04 (supplement)

Guideline No.	Study	Result (LC_{50})	Status, Classification & Comments	MRID #
OPPTS Series 885.4340	Cry1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: A Dietary Toxicity Study With Green Lacewing Larvae.	LC_{50} and NOEC > 480 ppm a.i (pollen expressing 32 ng Cry1F/mg pollen)	Green lacewing larvae fed a concentration of Bt Cry1F protein at 15x the expected rate found in corn pollen (pollen expressing 32 ng Cry1F/mg pollen) resulted in no mortality or signs of toxicity or abnormal behavior over a 13 day period (>20% control mortality period). The LC_{50} and NOEC was determined to be >15x the concentration of Cry1F found in pollen and the was determined to be > 480 ppm a.i (the test concentration). Mortality and pupation rate were comparable between the treatment and control group. Therefore Cry1F at concentrations <15x that found in corn pollen should have no detectable adverse effects on *Chrysoperla carnea* in the field. This study adequately addresses the green lacewing environmental hazard issue. Classification: Acceptable.	450201-09, 453078-01 (supplement)
OPPTS Series 885.4340	Cry1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: A Dietary Toxicity Study With the Parasitic Hymenoptera. (*Nasonia vitripennis*)	LC_{50} and NOEC > 320 ppm a.i (pollen expressing 32 ng Cry1F/mg pollen)	Parasitic Hymenoptera fed a concentration of Bt Cry1F protein 10x the expected rate found in corn pollen (expressing 32 ng Cry1F/mg pollen) showed no mortality or signs of toxicity or abnormal appearance or behavior of surviving wasps in the treatment or control group over a 12 day period. The test was terminated after 12 days because 20% mortality was reached in the negative control. The NOEC and the LC_{50} were determined to be > 320 ppm a.i (10x field rate when calculated for pollen expressing 32 ng Cry1F/mg pollen). Therefore no hazard at field use rates is expected from the cultivation of Cry1F containing corn. This study adequately addresses potential concerns for Cry1F protein expressed in corn to parasitic Hymenoptera. Classification: Acceptable.	450201-11, 453078-03 (supplement)

Guideline No.	Study	Result (LC_{50})	Status, Classification & Comments	MRID #
OPPTS Series 885.4340	Cry1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: A Dietary Toxicity Study With the Ladybird Beetle. (*Hippodamia convergens*).	LC_{50} and NOEC > 480 ppm a.i (pollen expressing 32 ng Cry1F/mg pollen)	Adult lady beetles fed a concentration of Bt Cry1F protein at 15x the expected rate found in corn pollen (pollen expressing 32 ng Cry1F/mg pollen) resulted in no mortality or signs of toxicity over a 29 day period. Therefore, the NOEC and the LC_{50} were determined to be >15x the concentration of Cry1F found in pollen determined to be > 480 ppm a.i (the test concentration). The test insects were exposed to a dose of active ingredient approximating the amount that would be ingested by the beetles feeding on aphids under field conditions. As a result, no discernible beneficial beetle population effects are expected from the proposed uses of the Cry1F producing corn. This study adequately address potential concerns for Cry1F protein expressed in corn to beneficial beetles. Classification: Acceptable.	450201-10, 453078-02 (supplement)
OPPTS Series 885.4340	Toxicity of the Cry1F Protein to Neonate Larvae of the Monarch Butterfly (*Danaus plexippus* (Linnaeus).	LC_{50} > 10,000 ng/mL. NOEC <10,000 ng/mL.	First instar larval weight and mortality were recorded after seven days of feeding. There was no mortality to monarchs fed 10,000 ng/mL diet, the highest rate tested. There was some growth inhibition at 10,000 ng/mL diet. The study is scientifically sound. Since doses equivalent to 10,000 ng/mL diet are not likely to occur in nature, it can be concluded that Cry1F protein will not pose a risk to monarchs. Classification: Acceptable. RECOMMENDATIONS: The conclusions should be confirmed by providing data showing that the amounts of Cry protein found on milkweed leaves in the field are at concentrations less than the 10.000 ng/mL diet used in this study. The NOEC also has to be determined.	451311-02

Guideline No.	Study	Result (LC_{50})	Status, Classification & Comments	MRID #
154-35	Field Survey	N/A	Sticky traps were set out weekly for six weeks. In addition, ten plants in the center row were visually evaluated for beneficial arthropods weekly for six weeks. Beneficial insects counted in this study were: lady beetles (*Cycloneda munda* & *Coleomegilla maculata*), predacious Carabids, brown lacewings (Hemerobiidae), green lacewings (*Chrysoperla plorabunda*), minute pirate bugs (*Orius insidiosus*), assassin bugs (Reduviidae), damsel bugs (Nabidae), Ichneumonid and Braconids (parasitic wasps), damselflies and dragonflies, and spiders. Data included counts of adult and larval lady beetles and lacewings when appreciable numbers of were collected. Visual counts showed no significant differences between the number of arthropods collected in Bt Cry1F corn and the non-transgenic isolines with two exceptions. There was a significantly greater number of lady beetles in the 1507 corn line and a significantly greater number of spiders in the 1360 line than the non-transgenic isolines. For lady beetles, there were an average of 1.2 beetles in the 1507 line compared to 0.6 beetles in the non-transgenic line. For spiders, the 1360 line averaged 1.8 spiders per ten plants and the non-transgenic isoline averaged 0.5 spiders per ten plants. In addition, significantly more *Orius* were found in the 1507 line then the non-transgenic line on two of the three sample dates. In general, the most beneficial insects found by visual counts were in the 1507 Cry1F corn line. Sticky trap counts showed that no significant differences between the number of most arthropod species in the transgenic corn and their respective isolines. However, the average number of parasitic Hymenoptera and *Orius* observed across all sample dates were significantly greater in the 1507 line then the non-transgenic isoline. For parasitic Hymenoptera, there was an average of 1.7 wasps per trap in the 1507 line compared to the average of 1 per trap in the non-transgenic isoline. For all arthropods, the average number of insects per trap in the non-transgenic 1507 isoline was 11.1 and the average number in the transgenic 1507 line was 8.7. The non-transgenic 1360 isoline averaged 9.5 insects per sticky trap while an average of 6.8 insects per sticky trap were collected in the 1360 transgenic line. The study is scientifically sound. There was no consistent pattern of differences in abundance of predatory insects on the Cry1F versus the control corn plots during the sampling period. This field census study adequately address potential concerns for Cry1F protein expressed in corn to non-target insect populations. Classification: Acceptable. RECOMMENDATIONS: Testing of larger plot sizes would, however, produce more significant results. Therefore it is recommended that the beneficial insect monitoring should continue into the first few years of commercial use of Cry1F corn crops to confirm the single season small plot "no effects" findings and make long range observations on non-target insect effects and abundance.	450201-13

Guideline No.	Study	Result (LC_{50})	Status, Classification & Comments	MRID #
N/A	Non-target Exposure and Risk Assessment for Environmental Dispersal of Cry1F Maize Pollen. (A probabilistic risk assessment)	N/A	To consider the exposure of non-target species including endangered Lepidoptera species to field corn pollen expressing the Cry1F delta endotoxin by evaluating pollen dissemination. The Cry1F concentration found in pollen occurring on milkweeds near the edge of Bt corn fields was predicted. Distance of pollen dispersal, levels of Cry1F expression in pollen, milkweed distribution and biomass from the edge of the field, pollen grain physical properties, and spatial-temporal availability of Cry1F to monarch larvae was determined. According to a probability-log plot demonstrating lepidopteran species susceptibility to Cry1F, 99% of lepidopteran species exhibit an LC_{50} of $0.06 µg g^{-1} which is 290-fold lower than the geometric mean LC_{50} (12.4 µg g^{-1}) and lower than the most sensitive lepidopteran species. The toxicity threshold, or no effect level for monarch neonates, for the Tier 1 risk assessment was determined to be 10 µg g^{-1} diet. When fed up to 10 µg g^{-1} Cry1F microbial toxin in diet, neonate monarch larvae were not affected. The toxicity threshold, or no effect level for monarch neonates, for the Tier 1 risk assessment was determined to be 10 µg g^{-1}. The log-probability plot of the Bt LC_{50} for lepidopteran species shows that the EEC does not exceed the LC_{50} for 98% of the intergenera population beyond 1 m from the field edge. The LC_{50} is not exceeded for 90% of the population 0.2 m from the edge. For monarch larvae, the no effect level (10 µg g^{-1}) occurs near the 50^{th} percentile intergenera LC_{50}. Since there is a rapid fall-off in exposure with distance, there is limited potential for non-target effects beyond the immediate field border. In addition, the estimated risk quotients (ratio of exposure to effect) demonstrate a lack of concern for monarchs (or other lepidopteran species) beyond 1 m from the field edge. The RQ in the corn field is 0.096. Classification: Acceptable.	450415-02

2) Effects on Mammals

The data submitted to EPA indicate that there is no significant toxicity to rodents from acute oral testing at the maximum hazard dose. In addition, there are reports of no adverse effects on livestock after several years of feeding with *Bt* corn. Mammalian wildlife exposure to the *Bt* Cry

proteins is considered likely; however, the mammalian toxicology information gathered to date does not show a hazard to wild or domesticated mammals.

