C. ENVIRONMENTAL ASSESSMENT

EPA has conducted an environment reassessment of the registered *Bt* plant-pesticides. The general topics covered include outcrossing and potential for weeds to develop if pollen from *Bt* crops plants was to fertilize other plants, horizontal gene transfer, expression of *Bt* Cry proteins in plant tissues, ecological effects, especially considering the currently available data on monarch butterflies, and fate of *Bt* 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, public literature, results of workshops and scientific seminars, and additional discussions with scientific experts. This preliminary environmental risk reassessment will be revised following the Scientific Advisory Panel when EPA has an opportunity to consider the SAP recommendations and all public comments.

1. Outcrossing and Weediness

The movement of transgenes from the host plant into weeds and other crops has been a significant concern for EPA due to the possibility of novel exposures to the pesticidal substance. This concern has been considered for each of the *Bt* plant-pesticides currently registered and EPA believes that these concerns have been satisfactorily addressed. The Agency has determined that as currently registered there is no significant risk of gene capture and expression of any *Bt* endotoxin by wild or weedy relatives of corn, cotton, 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.

Under FIFRA, EPA has reviewed the potential for gene capture and expression of the *Bt* endotoxins by wild or weedy relatives of corn, cotton and potatoes in the U.S., its possessions or territories. *Bt* plant-pesticides 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 (periodicity or timing of events within an organism's life cycle as related to climate, e.g., flowering time) and habitat. There is a possibility, however, of gene transfer from *Bt* cotton to wild or feral cotton relatives in Hawaii and Florida. Where feral populations of sexually compatible cotton species exist in Florida and Hawaii, EPA has prohibited or restricted the sale or distribution of *Bt* cotton in these areas. These containment measures prevent the movement of the registered *Bt* endotoxin from *Bt* cotton to wild or feral cotton relatives in Hawaii and Florida.

a. Bt Corn Plant-Pesticides

1) Gene Transfer - Outcrossing

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 outcrossing 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 which 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

A close relative of corn or maize is 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 an outcrossing 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 aren't 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 outcrossing 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 which 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 F1 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. Bt Cotton Plant-pesticides

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. There is a possibility for gene transfer in locations where wild or feral cotton relatives exist. Therefore, EPA requires stringent sales and distribution restrictions on *Bt* crops within these areas to preclude outcrossing or hybridization from the crop to sexually compatible relatives.

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[A]). 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, 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 (*i.e.*, southern Florida and Hawaii) 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 outcrossing 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 *Bt*-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- 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 three areas in the United States and its territories wherein cultivated cotton has the opportunity to outcross to wild or feral species which are genetically compatible: (1) southern Arizona, (2) Hawaiian islands, and (3) southern Florida (Stewart, 2000).

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 *Bt*-Cotton is restricted south of Interstate 60. There is currently no commercial cotton production in the southern part of Florida.

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

4) Pollination of Upland and Pima Cotton

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 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

[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*).

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 *Bt* cotton in Florida, restricting its use to those sites North of Tampa (Route 60). The Agency is satisfied that the planting restrictions on *Bt* cotton (*i.e*., no *Bt* cotton south of Tampa) will mitigate concerns for gene transfer to wild cotton:

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

5) Potential Impacts of Outcrossing in Cotton

The absence of any truly weedy characteristics in the genus *Gossypium* indicates that the potential for gene exchange to promote weed development is remote. The species present in the United States and its territories and possessions have existed here for at least 200 years for cultivated cottons and much longer for wild species. No evidence of genetic exchange influencing the competitive abilities of the recipient species exist for this group. As mentioned previously, the movement of genes from diploid to higher ploidy levels is not plausible and even though many *Gossypium* species have co-existed for millennia, they have largely remained individually recognizable genetic constitutions.

The fixation of a *Bt* gene into a natural landrace of cotton or feral population of the cultivated cottons would have a neutral effect on the population structure of the recipient (Stewart, 1997). There always exists the possibility that incorporation of a *Bt*-endotoxin gene or similar construct could provide some positive benefit to the recipient plant. Within the geopolitical boundaries covered in this review, there is no known example of an insect pest keeping a species of *Gossypium* in check through herbivory or parasitism. This is not the case, however, in Brazil where Moco cotton is on the decline following the arrival of the boll weevil.

c. *Bt* Potato Plant-Pesticides

EPA has reviewed the potential for gene capture and expression of the *Bt* Cry3A plant-pesticide 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 (Btt)* 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. 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).

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, den Nijs, Peloquin, and Hanneman, 1991). 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 an organic grower's non-*Bt* potato fields are in close proximity to *Bt* potato fields, cross-pollination would not result in the tubers containing the *Bt* gene since they are vegetatively propagated. Seed potato (*i.e.*, cut tuber pieces) production from such tubers would also be *Bt* 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, Tarn, and Davis, 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, Rueda, Kadane-Mariam, and Peloquin, 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.

Bacteria are generally recognized as more promiscuous than other organisms concerning DNA transfer, exist in the soil in large numbers, and the antibiotic resistance gene most often used in the transgenic crops, kanamycin (*nptII*) as well as the *cry* genes, are of bacterial origin. Therefore, it is likely that bacteria would have the highest probability of integrating and expressing these genes. Horizontal transfer from transgenic plants to soil bacteria has not been demonstrated.

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 the persistent fraction 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 higher concentrations of DNA, transformation of bacteria 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, et al., 1998; Nielsen, et al., 2000). Under conditions where homology does not occur, horizontal transfer has not been observed, even at extremely low frequencies of less than about10^{-9} to 10^{-17} (Nielsen, et al., 1998; Paget, et al., 1998)[3]. Therefore, DNA transfer occurs rarely if at all from plants to bacteria. Nielsen and coworkers (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. They caution, however, that other non-homologous transgenes, new to the recipient bacteria, 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, broad host range plasmids, highly conserved genes (e.g rDNA), do exist in nature. 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 or uncommon in soil organisms. In other words, the prevalence

[3] Data reviewed in Nielsen and coworkers (1998), section on experimental studies of horizontal gene transfer from plants to bacteria under optimized laboratory conditions. Units, where given, are transformants per recipient bacterial colony forming unit (CFU). In most cases, units of transformation are not given. Paget et al. (1998) gave no transformation frequencies.

of the trait in the soil prior to possible introduction by the transgenic plant should be considered. In the case of the transgenes of interest, or the traits they confer, that are used in the *Bt* crops, occurrence in soil bacteria or fungi has been well established. For example, Smalla and coworkers (Smalla et al. 1993) demonstrated that kanamycin resistant bacteria were common in soil and sewage. In the soils tested, numbers ranged from 6.6 $x10^3$ to 2.8 x 10^5 (0.01 to 0.56 percent of total bacteria)[4]. Bacteria containing *nptII* were not found in cultured bacterial species from soil, but were common in bacteria cultured from sewage (9.1 to 47.6 percent). This is not surprising, and does not demonstrate the absence of *nptII*-like genes from soil inhabiting bacteria. This is because only a small fraction of the bacteria found in soil can currently be cultured, and only a small subset of these were examined in this paper. In fact, two of three soils showed consistent *nptII* specific PCR amplifications from total soil extracted DNA (that is, from soil organisms or extracellular DNA)[5]. 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, 1998). The authors ... "conclude that deliberate releases of organisms carrying Tn*5* or *nptII* are not inherently more hazardous than the 'natural' releases discussed here." (Smalla et al., 1993)

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, beta-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, et al., 1998), the likelihood of horizontal gene transfer is typically extremely low. Coupled with the common occurrence in the soil, prior to commercialization, of the traits found in *Bt* crops, this low probability event makes adverse impact by these traits through horizontal transfer in the soil unlikely.

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 tissue bioactivity values.

[4] Units not given in reviewed paper.