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3) Avian

When administered by oral gavage at a dosage up to 2,000 mg protein/kg body weight, *Bt* corn has no apparent effect upon bobwhite quail after 14 days. A study with a non-commercial line of MON 80187 showed no mortality or differences in food consumption, body weight, or behavior when bobwhite quail were fed 50,000 or 100,000 ppm Cry1Ab in corn meal. In addition, there are reports of no adverse effects from the commercial poultry industry after several years of using *Bt* corn in poultry feeds.

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

These data indicate that the *Bt* Cry1 protein produced in corn does not show a hazard to birds. However, these data are not sufficient to make a final hazard assessment from repeated exposure(s) to higher doses of *Bt* corn. A six week study with 60 to 70% corn in the diet is necessary to assess hazards from chronic exposure of wild and domesticated fowl.

4) Aquatic

There is no evidence for sensitivity of aquatic (including endangered) species to Cry proteins. Toxicity studies with aquatic organisms show very limited hazard for fish or invertebrates exposed to either corn pollen or to bacterially expressed Cry protein. In addition, aquatic exposure from *Bt* crops is extremely small. A simple standard pond scenario (1 hectare pond, 2 meters deep draining a 10 hectare watershed planted with corn) was used to develop a worst case EEC for Cry1Ab and Cry1F protein (high protein expression level) on the basis of corn pollen loadings from airborne pollen deposition and agricultural runoff. Airborne pollen deposition will result in water concentrations less than 78 ng Cry1Ab protein/L when based on conservative estimates for pollen dispersal. The contribution of Cry1Ab to the pond through agricultural runoff is comparable (66 ng L-1 based on GENECC). Thus, total water concentration of less than 144 ng Cry1Ab protein/L is projected under worst case conditions (Wolt, 2000). Airborne pollen deposition results in water concentrations of approximately 1.25 ng Cry1F/mL and the contribution of Cry1F to the pond through agricultural runoff is <0.15 ng/mL. Thus, total water concentration of 1.4 ng Cry1F protein/L is projected under worst case conditions.

a. Aquatic Invertebrates

The major source of *Bt* Cry proteins in fresh water would be corn pollen. Toxicity studies with corn pollen containing Cry1Ab proteins conducted using *Daphnia magna* show an acute EC_{50} was greater than 100 mg/L in one study and in another150 mg/L. The LOEC (lowest observed effect concentration) was found to be 150 mg/L. The amount of pollen was considered to well exceed the 144 ng Cry1Ab protein /L projected aquatic exposure in the fields under worst case conditions. Toxicity studies with corn pollen containing Cry1F proteins conducted using the sensitive aquatic indicator species *Daphnia magna* show the no-mortality concentration and NOEC to be >100 mg a.i./L. There were no overt signs of toxicity to daphnids exposed to 100 mg Bt Cry1F pollen/L. The amount of pollen tested was considered to well exceed field exposure. These studies indicate that *Bt* Cry1Ab and Cry1F proteins expressed in pollen do not pose a hazard to *Daphnia magna*. In view of the above data, no freshwater aquatic invertebrate hazard is expected from the use of *Bt* Cry1Ab corn crops.

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

b. Fish

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

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

5) Plants

Since the active ingredient in this product is an insect toxin (*Bt* endotoxin) that has never shown any toxicity to plants, the plant toxicity studies have been waived.

6) Non-target Invertebrates

a) Honey Bees

1) Cry1Ab

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

Concerns have been raised regarding some honey bee larval studies submitted to the Agency. The question raised was whether the honey bee larvae that were dosed with pollen containing Cry proteins, were actually exposed to the proteins. Because the pollen has to be pre-digested by nurse bees (which, conversely, may also inactivate the Cry protein) in order to be palatable to larval honeybees (EcoStrat, 2000). However, small amounts of pollen are known to be fed directly by nurse bees in the hive (Winston, 1987). In addition, the Agency has other laboratory studies on file in which aqueous mixtures of purified Cry protein had been added to the diet of honeybee larvae maturing within honeycomb brood cells, or to a 1:1 (vol:vol) honey-water mixture for adult honeybees. No adverse effect was observed, neither on larvae, nor on adults. This conclusion has been confirmed by hive studies in the field.

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

2) Cry1F

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

b) Lady Beetles

1) Cry1Ab

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

2) Cry 1F

Adult lady beetles (*Hippodamia convergens)* fed a concentration of Bt Cry1F protein at 15x the expected rate found in corn pollen resulted in no mortality or signs of toxicity over a 29 day period. Therefore, the NOEC was determined to be >15x the concentration of Cry1F found in pollen and the LC_{50} was determined to be > 480 ppm a.i (the test concentration). The submitted study shows that corn containing the Cry1F protein should not cause significant adverse effects to lady bird beetle predators. The test insects were exposed to a dose of active ingredient approximating the amount that would be ingested by the beetles feeding on aphids under field conditions. As a result, no discernible beneficial beetle population effects are expected from the proposed uses of the Cry1F producing corn. This conclusion is confirmed by adult and larval lady beetle abundance found in the field census study. These studies adequately address potential concerns for Cry1F protein expressed in corn to beneficial beetles.

c) Parasitic Hymenoptera

1) Cry1Ab

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

2) Cry1F

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

d) Green Lacewing

1) Cry1Ab

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

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

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

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

Generally, these findings do not show any detrimental effects at Cry protein exposure levels in the field. The laboratory results were seen only at exposure to micro gram quantities, whereas in

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

2) Cry1F

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

e) Soil Invertebrates

The FIFRA Sciientific Advisoty Panel (USEPA, 2001) does not believe that Collembola and earthworms are appropriate indicator species for Cry1Ab testing because of the Lepidoptera-specific nature of the Cry1Ab protein. When it initially reviewed the applications for the products that were registered in 1995, EPA considered requiring studies evaluating effects upon the representative beneficial soil invertebrates Collembola and earthworms. EPA was concerned (1) that such soil organisms may be subject to long-term exposure as a result of soil incorporation of crop residues or when crop residues are left on the soil surface and (2) that adverse effects on such soil organisms could result in an accumulation of plant detritus in fields. Recent reports of exudation of Cry proteins by corn roots throughout the growth season add to this concern. However, EPA understands that routine agronomic practices have included the long term use of chemical insecticides, which have adverse effects on soil organisms, but there has not been an accumulation of significant amounts of plant detritus in soils (Pimentel& Raven, 2000). Thus, *Bt* crops, which are expected to have less impact on these species than chemical pesticides, should not result in any increased build up of plant detritus or Cry proteins at toxic levels. Supporting this conclusion are data required by the EPA which indicate that such proteins are known to degrade rapidly in field soils. Therefore the potential for significant soil

buildup and effects to non-target soil organisms are not anticipated. This has been confirmed by Saxena and Stotzky (2001), who report that *Bt* Cry protein released from root exudates and biomass of *Bt* corn has no apparent effect on earthworms, nematodes, protozoa, algae, bacteria, actinomyces and fungi in soil in spite of the fact that enough detectable Cry protein is bound to soil particles to show toxicity to the target pest. These results suggest that despite its presence in soil, the Cry protein released in root exudates of *Bt* corn, or from the degradation of the biomass of *Bt* corn, is not toxic to a variety of organisms in the soil environment. Stotzky (2000) also reported that the same degree of *Bt* Cry protein persistence takes place in soils that have been exposed to repeat *Bt* microbial spray applications. In addition, new plants grown in *Bt* containing soil do not take up the *Bt* protein.

i. Earthworms

Earthworm feeding studies submitted to the Agency for all of the registered Cry proteins demonstrate that the Cry proteins are not toxic at the expected environmental concentration. Concerns have been raised as to whether the earthworms actually ingested the *Bt* Cry proteins when these are incorporated into the soil in the test systems used (EcoStrat, 2000). This question is mainly of academic importance. For hazard assessment purposes it is sufficient to know that the earthworms were not harmed when presented with the *Bt* Cry proteins in their soil environment. If they do not ingest it in the test soil, likewise they will not ingest it in the field. The earthworms do, however, ingest the *Bt* Cry proteins with the soil without harmful effects. Saxena and Stotzky (2001) report that there were no significant differences in the percent mortality and weight of earthworms after 40 days in soil planted with *Bt* or non-*Bt* corn or not planted, or after 45 days in soil amended with biomass of *Bt* or non-*Bt* corn or not amended. However, the toxin was present in both the casts and guts of the worms in these tests.