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

Cry Protein Tissue Expression

Active Ingredient	Leaf	Root	Pollen	Pith	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 µg/g	_	< 90 ng Cry1Ab/ g dry wt. of pollen	_	0.19-0.39 µg/g (grain)	4.65 µg/g
Cry9C (006466)	44 µg/g	25.87 µg/g	0.24 µg/g	2.8 µg/g (stalk)	18.6 µg/g (kernel)	250 µg/g
Cry1Ac (006445)	2.04 µg/g	_	11.5 ng/g	_	1.62 µg/g	_
Cry3A (006432)	28.27 µg/g	0.39 µg/g (tuber)	_	_	_	3.3 µg/g

* 1994 Field Data

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

a. 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	The highest expression levels for Cry1Ab protein in Bt11 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. An acre of corn was assumed to contain 89,300 lbs. of fresh tissue and produce 0.57 lb. *Btk* protein/acre of corn. Nearly 90% of the *Btk* protein was in the leaf tissue according to these calculations. The highest determined values for *Btk* protein were obtained at the first assay point with leaf tissue from field corn grown in Hawaii. The values 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. Other values were presented in Table 1 but these were derived values corrected for the original weight of the tissue added. The standard error values and the original weight values are not included nor is the method for making the corrections adequately described. Therefore, this data is not analyzed. The European corn borer (ECB) bioassay data indicates that Bt11 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 Bt11 stalk tissue showed a similar trend but the decrease was not as marked. The bioassay values for purified *Btk* protein (Bt103) added to soil also decreased between week 0 and week 3 but again the decrease was not as marked. Western blot assays were preformed on boiling SDS buffer extracts of *Btk* spiked soil samples but no blots were provided. The company claims that *Btk* protein could be detected in the initial soil spiking sample but not thereafter. The company also states that *Btk* protein cannot be quantitatively extracted from spiked soil for ELISA and theorizes that *Btk* protein binds to soil particles and cannot be released by the standard extraction procedures. 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. CLASSIFICATION: ACCEPTABLE. If the company wants the entire submission considered, clarification of several points in the data need to be submitted first.	43696001

Btk protein in Bt 11 is most highly expressed in the leaf tissue, in the order of 3.3 micro g/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.

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	*Btk* Cry1Ab protein bioactivity, added to the soil as a component of corn line #754-10-1 tissue decreased with an estimated half life 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 half life of 25.6 days, and a DT_{90} of 40.7 days. The bioactivity of purified Cry1Ab protein in soil decreased with an estimated half life of 8.3 days and a DT_{90} of 32.5 days.	
Expression Levels	"B.t.k. HD-1 protein levels measured in the test (MON801) leaf averaged 1.3 micro g/g fresh weight, with a range of 0.84-2/36 micro g/g; seed averaged 0.57 micro g/g, with a range of 0.23-0.95 micro g/g; and whole plant averaged 1.77 micro g/g fresh weight, with a range of 1.44-2.01 micro g/g. In this study, B.t.k. toxin was below the limits of detectability in pollen..."	436960-01
Evaluation of Insect Protected Corn Lines in 1994 U.S. Field Locations	Cry1Ab protein levels measured in MON 810 in micro g protein/fresh weight: whole plant 4.15, grain 0.31, leaf 9.35, over season leaf 9.78, pollen 0.09	436655-02

1994 field data regarding MON 810 demonstrated expression levels of 0.18-0.39 micro g/g in grain, 7.93 -10.34 micro g/g in the leaf, 3.65-4.65 micro g/g in the whole plant, and 0.09 micro 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 micro g/g in the leaf, 2.3-4.5 micro g/g in forage, and 0.4-0.9 micro g/g in the grain. 1995 French field data showed 7.6-9.4 micro g/g in the leaf, 4.1-5.6 micro g/g in forage,

and 0.4-0.7 micro g/g in the kernel.

c. Exposure of Cry9C (006466)

1) Maximum Expression of Cry9C Protein in Various Corn Tissues

Study	Status, Classification & Comments	MRID #
Characterization of Cry9C and PAT protein levels in CBH-351 *Bt* corn under field conditions.	The study characterized the insecticidal Cry9C protein and a marker protein (PAT: phosphinothricin acetyltransferase, coded by a bar gene) in transgenic corn grown under actual field conditions in Johnston, Iowa. Corn plants which express Cry9C and PAT proteins have descended from transformation event CBH-351. The levels of all proteins were fairly stable during the first two sampling stages, vegetative and pollen shed, and declined with each of the two final stages, silage and harvest. This decline over time was true for four other tested strains although data for only two of the stages, vegetative and silage, were provided. The amounts of Cry9C protein in an acre of corn containing 25,000 corn plants/acre during the vegetative, pollen shed, silage, and harvest stages were 103, 334, 495, and 99 g (tissue dry weight basis), respectively. ADEQUACY OF STUDY: Acceptable (Supplementary)	442581-03
Environmental fate of Cry9C protein incorporated into soil.	Based on a bioassay with the tobacco budworm (*Heliothis virescens*), a target species, Cry9C protein incorporated into Drummer Ap loam soil [as corn plant tissue containing the Cry9C gene incorporated from *Bacillus thuringiensis* subsp. *tolworthi* (0.368 µg Cry9C/mg tissue)] biodegraded over a 42-day period with a half-life of approximately 4.5 days (range, 0.49-12.65 days). ADEQUACY OF STUDY: Acceptable (Core)	441617-01

Expression was found throughout the different plant tissues across the growing season. The level of the Cry9C and PAT proteins is stable in tissues and in whole plants during the first two stages - vegetative growth through pollen shed and declined with each of the two final stages, silage and harvest. The decline is less for the Cry9C protein than for the PAT protein. The amounts of Cry9C protein in whole plant for each stage were: vegetative - 250 µg Cry9C protein /g plant tissue on a dry weight basis), pollen shed - 230 (µg/g), silage - 96 (µg/g), harvest - 22 (µg/g). The amounts of Cry9C protein in an acre of corn containing 25,000 corn plants/acre during the vegetative, pollen shed, silage, and harvest stages were 103g , 334g, 495g, and 99g (tissue dry weight basis), respectively.

Based on data submitted, it appears that the expression of the Cry9C delta-endotoxin occurs at the highest levels in leaves, tassel, and whole plants. The maximum levels detected in any

individual samples were 175.0 µg/g dry weight for tassel, 44.0 µg/g for leaves, 25.87 µg/g for root, 18.6 µg/g for kernel, 2.8 µg/g for stalk, 0.24 µg/g for pollen and 250.0 µg/g for whole plants.

2) Estimated Half-Life and EEC

The submitted study establishes that the *Bt* protein was active in the test soil mix. Based on a bioassay with the tobacco budworm (*Heliothis virescens*), a target species, whole corn plant Cry9C proteins incorporated into test soils biodegraded over a 42-day period with a half-life of approximately 4.5 days (range, 0.49-12.65 days).

Given that the amount of Cry9C protein at harvest is 99 g/ac, the expected environmental concentration (EEC) of Cry9C will be 0.11 mg/kg dry soil (15 cm deep).

d. Exposure of Cry1Ac (006445)

The sites and levels of expression of the Cry1Ac delta endotoxin in cotton have been determined. The delta endotoxin is detectable in leaves, seeds, and whole plant assays. A total of 1.44 grams of protein would enter the soil per acre based upon estimates of 60,000 plants per acre. *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.

Study	Status, Classification & Comments	MRID #

Gene Expression	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 micro g per gram of fresh leaf tissue and from 0.49 to 1.62 micro g per gram fresh seed tissue. Based upon planting rates of 60,000 plants per acre and, 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. *Btk* 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. 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).	MRID is not availble
Soil Degradation	This study 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* produced half-lives of 9.3-20.2 d. Ground, lyophilized Cry1A(c) cotton line 931tissue produced a half-life of 41 d.	MRID is not available

e. Exposure of Cry3A (006432)

Study	Status, Classification & Comments	MRID #
Expression levels in field grown potatoes	Seven genetically modified lines of field grown potatoes were tested. 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 CryIIIA delta-endotoxin expression levels ranged from 0.40 to 2.00 micro g/g or 0.002-0.01% of the total tuber protein. 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

Delta-endotoxin bound to soil particles may be a route of exposure for some soil organisms. Experiments addressing amounts and persistence of delta-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 delta-endotoxin or transgenic plant material in soil in a laboratory setting.