a) Cry 1Ab

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

b) Cry1F

The submitted data show that Cry1F protein has no measurable deleterious effects on earthworms, a representative beneficial soil invertebrate species. This suggests that the proposed uses of the Cry1F protein in corn are not likely to have any measurable population effects on beneficial soil invertebrates. The one limit test concentration of 2.26 mg Cry1F/kg dry soil

represented more than 100X the estimated concentration present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants. This concentration is higher than any amount of Cry protein that may be present in the soil during any stage of the growing season (such as from root exudation). Based on the results of this study, Cry1F transgenic corn plantings will have no adverse effects on earthworms.

ii. Collembola

a) Cry1Ab

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

The Collembola studies submitted to the Agency for most of the registered Cry proteins showed no adverse effects at maximum hazard doses. Novartis (now Sygenta) had cited the MON 810 leaf tissue study to support their *Bt*11 corn plant-incorporated protectant. While this study is useful in characterizing effects of Cry1Ab corn tissue on Collembola and satisfies the requirement for MON 810 Cry1Ab corn, it does not adequately characterize the effect of *Bt*11 corn tissue on Collembola. The requirement for a Collembola study which includes control plant lyophilized leaf tissue from non-transgenic parental corn lines and lyophilized leaf tissue containing the *Bt*11 plant-incorporated protectant is not fulfilled. However, in light of recent recommendations by the FIFRA Scientific Advisory Panel (USEPA, 2001) that invertebrates known not to be affected by the Cry proteins specific for insects of different orders not be tested, this requirement can be waived.

b) Cry1F

Since Collembola feed on decaying plant material in the soil, they may be exposed to Cry1F protein in corn found in the field. A study was conducted to determine if there may be adverse

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

As discussed above, EPA does not believe that to date there are any valid data demonstrating specific adverse impacts of plants expressing *Bt* Cry 1 proteins on beneficial soil invertebrates. To the contrary, EPA believes that available scientific data and information indicate that cultivation of *Bt* crops has a positive effect on soil flora, when compared to the most likely alternative, use of non-selective synthetic chemical pesticides.

f) Non-Target Insect Abundance Studies

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

Pilcher et al. (1997b) conducted limited size field studies in two consecutive years with *Bt* corn. No differences were observed in the number of predators colonizing either isogenic control corn or *Bt* corn in 1994. In 1995 more predators were seen on *Bt* corn than on control corn. The authors concluded that *Bt* corn pollen did not affect predator abundance. However, they also concluded that the absence of significant differences may have resulted from plot size. Due to the small plot sizes separated by only one buffer row, pollen from *Bt* corn and isogenic corn may have been mixed by wind. They concluded that the inconsistent results between the two years indicate that larger scale studies are necessary for significant data.

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

Obrycki, (1997) performed a study to determine the effects of transgenic corn expressing *Bt* Cry protein on the abundance of predatory insects in corn fields. He found that the average number of predatory insects was not significantly different between the sprayed and unsprayed plots on four of the five observation days over a seven week period. Conventional pesticide spray drift

was suspected as the reason. No significant difference in abundance was found between the *Bt* and the non *Bt* plots on any of the five observation days. Similar numbers of Coccinellid eggs, larvae, and pupae were observed on the transgenic and the non-transformed corn plants. Higher numbers of Chrysopid eggs, *Orius insidious* (Anthocoridae), Nabidae, and Arachnida were observed on the *Bt* corn, but not at statistically significant levels. Aventis Crop Science currently is conducting a 5 year study on assessing non-target beneficial insects. In each of the first 3 years (through 1999) no differences were found in the numbers and types of insects in *Bt* and non-*Bt* fields.

Lozzia (1999) reports a biodiversity and structure of ground beetle assemblages (Coleoptera Carabidae) trial in Cry1Ab *Bt* corn and the effects on non-target insects conducted in 4 trials in North Italy over a 2 year period. No statistical difference was evident in the total number of carabids. There was no decreasing trend in the biodiversity indices from the first to the second year and considering the data as a whole, the two years appear comparable. The difference in biodiversity recorded for some indices was not due to the presence of transgenic corn. The aerial fauna as a whole for both years and both localities was not different. Similarly, abundance of aphids, leaf hoppers, other Homoptera, thrips, leaf beetles, spiders, lady beetles, parasitic Hymenoptera, other Hymenoptera, and Diptera were not different. The number of arthropods was higher, but not significant, in the transgenic corn. Several sampling methods and visual checking, show that there was no significant difference in abundance, composition or biodiversity of non target arthropods in isogenic and transgenic corn crops. The data show that the transgenic plants do not lead to an increase or decrease of any insect populations. It appears that Cry1Ab proteins do not directly affect the phytophagous species nor do they have "any indirect influence on other trophic levels or activities such as behavior, oviposition or predators-prey."

Nuessly & Hentz (1999) conducted 4 studies using Novartis (now Sygenta) Seeds' Attribute *Bt*11-derived Cry1Ab sweet corn hybrids and conventional sweet corn hybrids grown under local practices in 4 Florida locations. Noted in the reports were increases in species diversity in the corn plots, i.e. there were generally higher populations of beneficial and non-target insects as compared to the conventional control plots, associated with the significantly decreased use of broad-spectrum insecticides (organophosphates, carbamates and synthetic pyrethroids).

g) Non-Target Lepidoptera

The toxicity of *Bt* Cry1 proteins to certain Lepidoptera (moths and butterflies) is a well known and a widely published phenomenon. EPA risk assessments of *Bt* products rely on toxicity and exposure. Long term effects from the use of *Bt* microbial sprays to control gypsy moth have been studied in U.S. Forest Service sponsored research. Since the exposure to butterflies and moths from the agricultural uses of *Bt* Cry protein was not expected to be as high as in forest spraying (where no widespread/recurring or irreversible harm to lepidopteran insects was observed), *Bt* corn likewise was not expected to cause widespread or irreversible harm to non-target lepidopteran insects. Reports of toxicity of high doses of *Bt* Cry1Ab protein to monarch

butterflies in the laboratory do not necessarily mean that there will be exposure to toxic levels in the field. EPA has been participating in an aggressive research effort to assess the field significance of this finding. The following is a discussion of the studies available to date on the field toxicity of Cry1 proteins to non-target Lepidoptera species.

i. Black Swallowtail Butterfly

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

ii. Karner Blue Butterfly and Other Threatened or Endangered Species

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

EPA concluded that because Cry1 proteins are broadly active against Lepidoptera, some activity against the Karner blue would not be surprising. Toxicity testing of Karner blue larvae directly, however, is not possible due to its endangered status. Previous studies that tested the susceptibility of lepidopterans to Cry1 proteins resulted in different LC_{50} values for different species in the same genus. For example the Cry1Ab LC_{50} for *Spodoptera exigua* is estimated at 3,180 ng/mL diet and 95,890 ng/mL diet for *Spodoptera frugiperda* (Luttrell *et al*. 1999). Herms

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

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

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

Toxicity of pollen from the currently registered Cry1Ab *Bt* corn products to Karner blue larvae is estimated to be very low. At least 12 lepidopteran species have been tested to determine LC_{50} levels for Cry1Ab (MRID 455122-00). The most sensitive species tested is the monarch butterfly. Researchers have determined that the concentration producing no mortality whatsoever is greater than (>) 4000 pollen grains/cm^2 of leaf surface (Hellmich, et al, 2001). Thus the actual LD_{50} for monarchs is likely to be substantially higher. Since the EEC is 300

pollen grains /cm^2 or less at the field edge, and 200 at one meter and 75 at three meters,[5] the ratios of the EEC/LC_{50} (with >4000 pollen/cm^2 as that LC_{50}) have been conservatively calculated to be 1:>13.3 for Cry1Ab at the field edge, 1:>20 at one meter and 1:>53 at two meters from the field edge (Vaituzis, et al, 2001).

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

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

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

received a comment to the effect that "[t]he Karner Blue is documented as occurring adjacent to corn fields", examination of the cited reference proves the opposite.[6]

Overlap of corn pollen shed and larval emergence. Karner blue larvae are relatively less likely to be feeding during, or following the whole period of corn pollen shed. An analysis of pollen shed overlap with Karner blue larvae has been submitted to EPA and reviewed (MRID 455129-

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

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

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

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

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

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

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

iii. Monarch Butterflies

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

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

These reports were reviewed by the Agency. The reviews concluded that the preliminary controlled studies without exposure data are not conducive to conventional risk assessment procedures for Bt corn pollen effects on monarch butterflies without additional field study information. The reports of Bt corn pollen toxicity to monarch caterpillars did, however, result in a number of steps taken by the Agency to more fully assess and understand the possible effects of transgenic corn expressing an insecticidal protein from *Bacillus thuringiensis* (Bt corn) on non-target lepidopteran species, particularly monarch butterflies (*Danaus plexippus*). To help identify the level of exposure and other risks to monarch butterflies, on December 15, 1999 EPA issued a monarch butterfly adverse effects data call-in (DCI) notice to the registrants of Bt corn products under its FIFRA Section 3(c)(2)(B) authority. On December 9, 1999 (USEPA, 2000), and again on October 18-20, 2000 (USEPA, 2001), the Agency presented current and possible new data requirements to evaluate ecological effects, including the monarch question, to a FIFRA Scientific Advisory Panel for their recommendations. In addition, EPA consulted with monarch butterfly experts and USDA to better understand the effect of Bt corn pollen on monarch butterflies. Until more definitive data and information were available about the potential risks of Bt corn pollen to monarch butterflies and other lepidopterans, EPA requested that registrants instruct their customers who are planting non-Bt corn refuges (for resistance management) to place the non-Bt corn refuge between Bt corn and habitats such as prairies, forests, conservation areas, and roadsides as a precautionary measure. However, in light of the recently reviewed DCI research data showing that monarchs appear to breed on milkweed inside corn fields and that toxic levels of Bt pollen do not accumulate outside corn fields, this recommendation no longer appears necessary.