Cry1Ab produced estimated half lives of 1.6 days for Cry protein as expressed in transgenic corn tissue and 8.3 days for purified protein (Sims and Holden, 1996). Half-life data submitted in support of the registration of Cry9C averaged 4.5 days (range: 0.49 d to 12.65 d). Published data for Cry1Ab or Cry1Ac in cotton tissue or as purified protein produced half lives of 2.2 to 46 days, where measurable (in 4 of 11 experiments), with half lives in transgenic tissue shorter than for purified protein in two of three experiments (Palm, et al., 1996). None of the studies discussed above have been performed in agricultural fields, although most have used field soil in laboratory microcosms. EPA believes that these microcosm studies, which contain non-sterile soil, adequately represent conditions in the field for the purpose of risk assessment.

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). 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. However, this protection is not absolute, since degradation does in fact occur under several experimental conditions. 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).

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 , and the optimum range is pH 5.0-6.5. The optimal range for cotton is pH 6.0-6.5. 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, have not been explored.

Tapp and Stotzky (1998) propose that the reason for more rapid degradation of Cry 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 should be determined over sufficiently long periods to assure an accurate assessment of degradation. Several of the experiments examining persistence of Cry protein in soil have been performed until the Cry protein was either undetectable by sensitive bioassay or immunoassay, or was detectable only at very low concentrations. Therefore, these experiments were conducted for sufficient duration.

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 (such as protein degradation) 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 (Sims and Holden, 1996).

Some soil persistence experiments begin with very high concentrations of the Cry protein compared to the Cry protein amounts found in the plant (Koskella and Stotzky, 1997, Tapp and Stotzky, 1995). In addition, the bioassays or immunoassays used to detect the protein in the soil are very sensitive (around 5-10 ppb). Depending on the experiments, reductions of 10^3 to10^5 may be required to reduce the amounts of Cry protein added to soil to undetectable levels. Therefore, it is not 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.

To summarize, available data suggests that the "half-life" of Cry proteins incorporated into soil in corn plant residues, or as free delta endotoxin in non-sterile soil, are typically from approximately 1.6-22 days but have been measured to be as long as 46 days. As suggested by Palm and coworkers (Palm, et al., 1996), persistence or half-life 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 half lives. Binding of Cry protein to clays or humic acids reduces microbial degradation rates compared to other soil components or other media. Based on current data, however, it cannot be concluded from these results that persistence is generally greater than demonstrated in available half-life 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.

A previously unconsidered issue concerns the possibility that one of the roots of *Bt* crops, specifically Bt 11 corn, exude Cry1Ab protein into the soil (Saxena, et al., 1999). Exudation would likely cause continuous exposure of soil organisms to Cry1Ab protein, which differs from the conditions of previous risk assessments. Previous experiments examined the effects of a single incorporation of *Bt* plant material or Cry protein, as would occur with incorporation at the end of the growing season. It is also possible that soil organisms could be exposed to higher levels of Cry1Ab through continuous exudation than would occur with a single incorporation. Finally, since only Bt11 corn was examined in the Saxena et al. study, it is unknown whether the putative exudation could occur in other *Bt* crops.

It is important to consider whether Saxena and coworkers (Saxena, et al., 1999) demonstrated active secretion of Cry1Ab, as opposed to passive leakage of Cry protein from roots, because the former is expected to result in much higher levels of Cry protein deposition in the soil than the latter.

The amount of Cry protein deposited in the soil in the current experiments was not accurately measured, but was estimated to be in the tens of ng Cry1Ab/g soil range (Stotzky, 2000a; Stotzky, 2000b). This low concentration of Cry protein has not been shown to have any effect on non-lepidopteran non-target organisms; even concentrations 1000 to10,000 fold higher than are likely in the current experiments have not had clear deleterious effects on non-lepidopteran organisms or soil microbial communities, the latter over a period of four weeks for Cry1Ab, eight weeks for Cry1Ac, and seven weeks for Cry3A (Donegan, et al., 1995, Donegan, et al., 1996). On the other hand, if ingested, ng/g concentrations of Cry1Ab might have deleterious effects on sensitive non-target lepidopteran larva in the soil, if any of these species exist.

Some proteins are 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 or cell lysis) 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 in registered crops 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).

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). Therefore the report of Cry1Ab protein exudation in the "Nature" paper is unexpected and unexplained, if exudation is understood to mean an active deposition of Cry protein into the medium rather than incidental leakage. The estimated amounts of Cry protein exuded by the Bt11 corn plants used in the experiments were in the tens of ng Cry1Ab/g soil range (Stotzky, 2000a), although not specifically measured. 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 cells after several days growth (Borisjuk, et al., 1999). The average concentration of Cry1Ab in actively growing root tissue is 20.2 micro g/g total root protein. It is difficult to determine whether this root concentration is consistent with secretion of Cry1Ab from Bt11 roots, based on the roughly estimated concentrations found in the media in the Saxena et al. paper.

High dose and continuous feeding studies on Collembola, and an orbatid soil mite, *Oppia nitens* (Yu, et al., 1997), do not indicate likely adverse effects on non-lepidopteran species in the field. Several high dose acute toxicity studies of earthworms have also shown no observed adverse effects. MacIntosh and colleagues (MacIntosh, et al., 1990) tested active (trypsinated) Cry1Ab and Cry1Ac, and truncated Cry3A, on 18 non-target insects at 50 ppm for six days. Only lepidopteran species were affected. In addition, knowledge about the biochemistry and physiology of Cry protein toxicity and specificity suggest that it is unlikely that these proteins are toxic to organisms other than expected sensitive species. Although based on a limited data set, chronic exposure to low levels of Cry1Ab raises no concerns for non-target soil organisms.

Significant root exudation of Cry protein may pose some different routes or levels of exposure compared to single soil incorporation of transgenic plant material. Organisms that pass soil through their digestive systems, such as earthworms, could be exposed to higher levels of Cry protein due to exudation compared to a single incorporation of plant material. Organisms at higher trophic levels that feed on soil feeders may, secondarily, be exposed to toxin. Exposure of the outer integument of soil organisms to Cry proteins may be higher from free protein exuded into the soil compared to cuticular exposure from incorporated plant material. It is not possible to predict the effects of Cry protein binding to clay or humic acids regarding exposure through the outer surface of invertebrates. Toxicity to invertebrates by cuticular exposure has not been directly addressed for Cry proteins, but is highly unlikely considering the known mode of action of these proteins. Of 25 tests on non-target invertebrates, based on direct cuticular contact and using *Bacillus thuringiensis* bacterial formulations, only three showed significant mortality (above 10%) (Flexner, et al., 1986). Two of the species were specifically listed, and were an adult parasitic wasp (*Trichogramma* sp.) and a carabid beetle (*Bemdidion lampros*) (Flexner, et al., 1986). However, these studies may have little bearing on Cry protein effects, since mortality caused by bacterial formulations may be due to the infection process or other toxins, as well as inert ingredients. Mid-gut exposure due to contact with the invertebrate surface may occur in some cases through grooming.

Microorganisms living in soil may also be exposed directly to Cry protein through root exudation. However, no observable effects on microbial populations were found due to Cry1Ab or Cry1Ac protein exposure for up to 28 days for the former and 56 days for the latter in a single incorporation of 50 ng/g soil (Donegan, et al., 1995). Small transient changes in microbial populations have been associated with some *Bt* transgenic plant material (cotton), rather than the transgenic Cry protein itself, in experiments where transgenic plant tissue was used (Donegan, et al., 1995). It was suggested that the process of making the transgenic plant, e.g tissue culture which may introduce genotypic changes unrelated to the transgene, may have been responsible for these temporary population shifts. Other work comparing transgenic Cry3A potato and the non-transgenic isoline found no significant difference on the populations of several groups of soil microorganisms, including fungal species diversity and three plant pathogenic fungi, in this season long field study (Donegan, et al., 1996).

Many factors have been shown to cause changes in microbial populations in agricultural and other soils. Without better information regarding the range of what constitutes natural microbial communities or microbial communities in current agro-ecosystems, and the consequences of such changes, it is not possible to assign a significance to apparently minor changes in microbial populations if they occur. It is entirely possible, if not likely, that fluctuations of soil microbial communities are typical of most soil ecosystems. A recent report of as yet unpublished field data further indicates that Cry proteins are unlikely to have adverse impacts on soil organisms, including microorganisms (Stotzky, 2000d).

In the only examination of effects on plant pathogens (soil and leaves), Cry3A potato plants did not cause any significant differences in microbial populations or incidence of plant disease for a number of plant pathogens, compared to systemic insecticide treated plants (Donegan, et al., 1996)[6].

In summary, it is very difficult to determine the importance of shifts in the structure of microbial soil populations unless these changes can be associated with measurable ecological parameters. In most cases, such research has not been performed for microbial populations. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil, even at levels much higher than expected from the results of the Saxena et al. experiments.

In a presentation at the Workshop on Ecological Monitoring of Genetically Modified Crops (Stotzky, 2000d), it was reported that no differences were found in the types and numbers of microorganisms and enzymes in *Bt* soil. *Bt* soils show no effect on total biomass, bacteria, actinomyces, fungi, protozoa or nematodes. The C/N ratio is also not changed in the *Bt* soil. In addition, new plants grown in *Bt* containing soil do not take up the *Bt* toxin. It was also reported that the same degree of *Bt* protein persistence takes place in soils that have been exposed to repeat *Bt* spray applications compared to soil exposed to growing *Bt* crop. Live *Bt* cells were also recovered from the soils after spray applications for up to eight months of testing.

Exposure of soil organisms to Cry proteins from the roots themselves, during and after the growing season, must be considered. 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 0.39 micro g/g tissue or ~1.01 micro g/g tuber as reported by the NAS (National Academy of Sciences/National Research Council, 2000), and Cry9C is expressed at 27.87 micro g/g root tissue. Cry1Ab from Bt11 averaged 20.2 ng/mg total root protein, and 2.2 ng Cry1Ab/mg total

[6]Comparisons with untreated potato plants were not made due to high levels of insect damage to non-*Bt* plants not treated with insecticide.

root protein at senescence (day 119). 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). It is 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 during the growing season and afterwards, as well as from Cry protein incorporated into the soil from the above ground parts of the crop at the end of the growing season. Dead tissue containing Cry protein will also be available to detritus feeding organisms.

As discussed above, several soil invertebrates have been used to examine the toxicity of Cry proteins. Submitted studies have examined high dose acute toxicity to earthworms and Collembola, while published studies have examined the effects of longer term exposure to Collembola, orbatid mites (Yu, et al., 1997) and microorganisms (Donegan, et al., 1995; Donegan, et al., 1996). Also discussed above is new unpublished data (Stotzky, 2000d) that indicates no apparent impact on a range of soil microorganisms, total biomass, etc. Results of all these studies have revealed little or no adverse effects.

In summary, sufficient evidence exists to suggest that adverse impacts of currently commercialized *Bt* Cry1Ab and Cry1Ac proteins in the soil are not likely, although the levels of expression in the root, where not currently available, should be determined to assure that unexpectedly high levels of root expression are not found. Levels of root expression of Cry9C corn (27.87 micro g/g root tissue) are below levels used in toxicity tests for that protein, which were performed at multiples of the expected environmental concentration in the soil (0.11 micro g/g soil after incorporation to 15 cm) determined from whole plant concentrations of Cry9C. Root expression for Cry1Ab in Bt11 corn (20.2 ng Cry1Ab/mg total root protein) is also well below concentrations used in toxicity testing. 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 (0.39 micro g/g tuber, or ~ 1.01 micro g/g according to the NAS) are high enough that sensitive non-target Coleoptera (if any) exposed to these concentrations might be 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-pesticides in 1995, EPA conducted ecological risk assessments for all *Bt* endotoxins 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* endotoxins expressed in transgenic plants do not exhibit detrimental effects to non-target organisms in populations exposed to the levels of endotoxin 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 *Btk* 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* protein 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 *Bt* endotoxins, 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* endotoxin 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 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 endotoxin expression in the plant. For *Bt* plants, EPA has examined the

toxicity of the endotoxins to birds, fish, honeybees and certain other beneficial insects. Because of the extensive scientific literature on the susceptibility of lepidopteran species to *Bt*, EPA assumed the Cry 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 required data on *Collembola* (springtail) and earthworm species where crop residue exposure is a possibility to ascertain effects on beneficial soil invertebrates. In the honeybee study, effects studies on brood as well as adults are 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, are in progress. The preliminary reports from these ongoing studies are summarized below and in the supporting assessment document (Vaituzis, 2000).

Bt delta endotoxins are proteins, and not chemicals which might 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. 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. Corn

1) Summary of Non-Target Organism Toxicity Testing of Bt 11 (006444) and MON 810 (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 20ppm.	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 Hymenopteran	*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 20ppm. 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

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 *Bt*11 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 Bt11 corn. However, given the equivalent Cry1Ab expression in Bt11 and MON 810 corn (< 90 ng Cry1Ab/g dry wt.pollen) and the lack of treatment related effects seen in any *Bt* corn pollen *Daphnia magna* studies, the data requirement is satisfied for *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 Bt11. This 48-hour static renewal toxicity study of Event 176 maize pollen containing Bt Cry1Ab endotoxin 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 LOEC and NOEC were found to be 150 mg/L. These results indicate that Bt expressed in corn showed not toxicity at 150 mg/L to Daphnia magna. In view of the above results, no freshwater aquatic invertebrate hazard is expected from the use of this product. Bt11 pollen is preferred over Event 176 pollen as a test material in studies supporting Bt11 corn. However, given the low level of expression of Cry1Ab in Bt11 pollen [(< 0.55 micro g Cry1Ab/ g protein) or (< 90 ng Cry1Ab/g dry wt.pollen)] compared to Event 176 pollen [80.63 micro g Cry1Ab/g protein) or (12.36 micro g Cry1Ab/g dry wt. pollen)] and the lack of effects seen in the cited *Daphnia magna* study using event 176 pollen, the data requirement is satisfied for Bt11.	433236-10 442742-01

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 corn for testing, the test material was considered sufficiently similar to the Bt11 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.	43941601
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. 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-pesticide is still required. The Agency notes that the registrant has only been recently appraised of this finding.	MRID No. 442715-01

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 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

2) Summary of Non-Target Organism Toxicity Testing of Cry 9C (006466)

Study	Status, Classification & Comments	MRID #
Cry9C protein in corn pollen: A dietary toxicity study with the honey bee *(Apis mellifera*)	This study is acceptable, and determined that the dietary LC_{50} for honeybees exposed to Cry9C protein in corn pollen for 8 days was greater than 5.8 micro g Cry9C protein/L diet [24,000 micro g corn pollen/mL]. The no-observed-effect concentration was 5.8 micro g Cry9C protein/L diet.	443843-02
Corn Plant Powder Containing Cry9C Protein: A Dietary Toxicity Study with the Northern Bobwhite	This study was conducted according to accepted protocols and determined that the dietary LC_{50} for northern bobwhite exposed to corn plant powder containing Cry9C protein was greater than 58 micro g Cry9C protein/g diet, when administered in a diet containing 20% (w/w) of the powder, the only concentration tested. The no-observed-effect and no-mortality concentrations were 58 micro g Cry9C protein/g diet.	442581-14
Preparation and characterization of catfish pellets.	Based on results of a protein-specific ELISA analysis, no Cry9C protein was detectable in catfish pellets processed from corn kernels containing Cry9C protein.	443843-01
Freshwater Aquatic Invertebrate Acute Bioassay	The study was conducted according to accepted protocols and determined that the 48-hour EC_{50} of Cry9C protein in corn pollen is >0.036 µg Cry9C protein/L (150 mg Bt plus corn pollen/L). The NOEC and the no mortality/immobility concentration are 0.036 µg Cry9C protein/L (150 mg Bt plus corn pollen/L). Since the test and control solutions appeared cloudy with yellow particles in suspension and settled on the bottom of the test chambers, the study is graded supplemental since the amount of pollen that the *Daphnia* were exposed to could not be determined. However, the amount *Daphnia* were exposed to is considered far greater than the EEC and no adverse affects were noted.	442581-12