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

1. Monarch habitat

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

There have been several attempts made to determine monarch population levels. Swengel (1995) showed that from 1986-1994 there were significant changes in monarch counts including increases and decreases from five of eight year-pairs. Walton and Brower (2000) showed extreme variability in monarch counts in Cape May Point, NJ which is a major funnel point in September and October for monarchs migrating to Mexico. In Cape May, the 1999 counts were seven times greater than in 1998 and almost twice as high as any year since 1992 when the census began. Monarch Watch has conducted annual surveys since 1993. Surveys from 1993-1999 are available online at www.monarchwatch.org.

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

The DCI addresses the potential of monarch exposure to Bt corn pollen in the field and whether pollen densities encountered present a risk to these butterflies. Monarch larvae potentially feed on 14 different species of milkweeds. Seven of these milkweed species are fed on by monarchs in the Corn Belt. Common milkweed (*Asclepias syriaca*) is the predominant species oviposited

and fed on by monarchs. Whorled milkweed (*Asclepias verticillata*) may also be an important resource for monarchs (Hartzler and Buhler 2000). Milkweed densities vary and typically depend on the management practices of the habitat.

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

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

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

Corn pollen grains don't disperse far from its source because they are large (~90 to 100 microns). The majority of corn pollen stays within corn fields and only small quantities disperse

beyond 5 meters from the field edge. However, pollen levels are higher further into the corn field (e.g., 147.5 grains/cm^2 were found 25 m into the field) than close to the edge (e.g., 55.5 grains/cm^2 were found 3 m into the field) (Pleasants et al. 2001). Raynor et al. (1972) found that 63% of corn pollen remained within fields, 88% settled within eight meters of the field edge, and 98% settled within 60 meters. They also determined that there was only 0.2% of pollen deposited at 60 m from the corn field edge. Pleasants et al. (2001) found pollen densities at corn field edges were 50% of the level found within the field and densities were greater on milkweed plants within rows than between rows.

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

2. Corn pollen exposure

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

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

laboratory. Therefore, the Cry protein may breakdown more rapidly than seven days under field conditions.

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

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

Oberhauser et al. (2001) and Pleasants et al. (2001) showed that monarchs do occur on milkweeds in the field during pollen shed. The Oberhauser et al. (2001) study showed considerable overlap between the peak of the migratory monarch generation and pollen shed in Minnesota and Ontario. In Iowa and Maryland, the final generation of monarchs peaked prior to pollen shed. Four different monarch breeding regions (east-central Minnesota and west-central Wisconsin, central Iowa, coastal Maryland, and southern Ontario) were monitored when monarchs were present. Table1. shows the temporal overlap of monarch larvae and corn pollination.

Table 1.

State	% Overlap of Larvae & Anthesis	% Overlap of Migratory Gen. Larvae and Anthesis
Minnesota	20% to 68%	50%
Ontario	27% to 75%	50%
Maryland	0 to 36%	15%
Iowa	4% to 25%	15%

State	% Overlap of Larvae & Anthesis	% Overlap of Migratory Gen. Larvae and Anthesis
Wisconsin		50%

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

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

3. Bt Cry1Ab toxicity to monarchs

Since it has been established that monarch larvae can encounter and feed on Bt pollen in the field, it is important to know the Bt pollen toxicity level. Table 2. shows the LC_{50}s and the EC_{50}s (effect-eliciting concentration) for the various monarch larval stages fed purified trypsin resistant core of Bt Cry 1Ab proteins.

Table 2.

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

(Hellmich et al. 2001).

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

In nature, monarchs are not expected to get uniformly distributed doses of Bt as is observed in the laboratory. Unlike feeding on diet in the laboratory, monarchs would probably ingest varied

amounts of Bt in the field and also have the opportunity to avoid feeding on Bt altogether. Bt activity in pollen is also expected to decline over time in the field. Therefore, levels of Cry1Ab ingested by monarchs in the field are expected to be lower than levels fed to them in the Hellmich et al. (2001) laboratory study.

4. Bt pollen exposure and toxicity

Cry 1Ab in pollen of the currently registered Bt field corn hybrids (MON 810 and Bt11) is only found in trace quantities (<0.09 µg/g dry wt. pollen). In order to determine the exposure of monarch larvae to Bt pollen on milkweed leaves, five independent surveys were conducted during the 2000 growing season in Iowa, Maryland, and Ontario (Table 3). The highest average corn pollen density monitored in the field was 586 grains/cm^2 found three meters inside a Bt11 sweet corn field (Stanley-Horn et al. 2001).

Table 3. Mean pollen density on milkweed leaves inside a cornfield (Pleasants et al, 2001)

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

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

A study conducted by Hellmich et al. (2001) involved feeding first instar monarchs no pollen or known amounts of Bt (MON 810 and Bt11) and non-Bt pollen applied in the laboratory. Extremely high pollen levels (250 - 2000 grains/cm^2 for MON 810 and 150 - 4000 grains/cm^2 for Bt11) were fed to first instar monarch larvae in a controlled environment (since no pollen was

removed due to environmental factors, these conditions are considered a worst case scenario) and resulted in no significant effects on larval weight (Hellmich et al. 2001). From this data, it can be concluded that the NOEL for MON 810 is >2000 grains/cm^2 and for Bt11 is >4000 grains/cm^2 which is greater than levels that occur under natural field conditions.

However, assuming a worst case scenario where 1000 pollen/cm^2 would show weight loss effects, the effect of Bt corn pollen on monarch larvae would still be minimal since these levels are rare in the field, expected to occur at a 0.1% rate (Table 3). In addition, monarch larvae exposed to sub-lethal concentrations of Cry1Ab protein have been shown to mature into healthy adults (Jesse & Obrycki, 2000).

Table 3. Frequency distribution of pollen deposition density on milkweed leaves (Pleasants et al, 2001):

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

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

Stanley-Horn et al. (2001) studied the difference between first instar larvae feeding on Bt11 and on non-Bt pollen in the field (starting six days after initiation of pollen shed) for five days. The results showed no significant mortality, feeding, development, weight gain, % survival to pupation, days to pupation, pupal weight, emergence from pupae, adult weight and adult wing length. However, first instar monarchs feeding on milkweed sprayed with insecticides or subject to exposure from insecticide drift were adversely affected. There was 90% to 100% mortality of

monarchs feeding on milkweeds collected from within the field and 21% to 45% mortality from plants outside the field. This study also suggests that Bt sweet corn may provide a safer habitat for monarchs than fields requiring insecticide applications (Stanley-Horn et al. 2001, Vlachos and Roegner 1997).

5. Bt Cry1F toxicity to monarchs

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

Summary:

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

The submitted data demonstrate that levels of MON 810 and Bt11 corn pollen toxic to monarchs will probably not occur under natural field conditions. The mean pollen densities of all the studies was found to be 170 inside the corn fields and 63 grains/cm^2 at the edge. The highest average corn pollen densities monitored in the field were 586 in Maryland (Stanley-Horn et al. 2001) and 900 grains/cm^2 found in one Iowa corn field (Pleasants et al. 20001). In a worst case scenario, pollen deposition when no rainfall occurred was approximately 1400 grain/cm^2 (Pleasants et al. 2001). Research conducted in response to the DCI showed that Cry1Ab corn pollen densities of >4000 grains/cm^2 do not show mortality to monarchs (Hellmich et al. 2001). These studies have also shown that the order of monarch sensitivity to Cry proteins is Cry1Ab > Cry1Ac > Cry9C > Cry1F. Only pollen from Event 176 corn has been shown to adversely affect growth, fitness, and mortality of monarch butterflies (Losey et al. 1999, Jesse and Obrycki 2000, Stanley-Horn et al. 2001). However, this does not create a concern for monarchs since Event

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

Conclusions:

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

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

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

Based on the review of the submitted DCI data, the Agency concludes that the monarch toxicity information developed in the last two years does not give sufficient cause for undue concern of widespread risks to monarch butterflies at this time.[7] EPA will continue to closely monitor the results from further monarch butterfly research as a part of its regulatory oversight of Bt products.

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

b. Cotton

1) Summary of Non-Target Organism Toxicity Testing of Cry1Ac (006445)

Study	Status, Classification & Comments	MRID #
Avian Dietary LC50.	This study demonstrated that ground cottonseed expressing 0.9 ng *Btk* protein /g fresh wt showed no toxicity to northern bobwhite quail when fed at 10,000 ppm in the diet for 5 days.	431452-11
Stability of Cry1Ac protein	The Cry1c protein from the *Bacillus thuringiensis* subsp. *kurstaki* isolate HD-73 appears to be stable in honey and sucrose solutions.	431452-5
Honey bee larvae	The study adequately demonstrated that purified *Btk* HD-73 crystal protein (Cry1Ac) showed no toxicity to honey bee larvae when exposed to concentrations 1,700 and 10,000 times the levels found in pollen and nectar, respectively, of transgenic insect resistant cotton plants. The study was classified as core.	431452-6
Honey bee adults.	The study adequately demonstrates that purified *Btk* HD-73 crystal protein showed no toxicity to honey bee adults when fed a concentration for seven days 10,000 and 1,700 the amount of endotoxin detected in nectar and pollen, respectively, of transgenic insect resistant cotton plants. The study was classified as core.	431452-7
Parasitic hymenoptera *(Nasonia vitripennis).*	The study adequately demonstrates that purified *Btk* crystal protein showed no toxicity to adults of the parasitic Hymenoptera *Nasonia vitripennis* when fed levels 1,700 and 10,000 times the levels found in pollen and nectar, respectively, of transgenic insect resistant cotton plants. The study was classified as core.	431452-8
Ladybird beetles *(Hippodamia convergens).*	The study adequately demonstrates that purified *Btk* crystal protein showed no toxicity to adult ladybird beetles when fed levels 10,000 and 1,700 times the levels found in nectar and pollen, respectively, of insect resistant cotton plants. The study was classified as core.	431452-9
Green lacewing larvae *(Chrsopa carnea)*	The study adequately demonstrates that purified Cry1Ac protein showed no toxicity to green lacewing larvae when fed levels 1,700 and 10,000 times the levels found in pollen and nectar, respectively, of insect resistant cotton plants. The study was classified as core.	431452-10
Collembola	The data adequately demonstrated that Cry1Ac protein shows no toxicity to two species of Collembola at greater than 200 ppm. No adverse effects expected at field rates.	439416-1

2) Ecological Effects Testing Requirements for Cry 1Ac (006445)

EPA determined that the non-target organisms most likely to be exposed to the protein in transgenic cotton fields were beneficial insects feeding on cotton pollen and nectar, and upland birds feeding on cotton seed. Thus, tests were required utilizing representatives of those organisms. Waterfowl, fish, and aquatic invertebrate tests were waived due to probable lack of exposure.

3) Mammals

The data submitted to EPA indicate no toxicity to rodents during the acute oral testing at the maximum hazard dose. These data showed a lack of toxicity to mammals from exposure to high levels of Cry1Ac. No further testing was required.

4) Avian

Ten day old northern bobwhite quail, *Colinus virginianus,* were fed diets containing 100,000 ppm cottonseed meal from the transgenic cotton line 531, 100,000 ppm cottonseed from the control line C312, and straight basal diet, for five days. Following the five day exposure period untreated feed was given to the birds of all the groups for an additional three days. Three replications of 10 birds each were used for each treatment. Cotton seed tissue contained approximately 0.94 µg Cry1Ac protein/g fresh weight. No mortalities occurred in either the control or treatment groups. There were no significant differences in-either body weight or feed consumption of negative control, line C312 control, and line 531 treatment birds. There were no significant differences in-either body weight or feed consumption of negative control, line C312 control, and line 531 treatment birds. Planting and growing cotton expressing the Cry1Ac protein should not result in any detectable deleterious effects to upland birds.

5) Aquatic Species

EPA waived the data requirements for aquatic species testing because of a lack of exposure. Only limited amounts of pollen would be available for drift and exposure to aquatic invertebrates. There also are no reports of hazard from feeding of cotton seed meal to farmed fish.

6) Non-Target Invertebrates

a) Honeybee Toxicity/Pathogenicity Test

Apis mellifera larvae were exposed to 20 ppm Cry1Ac protein by delivering 5 µL/cell of protein-containing solution to cells containing 1-3 day old bee larvae. The other treatments were distilled water (negative control), heat attenuated Cry1Ac protein, and nothing (untreated control). After dosing the treated frames were returned to the hives for completion of larval development. After the treated cells were capped, hardware cloth emergence cages were placed over the treated areas. Upon adult emergence the bees were moved to adult holding cages, fed a

honey water solution, and held at 22-26C and 36-53% relative humidity. Each treatment was replicated four times, and at least 50 cells were included in each replicate. The test was scored for survival to capping, adult emergence, and adult survival. Each treatment was bioassayed against the target pest to confirm the presence and absence of biological activity. Percent capped cells of the untreated, distilled water, Cry1Ac and heat-attenuated Cry1Ac groups were 84, 86, 80, and 77%, respectively. Percent emergence of the above groups was 95, 64, 100, and 77%, respectively. Adult survival from emergence through trial termination of the above groups was 66, 59, 58, and 73%, respectively. Mortality differences, within the larval and post-emergence adult groups, between treatments were not statistically significant. All surviving adult bees were normal in behavior and appearance. It is apparent from the data presented that Cry1Ac protein has no measurable deleterious effects on honey bee larvae and adults.

b) Predatory Coleopteran–Lady Beetle.

Adult ladybird beetles were exposed to 20 ppm Cry1Ac protein in a honey water diet for 30 days. The predators were also exposed concurrently to an attenuated Cry1Ac control and a negative control. Two replicates of 25 predators each were used. The test diets were renewed every three days. The predators were observed for toxicity and mortality twice during the first four hours of the test, and once daily until the test was terminated at 30 days. Samples of the treatment and control diets were taken on test days 0, 9, 18 and 27 for verification of concentrations and stability of the test substances in the honey water diets. This was established by bioassay against *Heliothis virescens* larvae. There were no significant differences between mortalities observed in any of the test groups. After 30 days the mean total mortalities in the untreated control, attenuated toxin control, and toxin treatments were 20, 22, and 24%, respectively. There was no statistically significant increase in the rate of mortality observed in these groups when compared to the negative control group. The mortality, immobility and lethargy observed during the test were not considered to be treatment related. The LC50 value and the NOEL for ladybird beetles was 20 ppm. Cry1Ac has no measurable deleterious effects on the predaceous coleopteran.

c) Parasitic Hymenoptera.

Adult *Nasonia vitripennis* were exposed to 20 ppm Cry1Ac protein in a honey water diet for 23 days. The parasites were also exposed concurrently to an attenuated Cry1Ac control and a negative control. Two replicates of 25 parasites each were used. The test diets were renewed every three days. The parasites were observed for toxicity and mortality twice during the first four hours of the test, and once daily until the test was terminated at 23 days. Samples of the treatment and control diets were taken on test days 0, 9, 18 and 23 for verification of concentrations and stability of the test substances in the honey water diets. This was established by bioassay against *Heliothis virescens* larvae. There were no significant differences between mortalities observed in any of the test groups. After 23 days the mean total mortalities in the non-treated control, attenuated toxin control, and toxin treatments were 26, 38, and 20%, respectively. In the negative control group one wasp was observed to be immobile on test day

18. In the toxin treatment group one wasp was observed to be immobile on test day 0, and two wasps were noted to be lethargic on test day 22. All other surviving wasps in all treatment groups were normal in behavior and appearance. Until test day 17, mortalities in all the groups were 10% or below, indicating that the wasps were reaching their maximum life span under the test conditions at that time. The LC_{50} for parasitic hymenoptera exposed to Cry1Ac for 23 days was determined to be greater than 20 ppm. The NOEL was 20 ppm. This test demonstrates that Cry1Ac protein has no detectable deleterious effects on the parasitic hymenopteran *N. vitripennis* when fed the dosage of 20 ppm for 23 days.

d) Green Lacewing

Green lacewing larvae were exposed to 20 ppm Cry1Ac protein in a paste of *Sitotroga* sp. eggs for 11 days. The predators were also exposed concurrently to an attenuated Cry1Ac control and a negative control. Thirty larvae were exposed to each treatment. Fresh diet was prepared daily. The predators were observed for toxicity and mortality once during the first four hours of the test, and once daily until the test was terminated at 11 days, when the larvae began pupating. Samples of the treatment and control diets were taken on test days 0, 9, and 11 for verification of concentrations and stability of the test substances in the egg paste diets. This was established by bioassay against *Heliothis virescens* larvae. There were no significant differences between mortalities observed in any of the test groups. After 11 days the mean total mortalities in the untreated control, attenuated toxin control, and toxin treatments were 20, 20, and 10%, respectively. At test termination percent pupation in the untreated control, attenuated toxin control, and toxin treatments were 7, 10, and 27%, respectively. There was no statistically significant increase in mortality in either the attenuated control group or the 20 ppm treatment group when compared to the negative control group. The LC50 value and the NOEL for green lacewing larvae exposed to Cry1Ac for 11 days was determined to be greater than 20 ppm. Cry1Ac has no detectable deleterious effects on the predaceous neuropteran *C. carnea* when fed the dosage of 20 ppm for 11 days.

e) Non-Target Insect Abundance Studies

Wilson et al. (1992) studied the effects of Monsanto's experimental lines '62', '65', and '82' expressing Cry1Ab protein in cotton on pink bollworm (Lepidoptera: Gelechiidae) and other insects. They report that the transgenic cotton lines had no significant effect on populations of beneficial predator insects.