Chronic Exposure of *Folsomia candida* to Corn Tissue or Bacteria Expressing Cry9C Protein.	This study was conducted according to an acceptable protocol and determined that the LD_{50} of corn plant Cry9C protein and bacterial Cry9C protein to Collembola (*Folsomia candida*) over a 28-day exposure period is greater than 50% (by weight) of the diet [brewers yeast and test material], the highest dose tested. The no-effect-level for corn plant Cry9C protein was 50% of the diet (180 mg/kg dry soil) . The no-effect-level for bacterial Cry9C protein was 5% of the diet (20 gm/kg dry soil). Little mortality was observed in the two Cry9C-amended treatments. There was less than 15% mortality in either of the two 50% Cry9C treatments. There was no statistical difference in mortality rates between any treatment with the test materials and the control treatment. With the exception of the thiodicarb treatment, large numbers of Collembola were produced. There were no significant differences in the number of Collembola produced between test material treatments and the control for the two lower rates (0.5 and 5%). There was a significantly higher number of Collembola recovered from the control treatment than from either of the corn plant Cry9C, the bacterial Cry9C or control plant treatments at the highest rate (50%). Recoveries from each of the 50% Cry9C treatments were statistically similar. The only statistically significant Collembola increase rate reduction was from the 50% bacterial Cry9C treatment. Even at the very high rates of exposure used in this test (up to 5 orders of magnitude higher than the EEC), Collembola were not acutely affected by chronic exposure to Cry9C protein. Notation is being made that the study results stated that mortality in the yeast control replicates ranged up to 15.4%. Data in Appendix D (MRID 44258110, p. 65) indicate that mortality in the yeast-only controls ranged from 0.0 to 16.7%. This issue is not expected to have any effect on the conclusions reached from this study.	442581-10
Cry9C Protein in Plant Powder: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate.	This study was conducted according to OECD Guideline 207 and determined that the 14-day LC_{50} value for earthworms exposed to corn plant powder containing Cry9C protein in an artificial soil substrate was greater than 1.84 mg Cry9C protein/kg dry soil, the only concentration tested. The no-observed-effect concentration was determined to be 1.84 mg Cry9C protein/kg dry soil.	442581-13
Cry9C Protein in Corn Pollen: A Dietary Toxicity Study with the Ladybird Beetle (*Hippodamia convergens*).	The dietary LC_{50} for Cry9C protein in corn pollen when fed to ladybird beetles for 21 days was determined to be greater than 0.36 micro g Cry9C protein/L diet (1500 micro g corn pollen/mL), the only concentration tested. The no-observed-effect concentration was 0.36 micro g Cry9C protein/L diet.	442581-11

Insect Host Range Comparison of Cry9C Protein.	This non-guideline study is classified supplementary; it was conducted following GLP regulations 40 CFR 160. The study compared endotoxin activity of Cry9C from three sources (bacteria, corn plant tissue, and corn pollen) by determining the relative sensitivities of larvae of four species of insects. European corn borer, tobacco budworm, and diamondback moth were sensitive to Cry9C, but corn earworm was not sensitive to Cry9C.	442581-06
Effects of Cry9C Corn on Predatory Non-Target Beneficial Insects and Endangered Species; Determination of Predatory Non-Target Beneficial Insect Study/Pollen Production Study.	This study is classified as supplementary. It was not conducted in accordance with FIFRA Good Laboratory Practices Standards (GLPs) (40 CFR Part 160, 17 August 1989); however, it was conducted in a manner similar to the GLPs. Deviations from protocol did not affect the quality or the results of the study. There were no significant differences in the numbers of predatory insects trapped or observed on the sprayed versus the unsprayed corn plots (used a non-specific insecticide - Pounce™). There was no consistent pattern of differences in abundance of predatory insects on the Bt+ versus the Bt- corn plots. The most common predator observed was *Coleomegilla maculata*. Trapping height had no significant impact on the number of predators captured. Aventis is currently in the 4th year of 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.	442581-15

3) 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 no reports of adverse effects on livestock after several years of feeding with Bt corn. Mammalian wildlife exposure to the *Bt* Cry endotoxins is considered likely; however, the mammalian toxicology information reported in the previous section, does not show a hazard to mammalian wildlife.

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4) 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. Six week broiler chicken studies with Bt corn show comparable results. Consumption of Cry9C protein in corn had no measurable adverse effects on bobwhite quail. The dietary LC_{50} for northern bobwhite exposed to corn plant powder containing Cry9C protein was greater than 58 micro g Cry9C protein/g diet, when administered in a diet containing 20% (w/w) of the powder. In addition,

there are no reports of adverse effects from the commercial poultry industry after several years of using Bt corn in poultry feeds. These data indicate that the Bt Cry protein produced in corn does not show a hazard to birds. In view of the lack of toxicity with Bt corn, no avian hazard is expected from the continued uses of Bt crops.

5) 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 endotoxin. 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 protein (high protein 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 endotoxin/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 endotoxin/L is projected under worst case conditions (Wolt, 2000).

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. There was no mortality or behavioral effects observed in the first 24 hours with 100 mg/L although mortality occurred in both test and control treatments within 48 hours. The LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) were found to be 150 mg/L in a second study. A 48-hour static renewal toxicity study with pollen from Cry9C showed no treatment mortality or behavior change between treated and control replicates. Although the amount of pollen and protein were not quantified, the solutions were cloudy with pollen in suspension and on the bottom of the test chambers. The amount of pollen was considered to well exceed field exposure. These studies indicate that *B.t.* Cry 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 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 endotoxin 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 corn crops.

6) Non-target Invertebrates

a) Honey Bees

Tests were conducted on both honey bee larvae and adults for Cry1Ab proteins. At a single dose, 20 ppm showed no adverse effects to larval honey bees under the test conditions. Because there was only one dose, an LC_{50} could not be calculated, but the NOEL was determined to be greater than 20 ppm. In adult honey bees there were no statistically significant differences among the various treatment and control groups.

Recently concerns have been raised regarding some honey bee larva studies submitted to the Agency. The question raised was whether the honey bee larvae 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 is confirmed by whole 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 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 corn pollen.

For Cry9C the reviewed honey bee study suggests that at the expected environmental exposure the proposed use of Cry9C protein in corn is not likely to have any measurable deleterious effects on honey bees. The data showed no treatment mortality or behavior change between the dosed and control replicates. The NOEL was 5.8 ug Cry9C protein/L diet (24,000 ug corn pollen/ml) for 8 days. This is more pollen than the bees would be expected to consume under field conditions. Foraging honey bees do not usually visit corn plants because corn is wind pollinated.

b) Lady Bird Beetles

Lady beetle predator toxicity studies submitted at the time of registration demonstrate that corn pollen containing the anti-lepidopteran Cry proteins do not cause significant adverse effects to lady beetles. Purified Cry1Ab protein at 20 ppm showed no adverse effects to lady beetles such as *Hippodamia convergens* under the study conditions. The NOEL is greater than 20 ppm. The reviewed study for Cry9C showed no adverse effects to *Hippodamia convergens* in either mortality or behavior changes at greater than 0.36 ug Cry9C protein/L (1.5 g corn pollen/ml), the highest dose tested. The test insects were exposed to a dose of active ingredient at approximately the dose that would be ingested by the beetles consuming aphids under field conditions.

c) Parasitic Hymenoptera

No adverse effects were observed when a maximum hazard dose of 20 ppm Cry 1Ab was tested on *Brachymeria intermedia.* The NOEL is greater than 20 ppm, but since this was a single dose study, a LC50 cannot be calculated. No adverse effect to Hymenoptera are expected from exposure to Cry1Ab. EPA did not require a parasitic hymenoptera study for Cry 9C; the non-target abundance study done under field conditions fulfilled the data requirement.

d) Green Lacewing

The studies submitted to support the initial registration showed no adverse effects to green lacewing larvae at a maximum hazard dose of 16.7 ppm Cry1Ab protein after 7 days. The NOEL is greater than 16.7 ppm. No adverse effect to green lacewing was expected as a result of exposure to Cry1Ab. EPA did not require a green lacewing study for Cry 9C; the non-target abundance study done under field conditions fulfilled the data requirement.