Fitt et al. (1994) did a limited scope field evaluation for potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia. They tested 6 cotton varieties transformed with the Cry1Ab and their respective control lines. They found the numbers of beneficial insects were similar on control and transgenic plants. The impact of chemical spray drift clearly affected beneficial insect abundance. As expected from the known toxicity spectrum of *Bt*, there was little or no impact on the abundance of beneficial insects. Studies on larger plots are recommended for full effects evaluation.

Sims (1995) reports that Cry1Ac protein expressed in transgenic cotton produced no toxic effects on the four species of beneficial insects (*N. vitripennis, H. convergens, C. carnea*, *A. melliferae* adults, *A. melliferae* larvae). All surviving insects were normal in appearance and behavior during the tests. Overall the data presented in the study support the conclusion that Cry1Ac proteins expressed in the tissues of transgenic cotton have no activity against beneficial or non-target insects other than those in the order Lepidotpera.

Hardee & Bryan (1997) report a 2 year cotton field study on insect populations with emphasis on the tarnished plant bug (Heteroptera: Miridae). The study involved different *Bt* varieties: (1) nectar-less/high fibered variety MD51ne MD51ne and (2) *Bt* line 757 NuCotn33. In 1994 fewer beneficials were recorded in line 757 than in grower varieties (not significant) and significantly fewer in MD51ne in 1995 than in other varieties. Analyses for 'location by treatment interaction' each year showed that the interaction was seldom significant, indicating the validity of using plots at different locations as replications. The transgenic character itself did not cause an increase of any insect pest population.

f) Soil Invertebrates

Studies on the effects of earthworms were not required. It was originally thought that because long-term exposure of soil organisms such as earthworms is possible when crop residues are incorporated or left upon the soil surface, EPA would require studies evaluating effects upon earthworms. Data submitted by Monsanto indicate that Cry protein production ceases at senescence, allowing some time for protein degradation prior to harvest. Additionally, as the environmental fate data indicate that only 1.44 grams of Cry1Ac protein per acre would enter the soil as a result of post harvest incorporation of *Bt* cotton, and such proteins degrade rapidly, the potential for effects to non-target soil organisms is not anticipated. Thus, an observable deleterious effect on earthworms is not expected to result from the growing of Cry1Ac protein containing cotton plants.

Two Collembola species, *Folsomia candida* and *Xenylla grisea* were fed a test diet prepared by suspending 1.0 g of Bakers yeast in 3.0 mL of distilled water containing 200micro g of Cry1Ac protein. The dose concentration was confirmed by ELISA and/or insect bioassay techniques. Positive control consisted of chlorpyrifos added to yeast to obtain 200, 20, 2, 0.2 and 0.0 (negative control) ppm concentrations. There were ten insects per 5 replicates each for treatment and control groups for *Folsomia candida* and 6 replicates for *Xenylla grisea* in the test system. The test lasted for 21 days with fresh diet being added on days 0, 7 and day 14. The chlorpyrifos response system consisted of 4 replicates per concentration. The Cry1Ac protein tested did not have a detrimental effect on the survival or reproduction of *F. candida* or *X. grisea*. The NOEC therefore was >200 ppm. Adults and progeny of *X. grisea* were combined for the statistical analysis because of difficulty in discriminating between the initial adults and older progeny. For the chlorpyrifos control the no observed effect (NOEC) for *F. candida* was 2.0 ppm. Progeny production among the survivors at 2.0 ppm was not significantly different from the control. *X. grisea* was considerably less susceptible to chlorpyrifos. The NOEC was >200 ppm. The

survival and reproduction appear reduced at 20 and 200 ppm, however these were not statistically significant. The study was scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. No adverse effects were seen to Collembola by chronic exposure to purified Cry1Ac protein at a maximum hazard dose of 200 ppm. The study showed that at field use rates survival and reproduction of the test insects would not be impaired. The study also shows that *X. grisea* did not exhibit any detrimental effects. This study adequately address potential concerns for Cry1Ac protein expressed in transgenic cotton to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry1Ac proteins found in transgenic cotton pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial soil insects.

g) Endangered Species Considerations

Cotton is an insect pollinated crop, and only very small amounts of pollen containing the Cry1Ac protein can drift out of fields. Pollen containing Cry1Ac protein, at relatively very high dosages, was not toxic to the test species representative of organisms likely to be exposed to such pollen (e.g. lady beetles, green lacewings, honeybees). The larvae of endangered Lepidoptera species in cotton growing counties (Quino Checkerspot butterfly, Saint Francis' Satyr butterfly and Kern Primrose Sphinx moth) are not going to be exposed to Cry1Ac protein because their habitats do not overlap with cotton fields. The Quino Checkerspot butterfly is found only in the coastal sage scrub habitat in southern California, the Kern Primrose Sphinx moth (threatened, not endangered) is found only on a privately owned ranch in the Walker Basin, Kern County California. Finally, the only known population of St. Francis' Satyr butterfly is found in wetlands dominated by sedges and grasses on Department of Defense property in North Carolina. None of the larvae of these insects feed on cotton, and will not be exposed to Cry protein in pollen. The amount of pollen that would drift from these cotton plants onto plants fed upon by endangered/threatened species, would be very small (if measurable) compared to the levels fed to the test species. Therefore, EPA does not expect that any endangered/threatened species will be affected by pollen containing the Cry1Ac protein.

In addition, because EPA is imposing conditions for geographic areas (Hawaii and Florida) that have sexually compatible wild or weedy relatives of cotton, the Cry1Ac protein gene cannot escape into related wild plants which could serve as a source of Bt pollen for plants on which endangered/threatened species may feed on in these areas.

Because EPA expects that no listed endangered species of Lepidoptera will be exposed to the *Bt* Cry protein expressed in cotton plants, and because the most probable exposure scenario does not appear to affect listed species, EPA believes that this action will have no effect on listed species.

c. Potatoes

1) Summary of Non-Target Organism Toxicity Testing of Cry3A (006432)

Study	Status, Classification & Comments	MRID #
Avian Data	Monsanto conducted two dietary avian toxicity studies using the bobwhite quail and seven different potato lines producing the *Bt* Cry3A protein. The studies were both scientifically sound and no treatment mortality, differences in food consumption or behavior was observed between the dosed (50,000 ppm from potato tubers) and control birds. These studies adequately address potential avian toxicity concerns for *Bt* Cry3A protein produced in potato. No additional avian studies should be needed.	429322-14 429322-15
Cry3A Protein Comparison	To ensure that the truncated *Bt* Cry3A protein produced in the potato plants will not have an altered host-range of susceptible insects relative to the native full-length protein, comparative insect host-range studies have been submitted by Monsanto. The data consisted of SDS-PAGE co-migration, Western blot analysis, staining for carbohydrate residues, N-terminal amino acid sequence analysis, and biological equivalence. The results demonstrated that the *Bt* Cry3A protein with respect to the parameters tested was equivalent to the natural protein.	429322-03
Non-Target and Beneficial Insects	Monsanto submitted three standard non-target insect studies (parasitic wasp, ladybird beetle and green lacewing). The results of these studies indicated that the *Bt* Cry3A protein produced in potato plants showed no toxicity to parasitic hymenoptera (*Nasonia vitripennis*), green lacewing (*Chrysopa carnea*) and lady bird beetle (*Hippodamia convergens*).	429322-11 429322-12 429332-13
Honeybee Toxicity Study	The adult and larval honeybees were dosed with *Bt* in a sucrose and honey solution. The testing indicated that there was no significant loss of *Bt* protein bioactivity in honey or sucrose solutions when maintained for up to 7 days at a approximately 28 C. The adult honeybee study was found to be invalid due to excessive mortality in the controls. Since the adult honey bee study was not required, it will not have to be repeated. The larval honeybee study produced useable results and indicated that *Bt* Cry3A protein in potato showed no toxicity to honeybee larvae.	429322-09 429322-10
Evaluation of the Dietary Effects of Purified *Btt* Protein on Honey Bee Larvae	The honey bee study adequately demonstrated that purified *Btt* protein (Cry3A) shows no toxicity to honey bee larvae when exposed to 100 ppm protein. This dose far exceeds the amount expected to be encountered under actual field conditions.	441247-02
Earthworm	The data adequately demonstrated that purified Cry3A protein shows no toxicity to earthworms at levels greater than 100 mg protein/kg soil in a 14 day study.	441247-01

Study	Status, Classification & Comments	MRID #
Collembola	The data adequately demonstrated that Cry3A protein shows no toxicity to two species of Collembola at greater than 200 ppm. No adverse effects expected at field rates.	439416-1

2) Mammals

Monsanto submitted an acute feeding mammalian toxicity study reviewed in the Toxicity Assessment above. The Cry3A protein was found to be nontoxic to mice. EPA has determined that Cry3A is nontoxic to non-target mammalian species.