Since that time, there have been several publications proposing that transgenic *B.t.* plants may create serious impacts on non-target organisms that feed on pests exposed to the transgenic proteins. The reported effects of *B.t.* corn on larvae of the beneficial predatory insect, lacewing, stem largely from reports of work by Hilbeck et al. (1998a 1998b, 1999). EPA performed a formal review of the first two two studies on the effects of *B.t.* corn intoxicated prey and pure *B.t.* corn protein on lacewing (DP Barcode D236803 and D250457). While the authors report detrimental effects on lacewing larvae from consumption of *B.t.* corn protein, their data show that lacewing mortality and developmental effects more likely are related to the study diet, not to any potential *B.t.* endotoxin effects. Moreover, even if the reported 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 *B.t.* intoxicated prey (the European corn borer - ECB). The experimental design of the study, however, did not permit a distinction between a direct effect due to the *B.t.* protein on the predator versus an indirect effect of consuming a sub-optimal diet consisting only of sick or dying prey that had succumbed to the *B.t.* protein. The dead or dying prey may have been septicemic (and therefore indirectly toxic), of limited nutritional value, or unpalatable to the lacewing. The lacewing was not given a choice in diet, which it has in a field setting. In nature the lacewing does not rely upon a single food source for development (lacewing prefers insect eggs and aphids). In addition, the study has a high control mortality (34%, which is indicative of an unhealthy test system) and no prey consumption data. Also, there was no control with the purified *B.t.* endotoxin. Generally, the findings are inconclusive and the laboratory report results are not directly transferable to the field use setting. 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) In addition, all available Agency in-house and published field data do not show significant detrimental effects due to B.t. endotoxin on the lacewing.

Moreover, the authors subsequently reassessed the results of the study on reproductive effects on beneficial non-target organisms exposed to *B.t.* corn in the laboratory (Hilbeck et. al. 1998b). According to Hilbeck, there are no significant reproductive effects from *B.t.* corn protein. The authors thus 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 *B.t.* 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 massive (100 micro g /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. Also, since in the field setting the lacewing larvae have a choice of other insects or eggs to feed on, field exposure will be intermittent, rather than continuous. Furthermore, in high-dose *B.t.* corn fields intoxicated insects such as the ECB will not be available to the lacewings, since the ECB will be practically eliminated early by the *B.t.* protein in corn plants. (The first instars die as soon as they start eating Bt corn tissue.) 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).

As noted in EPA's review of the first study (Hilbeck et al. 1998a), the lack of *B.t.* consumption data by the larvae makes it impossible to determine correlation between exposure to *B.t.* and the observed responses. No data were presented to show the amount of prey consumed by each test group to make an independent assessment of unpalatability and sick prey effects that might be the result of food avoidance. The same is also true of the second study (Hilbeck et al. 1998b), in that it is not reported how much of a reduction in consumption of *B.t.* protein occurred in the replicates receiving a choice in diet. It is clear in this study also that there is a detrimental effect of the artificial study diet because data are presented that show an increase in mortality and development time in larvae reared exclusively on an artificial diet. Thus, the results of these studies do not support the conclusion that the *B.t.* protein was directly responsible for the observed differences in lacewing mortalities.

Environmental influences were also not considered in the speculation that *B.t.* corn may pose a risk to beneficial insects. In a field setting it is highly improbable that lacewing larvae will mature exclusively on a diet of prey larvae that have been exposed to *B.t.* endotoxin. Therefore it is highly unlikely that in the field the lacewings, or other beneficial insects, will ingest the amounts of *B.t.* that the larvae were forced to consume in the laboratory study (i.e. there is a very low field exposure to the *B.t.* protein). The reported laboratory findings are not representative of the feeding environment by predatory insects in the open ecosystem, nor is the laboratory exposure to *B*.t. endotoxin consistent with exposure that would be expected in the field.

In general the reported laboratory findings do not show significant detrimental effects and do not provide data that show a risk to beneficial insects in a field use situation. The author, A. Hilbeck, agrees with this by stating 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 *B.t.* crops on insect predators showing no significant differences in the density of beneficial insects, including green lacewings.

e) Soil Invertebrates

i. Earthworms

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* 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.

The submitted data for Cry9C demonstrated no adverse effects for earthworms observed at a maximum hazard dose of 16.7 times the field concentration. The study was scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. The 14-Day LC_{50} value and no observed effect concentration (NOEC) for earthworms exposed to Cry9C protein is greater than 1.84 mg a.i./kg dry soil. Given that the amount of Cry9C protein at harvest is 99 g/acre, the expected environmental concentration of Cry9C will be 0.11 mg/kg dry soil (15 cm deep). The 1.84 mg test dose represents a level of exposure >16.7 times the actual contact the earthworm would have under field conditions.

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 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 without harmful effects. In a recent presentation at the Workshop on Ecological Monitoring of Genetically Modified Crops (Stotzky, 2000d), data were presented showing that the earthworms do indeed ingest Bt protein with the soil. The Bt proteins were shown to be present in the frass for up to 48 hours after exposure, without any detrimental effects.

ii. Collembola

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. Thiodicarb was used as a positive control or reference substance. Test substances included corn powder at 0.5, 5.0, and 50% of the diet and Thiodicarb added to the diet at 1, 10 , 100, 1,000, and 10,000 ppm. 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. However, all Collembola exposed to the two highest rates of thiodicarb (1000 and 10,000 ppm) died within 14 days. At the lower rates of 1, 10, and 100 ppm, respective percent mortalities were 0, 7.5, and 45. 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. One study, which was considered an anomaly, showed toxic to a Collembolan species (*Folsomia candida*) at a high test dose. This study used Cry1Ab plant extract as the test material. However, when the maximum possible Cry protein concentration in the soil was factored in, the toxic level exceeded the level in the soil by a factor of 196. Therefore, no toxicity to Collembola in the field was expected. Novartis had cited the MON 810 leaf tissue study to support their Bt11 corn plant-pesticide. 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 Bt11 corn tissue on Collembola since possible treatment related effects were observed in a Bt corn Collembola study. 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-pesticide is still required.

The study submitted by Aventis adequately addressed potential concerns for Cry9C protein expressed in corn to Collembola (*Folsomia candida*). There was no toxicity observed in a chronic exposure test over a 28-day period where the Collembola were exposed to Cry9C from plants or bacteria. The NOEL for Cry9C protein derived from plant tissue was given to the Collembola at greater than 50% (by weight) in the diet (equivalent to 180 mg/kg dry soil) and the NOEL for bacterial Cry9C protein was 5% of the diet (equivalent to 20 gm/kg dry soil). Given that the amount of Cry9C protein at harvest is 99 g/acre, the expected environmental concentration (EEC) of Cry9C will be 0.11 mg/kg dry soil (15 cm deep). The 5% bacterial and the 50% plant Cry9C protein test doses represent a level of exposure 1.8 X 10^5 and 1,637 times the actual contact (EEC) that Collembola would have under actual field conditions after harvest. Cry9C protein in corn is not likely to have measurable effects to a substantial number of individual non-target beneficial soil insects in populations exposed to the levels of endotoxin found in plant tissue.

When it initially reviewed the applications for the products that were registered in 1995, EPA considered requiring studies evaluating effects upon the representative soil organisms 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. 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, *B.t.* 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. Supporting this conclusion are data which indicate that endotoxin production ceases at plant senescence, which allows time for protein degradation prior to harvest. Additionally, the environmental fate data indicate that only <1 to 90 grams of *B.t.* Cry protein per acre would enter the soil as a result of post harvest incorporation of *B.t.* plants, and since such proteins are known to degrade rapidly in field soils, the potential for significant soil buildup and effects to non-target soil organisms is not anticipated. This has been confirmed by in-house environmental fate data, published single species studies, and the field studies cited in this document.

In a recent presentation at the Workshop on Ecological Monitoring of Genetically Modified Crops, (Stotzky, 2000d) it was reported that no differences were found in the types and numbers of microorganisms and enzymes in Bt soil than control samples. Bt soils show no effect on total biomass, bacteria, actinomyces, fungi, protozoa or nematodes. The C/N ratio is also not affected. In addition, new plants grown in Bt containing soil do not take up the Bt protein. It was also reported that the same degree of Bt Cry protein persistence takes place in soils that have been exposed to repeat Bt spray applications. Live Bt cells were also recovered from the soils after spray applications for up to eight months of testing.

As discussed above, EPA does not believe that there are any valid data demonstrating specific adverse impacts of plants expressing B.t. endotoxins on beneficial soil invertebrates. To the contrary, EPA believes that available scientific data and information indicates that cultivation of B.t. 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

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. 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 only food source.

Pilcher et al. (1997) 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.

Aventis submitted a field study on Cry9C corn prior to registration. One objective of the study was to determine the effects of transgenic corn expressing Cry9C protein on the abundance of predatory insects in corn fields (Obrycki, J. 1997). 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 currently is in the 4-th year of 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 bird beetles, Hymenoptera parasitoids, 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 Seeds' Attribute Bt11-derived 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 *Bt* 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* 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 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* to monarchs in the laboratory do not necessarily translate into exposure at toxic levels in the field. EPA is participating in an aggressive research effort to reassess this finding.

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 *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). Although many of the larvae died during the 7 days that the experiments were run, 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/cm2), a level that far exceeded the highest pollen density observed in the field (200 grains/cm2). The authors conclude that *Bt* pollen of the variety tested is unlikely to affect wild populations of black swallowtails. The results suggest that undesirable effects on non-target insects from the use of transgenic plants may be manageable.

ii. Karner Blue Butterfly and Other Threatened or Endangered Species

The potential for off-site pollen flow from *Bt* corn fields has further implications to endangered species. Because of the selectivity of Cry1Ab endotoxin for lepidopteran species, endangered species concerns are mainly restricted to the order Lepidoptera. The majority of endangered lepidopteran species have very restricted habitat ranges. Examination of an overlay map showing the county level distribution of endangered 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, endangered 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.

Because Cry1 and Cry9C proteins are broadly active against Lepidoptera, some activity against the Karner blue would not be surprising. Testing of Karner blue *(Lyceides melissa samuelis)* larvae directly is difficult due to its endangered status.

One study was performed demonstrating that the Karner blue larvae were susceptible to a formulated microbial *Bt* product based on the *B.t. kurstaki* HD-1 strain that contains Cry1 proteins (Herms et al., 1997). The sensitivity level was similar to that of Gypsy moth larvae. The Karner blue requires wild lupine *(Lupinus perennis)* as an oviposition substrate and larval food source. In the absence of definitive information indicating whether Cry1Ab or Cry9C proteins are inherently toxic to Karner blue larvae, the key relevant issues regarding the potential for *Bt* corn pollen to impact the Karner blue are exposure issues: (1) whether Karner blue larvae and wild lupine plants occur in close proximity to corn fields, and (2) if so, whether Bt corn pollen may be present on wild lupine plants during the period when Karner blue larvae are feeding. The butterfly is found along the northern extent of the range of wild lupine, 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).

Wild lupine does not occur in corn fields, although there are anecdotal reports of wild lupine growing within a couple of hundred meters of corn fields. Moreover, it is important to note that Karner blue larvae are not likely to be feeding during or following the time of corn pollen shed. Karner blue eggs typically hatch twice a year. The first brood of larvae hatches in mid-April, and the adults emerge in late May to early June. The second brood of larvae feed through mid-July, and the adults emerge through mid-August (USFWS, 2000b). In the northern corn maturity zones where Karner blue occurs, corn plants (field corn, sweet corn or popcorn) typically shed pollen after mid-July, and more commonly not before late July or early August (Foster and Flood, 1995). Therefore, even if wild lupine plants were to occur in close proximity to corn, it is highly unlikely that any corn pollen would be present on them at the time Karner blue larvae are actively feeding. Degree-day effects that might result in earlier or later pollen shed would also tend to

correspondingly affect the rate of Karner blue development, and therefore not contribute to any greater likelihood of Bt pollen exposure. Based on the multiple factors cited above, the risk of *Bt* corn pollen affecting Karner blue larvae is not likely to exist.

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: Losey, et al (Cornell) and Hansen and Obrycki (Iowa State). In the Losey et al. study, pollen collected from *Bt* corn was applied by gently tapping a spatula of pollen over milkweed leaves 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%).

Hansen & Obrycki used *Bt* field corn pollen covered leaf samples taken from within and at the edge of the corn field were used 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 study data by themselves are not useful for risk assessment of widespread or recurring *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 actual risks to monarch butterflies, on December, 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, 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 (USEPA, 2000). 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 refugia (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.

The DCI called for information in five basic areas relating to the potential of *Bt* corn to impact non-target lepidopterans, particularly monarchs. 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 DCI data already submitted and incorporated that data into this preliminary assessment. All of the DCI data is due by March, 2001. In addition, there is a new USDA coordinated research effort underway and additional research by independent university and government researchers. The Agency will incorporate the results of these studies into the final risk assessment.

✳ **Milkweed**

Milkweed issues are addressed because milkweeds are the host plants for monarch larvae, so their distribution relative to corn agriculture represents an important component for evaluating the potential for effects of *Bt* corn on monarchs. Milkweeds are considered a weed in corn agriculture, and are therefore subject to control measures by cultural practices (e.g., tillage, cultivation, herbicides). In areas where weed control is practiced this may result in much higher milkweed densities in non-corn areas, such as pastures, roadsides, and fallow fields. The larvae of approximately 90% of the monarch butterflies passing through the corn belt will feed on the 7 most common milkweed species found in that area (Monarch Watch, 2000).

Roughly 50% of the monarchs in the US may pass through the corn belt each year (Wassenaar and Hobson 1998). Recent estimates (MBRS, 1999; USDA, 2000) are that approximately 1.5 million square miles represent the summer monarch breeding area, with 10.5% of this area comprised of corn fields. In the corn belt, 16.4% of the potential summer monarch breeding range is estimated to comprise corn fields. More recent estimates are in the 10% range (USDA, 2000).

Based on data from the 1997 US Census of Agriculture, the total area under corn cultivation in states that have been identified as breeding areas for the monarch butterfly is approximately 105,174 square miles. This represents 18 percent of the 570,045 square miles of crop, pasture, and range land associated with monarch breeding sites. If hazard to monarchs is limited to milkweed at the field edge, the analysis indicates that a 1-m field edge margin typically represents a 1% increment of the planted field area. The field edge habitat estimate may have minor significance in light of new information collected in the summer of 2000. The new studies show that milkweed grows well between corn rows and that monarch larvae were seen on these plants during the peak breeding period (Marcotty, 2000). This would indicate that monarch larva exposure to *Bt* pollen would take place in *Bt* corn fields in geographical locations where there is

an overlap of pollen shed and monarch breeding. Here one needs to factor in the preliminary data showing that there is no pollen shed and monarch breeding overlap in most of the corn belt, except in the northern range. And in assessing hazard in the northern corn belt one needs to look at the findings that MON810, CBH351, and Bt11 corn pollen at levels found in the fields show no detrimental effect on monarch development.

✳ **Pollen Movement**

The off-field movement of corn pollen represents another key component of the potential exposure of non-target lepidopterans. Corn pollen is relatively heavy compared to many other types of pollen, which limits off-field transport. Under low to moderate wind speeds, the vast majority of pollen movement away from the field falls within a few meters of the field edge. Results reported by Raynor et al. (1972), show that 63 percent of the pollen remained inside the confines of the field, 88 percent settled within 8 meters, 98 percent settled within 60 meters, and deposition at 60 meters was only 0.2 percent of that near the source. The quantity settling per unit area decreased rapidly with distance, such that the average deposition per square meter outside the field (0 to 60 m from the field edge) was less than 2 percent of that within the field. The steep deposition gradient is influenced by the very large size of corn pollen (pollen diameters are typically 90 to 100 microns) and release from a low-level source. Dispersion over long distances is possible in certain weather conditions (e.g., thunderstorms and accompanying updrafts) but the large size of corn pollen produces steep declines in concentrations with distance from the source and limited upward spread of the plume (Emberlin et al., 1999).

While it has been suggested in published literature that 60 m from the edge of a corn field represents the zone in which monarchs feeding on milkweeds may be exposed to pollen levels that could affect monarchs, the actual field edge zone where pollen may be present in densities that may impact monarchs is much narrower. Studies by several independent researchers consistently confirm that the majority of corn pollen is deposited within the corn field. Significant exposure of non-target lepidopterans outside the corn fields depends not only on the possible distance that corn pollen may move off-field, but also on a more important factor which is the density of pollen on a surface, such as a milkweed leaf. Data on pollen movement and Bt (Cry) protein activity in different Bt corn transformation events indicate that corn pollen is only present at distances of up to 1 to 5 m outside of a corn field in densities that could represent significant exposure to feeding larvae. This edge area represents less than 0.25% of the total corn acreage, and a correspondingly small percentage of non-corn milkweed habitat. Monarch exposure to B.t. corn pollen outside of the corn field, therefore, appears to be minimal. Data on retention of pollen by milkweed plants indicate that milkweeds retain only approximately 30% of the pollen that impinges on leaves, and that this pollen is readily dislodged (90%) by rain events and wind, thereby limiting exposure to possibly harmful pollen levels within the corn field system.