3) Avian Species

Monsanto conducted two dietary avian toxicity studies using bobwhite quail and 7 different potato lines producing Cry3A protein. The studies were both scientifically sound and no treatment mortality, differences in food consumption or behavior was observed between the dosed (50,000 ppm from potato tubers) and control birds. These studies adequately address potential avian toxicity concerns for Cry3A produced in potato.

4) Aquatic Species

Studies for aquatic species were waived because of expected lack of exposure. Potatoes are not used as fish food. Most *Bt* potato varieties produce a minium amount of pollen and the amount of pollen drops to very low levels within a few meters of the pollen source (Dale et al, 1992) so pollen drift to aquatic sites is minimal to non-existent.

5) Non-target Invertebrates

a) Honeybees

The registrant was required to submit a larval honeybee study. The registrant also submitted an adult honeybee study which was not required for registration. The adult and larval honeybees were dosed with Cry3A protein in a sucrose and honey solution. The registrant wanted to ensure that the Cry3A protein was stable in this type of solution. Testing indicated that there was no significant loss of Cry3A protein bioactivity in honey or sucrose solutions when maintained for up to 7 days at approximately 28°C. The larval honeybee study was scientifically sound and demonstrated that *Btt* in potato has no detectable deleterious effects on honeybee larvae. The adult honeybee study was found to be invalid due to excessive mortality in the controls. Since this study was not required, EPA did not require the study to be repeated. No adverse effects on larval or adult honeybees has been reported since registration in 1995.

b) Predatory, Parasitic and other Non-target Insects

The registrant submitted the three standard non-target insect studies (parasitic wasp, ladybird beetle, and green lacewing). The results of these studies indicated that Cry3A has no observable adverse effects on parasitic Hymenoptera (*Nasonia vitripennis),* green lacewing *(Chrysopa carnea*) and lady beetle (*Hippodamia convergens*).

An additional field study on the comparative impacts of foliar-applied microbial *Btt.*, transgenic potato plants, and conventional insecticides on non-target arthropods was submitted by the registrant. Beneficial arthropods (i.e. lady beetles, damsel bugs, flower flies, soldier beetles, big-eyed bugs, spiders, minute pirate bugs, green lacewings, brown lacewings, stink bugs, and ground beetles) were significantly more abundant in plots containing genetically modified potato plants and foliar-applied microbial *B.t.t.* than in those treated with conventional chemical insecticides. Aphid control was achieved in the plots containing transgenic potatoes solely through predation by natural enemies, while aphid populations rose to high levels in plots where beneficial arthropods were eliminated and no chemical aphid control was applied.

The registrant also submitted a study which tested the sensitivity of selected insect species to the Cry3A protein produced in the potato plants. The tested species were as follows: 3 rootworms; 4 lepidopterans- European corn borer, tobacco hornworm, corn earworm, and tobacco budworm; 1 dipteran-yellow fever mosquito; 1 orthopteran-German cockroach; and 1 hemipteran-green peach aphid. The results demonstrated that no species other than the Colorado potato beetle (*Leptinotarsa decemlineata*) displayed significant mortality. There was a slight reduction in the amount of honeydew produced by the Green peach aphid which was an indication of reduced feeding.

These studies indicate that Cry3A protein produced in potato plants should not adversely affect the non-target insects studied in these tests. Since Cry3A is specific to coleopterans it is not surprising that the non-target coleopteran insects that feed on these potato plants will, in all likelihood, be adversely affected by the Cry3A. Since any coleopteran insect that feeds on these plants would be considered a plant pest, this should not present a risk to non-target, non-pest insects.

c) Soil Invertebrates

Three hundred and five adult earthworms (*Eisenia fetida*) were acclimated for 24 hours to an artificial soil substrate. The worms were rinsed with deionized water and randomly distributed into groups of 10. The worms were not fed during testing. The moisture content of the soil substrate was 33%; the relative humidity of the test chamber was 86%; and the pH of the soil was 6.8/6.9 at day 0, and 7.4 at day 14. The soil was analyzed for actual active ingredient content. Earthworms were exposed to a single test concentration of 100 mg a.i. per kg of soil (approximately 120-fold the amount Cry3A protein estimated to be present in a kg of soil), and observed for mortality and signs of toxicity on day 7 and day 14 of the test. A negative and a

positive control group were maintained concurrently. The no observed effect concentration was determined by visual examination of the mortality, body weight and clinical observation data. The worms exhibited no aversion to the test or control soils. All the worms were normal in appearance and behavior during the course of the study. There were no treatment related effects on body weights. The LC_{50} of Cry3A protein for earthworms (*Eisenia fetida*) as representative beneficial invertebrate soil species is >100 mg a.i./kg dry soil in a 14-day exposure study. The no observed effect concentration is >100 mg a.i./kg dry soil. The 100 mg dose represents a level of exposure 120 times greater than the actual contact the earthworm would have under field conditions.

Two Collembola species, *Folsomia candida* and *Xenylla grisea* were fed a test diet prepared by suspending 1.0 g of Bakers yeast in 3.0 mL of distilled water containing 200 micro g of Cry3A protein. The dose concentration was confirmed by ELISA and/or insect bioassay techniques. Positive control consisted of chlorpyrifos added to yeast to obtain 200, 20, 2, 0.2 and 0.0 (negative control) ppm concentrations. There were ten insects per 5 replicates each for treatment and control groups for *Folsomia candida* and 6 replicates for *Xenylla grisea* in the test system. The test lasted for 21 days with fresh diet being added on days 0, 7 and day 14. The chlorpyrifos response system consisted of 4 replicates per concentration. The Cry3A protein tested did not have a detrimental effect on the survival or reproduction of *F. candida* or *X. grisea*. The NOEC therefore was >200 ppm. Adults and progeny of *X. grisea* were combined for the statistical analysis because of difficulty in discriminating between the initial adults and older progeny. For the chlorpyrifos control the no observed effect (NOEC) for *F. candida* was 2.0 ppm. Progeny production among the survivors at 2.0 ppm was not significantly different from the control. *X. grisea* was considerably less susceptible to chlorpyrifos. The NOEC was >200 ppm. The survival and reproduction appear reduced at 20 and 200 ppm, however these were not statistically significant. The study was scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. No adverse effects were seen to Collembola by chronic exposure to purified Cry3A protein at a maximum hazard dose of 200 ppm. The study showed that at field use rates survival and reproduction of the test insects would not be impaired. The study also shows that *X. grisea* did not exhibit any detrimental effects. This study adequately address potential concerns for Cry3A protein expressed in transgenic potatoes to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry3A proteins found in transgenic potatoes pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial soil insects.

6) Threatened and Endangered Species

EPA has determined that Cry3A potatoes will not affect any threatened or endangered species. The known host range for the Cry3A protein is restricted to Coleoptera species. The listed coleopteran threatened/endangered species in potato growing areas are: the American burying beetle, Hungerford's crawling water beetle, Mount Hermon June beetle, Northeastern Beach Tiger beetle, Puritan Tiger beetle and the Valley Elderberry Longhorn beetle. These are not

going to be exposed to Cry3A protein because their habitat does not overlap with potato fields and/or their larvae do not feed on potato tissue and will not be exposed to Cry protein in pollen, or to toxic Cry3A levels in the soil. The amount of pollen that would drift from the potato plants onto plants fed upon by endangered/threatened species would be very small compared to the levels fed to the test species. Submitted data confirm that some coleopteran species tested are not affected, including lady beetles. Generally potatoes do not produce large amounts of pollen which limits exposure. No endangered or threatened avian species feed on potatoes and no aquatic species are known to feed on potato plants.

5. Environmental Reassessment Summary

This reassessment finds no hazard to the environment at the present time from MON810 and *Bt*11 transformation events in corn, Cry1Ac in cotton and Cry3A in potatoes as currently registered. The reassessment considered the following issues.

a) Gene Flow and Weediness

The movement of transgenes from the host plant into weeds and other crops has been considered for each of the *Bt* plant-incorporated protectants currently registered. The Agency has determined that as currently registered there is no significant risk of gene capture and expression of any *Bt* Cry protein by wild or weedy relatives of corn and potatoes in the U.S., its possessions or territories.

There is a possibility for gene transfer in locations where wild or feral cotton relatives exist. If complete isolation and prevention of gene flow for *Bt* cotton is desired, then plantings of *Bt* cotton in Hawaii, Puerto Rico, and the U.S. Virgin Islands may require a minimum 3 mile distance from *Gossypium* spp. with 24 border rows of non-*Bt* cotton surrounding the plots. Monitoring of native populations of established *Gossypium* spp. may be necessary to assess the efficacy of this isolation procedure for *Bt* cotton. This would entail monitoring of wild populations for evidence of gene introgression through PCR or similar sensitive methods. Alternatively, the absolute restriction of planting *Bt* cotton in Puerto Rico, Hawaii, and the U.S. Virgin Islands, would, of course, alleviate any concerns over gene flow.

The current restriction on the planting of *Bt*-cotton in Florida south of Route 60, near Tampa, precludes any chance of outcrossing with feral *Gossypium* spp. due to the very large distance between any commercial plantings and wild populations in the extreme south of the state.

b) Fate in Soils and Indirect Effects on Soil Biota

Most of the Cry protein deposited into soil by *Bt* crops is quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time. It is also reported that the same degree of *Bt* Cry protein persistence takes place in soils that have been

exposed to repeat *Bt* spray applications when compared to soil exposed to growing *Bt* crop. Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are needed to yield relevant data on persistence and natural variation. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil. Horizontal transfer from transgenic plants to soil bacteria has not been demonstrated. Current studies of *Bt* in soil show no effect on bacteria, actinomyces, fungi, protozoa, algae, nematodes, springtails or earthworms. In addition, new plants planted in *Bt* Cry protein containing soil do not take up the *Bt* protein.

c) Direct Effects on Non-target Wildlife

In light of recent environmental effects concerns from commercialization of *Bt* crops the Agency has reviewed new and existing data regarding non-target wildlife effects for *Bt* crops with a special emphasis on Lepidoptera and monarch butterflies and reevaluated the sufficiency of data to support continued registration of *Bt* crops. The weight of evidence from the reviewed data indicate that there is no hazard to non-target wildlife from the continued registration of *Bt* crops.

1) Mammals

The reviewed data indicate that there is no significant toxicity to rodents from acute oral testing at the maximum hazard dose. In addition, there are no reports of adverse effects on livestock after several years of feeding with *Bt* corn. In light of the above toxicology information, no detectable adverse effects are expected to mammalian wildlife.

2) Avian

Direct testing has demonstrated a lack of acute toxicity with *Bt* corn, potatoes and cotton seed to avian species. Therefore, no avian hazard is expected from the continued uses of *Bt* crops. However, submitted avian toxicity data on Cry1Ab *Bt* corn are not sufficient to make a final hazard assessment from repeated exposure(s) to higher doses of *Bt* corn. Hazards from chronic exposure of wild and domesticated fowl cannot be determined without a six week study with 60 to 70% corn in the diet.

3) Aquatic

There is no evidence for sensitivity of aquatic (including endangered) species to Cry proteins from direct testing and the anticipated lack of significant exposure. Toxicity studies with aquatic organisms do not show a hazard for fish or invertebrates exposed to either *Bt* corn pollen or to bacterially expressed Cry protein. It was also demonstrated that farm fish food mix made from corn or cotton seed containing the *Bt* protein does not contain detectable active *Bt* Cry protein. Therefore farmed fish would not be exposed to *Bt* Cry proteins.

4) Insects

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

5) Lepidoptera

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

The weight of evidence of the published and recent research data reviewed indicate that milkweeds in the corn fields and to within 1 meter of cornfields are unlikely to be dusted with toxic levels of *Bt* pollen from the currently registered *Bt* corn varieties, MON810, *Bt*11 and TC1507. In addition, the distribution of corn pollen within and outside of corn fields, the distribution of milkweeds within corn habitat and other types of habitat, monarch oviposition and feeding behavior, limited temporal overlap between monarch larvae and pollen shed (and similar issues) in much of the corn growing regions of the United States indicate a low probability of demonstrable adverse effects of *Bt* corn pollen on monarch larvae.

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

6) Endangered Species

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

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

7) Conclusions

In general, the reviewed publications, recent research data, and information submitted as a result of the data call in (DCI) provide a weight of evidence assessment indicating no unreasonable

adverse effects of *Bt* Cry proteins expressed in plants to non-target wildlife or beneficial invertebrates, whether they are earthworms, springtails, parasites, predators, pollinators or soil microbial and invertebrate flora. Published field testing results and field test data submitted to EPA show minimal to undetectable to beneficial changes in the non-target insect populations. EPA is, however, continuing to participate in research and review the pertinent scientific literature for the purpose of reevaluating the Agency's Ecological Risk Assessment of the *Bt* crop registrations in the event that unexpected long range population, community or ecosystem effects are detected.

EPA believes that cultivation of transgenic plants expressing *Bt* Cry proteins may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, *Bt* crops require substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms. Many of these beneficial organisms are important integrated pest management controls (IPM) for secondary pests such as aphids and leafhoppers. The overall result of cultivation of plants expressing *Bt* Cry proteins is that the number of chemical insecticide applications for non-target pest control is reduced for crops with multiple pest problems.

Since the September 2000 version of the risk and benefits assessments and the October 2000 SAP meeting, this section has been updated to indicate 1) Collembola and earthworm tests are no longer necessary to evaluate risk to soil non-target organisms from *Bt* crops, 2) additional *Bt* corn avian data with a higher percentage of *Bt* corn in the diet is necessary for a more thorough assessment of chronic risk, 3) continuing non-target insect census data is necessary for long-range risk characterization, 4) additional Cry protein soil accumulation data is necessary for a more complete exposure characterization, and 5) *Bt* cotton isolation distances may need revision in Hawaii, Puerto Rico, and the Virgin Islands due to gene flow concerns.

EPA has sufficient information to believe that there is no risk from the registered uses of Bt potatoes, Bt cotton and Bt11, MON810 or TC1507 corn to non-target wildlife, aquatic and soil organisms and domesticated fowl and animals. Supplementary studies suggested by the Scientific Advisory Panel, and others, would provide additional weight to support the Agency's conclusions. Therefore, EPA is considering requesting the following supplementary data.

Confirmatory Studies Needed to Complete the Database of Registered Plant-incorporated protectants

Common Name and Cry Protein	OPP Chemical Code	Study Types
Cry1Ac *Bt* Cotton	6445	1) Cry1Ac expression levels in the root. 2) Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are needed to yield relevant data on persistence and natural variation. 3) Yearly insect census estimates from representative fields.
*Bt*11, Cry1Ab *Bt* Corn	6444	1) The data regarding Cry1Ab expression levels in the root were expressed as ng Cry1Ab/mg plant protein. An estimate of Cry1Ab protein/gm dry wt. of root tissue is needed based on historical data for total protein in roots. 2) While much of the data required in the *Bt* corn data call in has been submitted, final published reports from ongoing research are still required for the following categories of data: the distribution of monarch butterflies, milkweed plants and corn; corn pollen release and distribution in the environment; toxicity of *Bt* corn Cry proteins and *Bt* corn pollen to lepidopterans; monarch egg laying and feeding behavior; and monarch population monitoring. 3) Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are needed to yield relevant data on persistence and natural variation. 4) Submitted avian toxicity data on Cry1Ab *Bt* corn are not sufficient to make a final hazard assessment from repeated exposure(s) to higher doses of *Bt* corn. A six week study with 60 to 70% corn in the diet is necessary to assess hazards from chronic exposure of wild and domesticated fowl. 5) Yearly insect census estimates from representative fields.

Common Name and Cry Protein	OPP Chemical Code	Study Types
MON810, Cry1Ab *Bt* Corn	6430	1) Cry1Ab expression levels in the root. 2) While much of the data required in the *Bt* corn data call in has been submitted, final published reports and updates from ongoing research is still required for the following categories of data: the distribution of monarch butterflies, milkweed plants and corn; corn pollen release and distribution in the environment; toxicity of *Bt* corn Cry proteins and *Bt* corn pollen to lepidopterans; monarch egg laying and feeding behavior; and monarch population monitoring. 3) Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are needed to yield relevant data on persistence and natural variation. 4) Submitted avian toxicity data on Cry1Ab *Bt* corn are not sufficient to make a final hazard assessment from repeated exposure(s) to higher doses of *Bt* corn. A six week study with 60 to 70% corn in the diet is necessary to assess hazards from chronic exposure of wild and domesticated fowl. 5) Yearly insect census estimates from representative fields.
Cry3A Potato	6432	1) Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are needed to yield relevant data on persistence and natural variation. 3) Yearly insect census estimates from representative fields.

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