Duration of pollen shed typically occurs over a 10 to 14 day period within a given field. Pollen

shed usually starts two to three days prior to silk emergence (R1 stage) and continues for five to eight days; peak shed occurs on approximately the third day. Pollen shed on a typical midsummer day occurs between 9:00 and 11:00 a.m. Cool, cloudy, or humid conditions may delay the daily onset of pollen shed. A whole field may take as long as 14 days to complete pollen shed due to natural field variability in plant development. Under favorable environmental conditions the vast majority of pollen will be shed in a 1 to 2 day period at the middle of this interval.

✳ Bt Corn Pollen Toxicity

The toxicity of *Bt* corn pollen to non-target lepidopterans represents the third major component for evaluating the potential of *Bt* corn to affect non-target lepidopterans. It is the larval stages of monarchs, not the adults, that are potentially affected by Bt corn pollen because it is the larvae that may ingest *Bt* corn pollen. The Cry proteins incorporated into Bt corn need to be ingested to exert their toxicity; *Bt* corn products do not represent contact toxins. New data on monarch larval neonate susceptibility to purified Cry proteins was presented at the Monarch Butterfly Research Symposium, Chicago, IL (MBRS, 1999). LC_{50} and LC_{90} values were determined using diet incorporation assays with the purified trypsin-resistant core of various *Bt* Cry proteins. Seven day exposures showed that Cry1F protein has no detectable impact on monarch larvae, while the Cry9C protein is about 230-fold lower in toxicity than the Cry1Ab protein, and 23-fold lower than the Cry1Ac protein. Therefore, the order of sensitivity is Cry1Ab > Cry1Ac > Cry9C > Cry1F (Table C-1).

Table C-1. Toxicity of purified Cry proteins against monarch neonates after 7-day exposures

Protein	LC_{50} (ng Cry protein/ml diet)	LC_{90} (ng Cry protein/ml diet)
Cry1Ab	1.5	6
Cry1Ac	15	71
Cry9C	320	750
Cry1F	>10,000	>10,000

Monarch larval sub-lethal effects were also evaluated for four Bt Cry proteins. Bt Cry protein concentrations of about an order of magnitude lower than those causing mortality were associated with larval growth inhibition. The exception was Cry1F protein, which even at the highest concentrations tested (10,000 ng/ml) only caused partial growth inhibition. At sub-lethal levels, or when the source of toxin was removed, the larvae proceeded to complete the life cycle (Hansen & Obrycki, 2000).

Table C-2. Levels of Bt Cry protein in corn hybrids covered by this review.

Bt Corn Event / Protein	Mean Bt Cry protein level in pollen
MON 810 / Cry1Ab	<90 ng / g dry wt. pollen
Bt11 / Cry1Ab	<90 ng / g dry wt. pollen
CBH-351 / Cry9C	c.400 ng / g dry wt. pollen

Pollen from these events are unlikely to be found in densities that may affect non-target lepidopterans, even on milkweeds within a corn field. Additionally, modeling work on the overlap of pollen shed timing with the presence of monarchs indicates that, for most of the corn belt, except for the northern range, the monarch larvae are not present during pollen shed. Biological activity of Bt corn pollen against sensitive lepidopteran larvae is significantly reduced within approximately one week, or less in wind and rain, after pollen shed.

For MON810, Bt11 and CBH-351 no effects on larval survival were observed at pollen concentrations up to 600 grains/cm^2, although slight-to-moderate effects on larval weight were seen. No effects on larval weight were observed at 150 or 60 grains/cm^2 (Table C-3). In another study no effects were seen on larval growth or survival at 400 grains/cm^2 for MON810. It is reasonable to expect that Bt11 would yield comparable results as MON810, in that both events have only trace levels (<90 ng/g dry wt.) of Cry1Ab protein in pollen (Table C-2).

Table C-3. Monarch larval mortality and sub-lethal effects at various Bt corn pollen densities on milkweed leaf surfaces (USDA, 2000).

	600 grains/cm2		150 grains/cm2		60 grains/cm2	
Event	Mortality	Weight	Mortality	Weight	Mortality	Weight
MON 810	-	85%	-	-	-	-
CBH-351 (Cry9C)	-	56%	-	-	-	-

(-) No significant effect

The level of Bt Cry protein in pollen from inbred lines (which are homozygous for the Bt gene) would be expected to be about twice the level found in pollen from hybrids (which are hemizygous for the Bt gene), but inbreds are only found in smaller isolated seed production fields, thus reducing the level of exposure. Along with long isolation distances, these fields are generally 'cleaner' due to careful weed management, represent less than 1% of corn acreage, and produce less pollen than hybrid corn plants. For these reasons, inbred corn pollen does not represent any

greater risk than hybrid corn pollen.

Inbred and hybrid Bt sweet corn (registered under EPA Reg. No. 65268-1) presents a negligible risk in that the total US acreage for sweet corn is only 1% of the total field corn acreage (USDA – NASS, 1997). Further, the frequent insecticidal treatments in sweet corn (Vlachos and Roegner, 1997) are expected to have a higher negative impact on monarchs (adult as well as larval stages) than any impact from the small amount of Bt sweet corn pollen.

An approximate No Observable Effect Concentrations (NOEC) (the concentration of pollen grains/sq cm that did not result in larval mortality or developmental delays) of Bt corn pollen for monarch larvae is estimated at 150 pollen grains/sq cm for MON810 and Bt 11 Cry1Ab Bt corn hybrids. NOEC for Cry9C is substantially less. In Maryland the highest level of pollen deposition was inside and at the edge of the corn field, where pollen was found at about 50 grains/sq cm of leaf area. In the Nebraska study pollen deposition ranged from 6.0 grains/sq cm at the field edge to less than 1.0 grain/sq cm beyond 10 meters from the field. Pollen concentrations on milkweed leaves in Ontario were very similar to those reported in Maryland. Samples collected from fields in Ontario immediately following the period of peak pollen shed showed pollen concentrations averaged 78 grains/sq cm at the field edge; 28 grains/sq cm at 1 meter from the field edge; and 1.4 grains/sq cm at 5 meters from the field edge. Thus, approximately 90 percent of the pollen settled within 5 meters of the field edge. The researchers used the studies on corn pollen deposition on milkweed leaves under field conditions in Iowa, Ontario, Canada, and Maryland to determine approximate No Observable Effect Concentrations (NOEC) of Bt corn pollen for monarch larvae. Bt corn pollen was typically below (and only rarely exceeded) the NOEC (within 3 meters of the corn field) at all locations evaluated (Table C-4).

Table C-4. Exposure probability with distance for pollen deposits (150 grains/cm^2 pollen density) on milkweed leaves. Data for seven corn fields in Iowa (USDA, 2000).

Distance from field (meters)	Probability that pollen levels exceed 150 grains/cm^2
0	0.20
1	0.12
>2	0.00

In addition, viability of corn pollen grains only lasts a few hours after their release from a corn plant. These changes, in combination with the known tendency of Bt Cry proteins to rapidly break down when exposed to ultraviolet light, heat or biological activity, suggest that any insecticidal activity of Bt corn pollen should degrade rapidly (Gelertner, 1990). Studies using pollen that contains Cry1Ab from the MON810 event exposed to natural light indicated a loss of

any detectable bioactivity within 7 days (USDA, 2000).

✳ Monarch Biology

Data indicate that monarchs most frequently lay their eggs on the underside of tender, young milkweed leaves, and that first instar larvae begin feeding close to where the eggs are laid. The leaves selected for oviposition are more frequently at the tops of smaller (less than one foot tall) milkweeds; these upper leaves retain less pollen than lower leaves because of less surface area, and they are oriented more vertically than lower leaves. Older larvae are quite mobile and very often move from one milkweed plant to another. These behavioral and physical considerations further reduce potential larval exposure to pollen.

The likelihood that monarchs will oviposit eggs on milkweed plants dusted with corn pollen has a direct effect on the level of monarch larvae exposure to Bt protein. Preliminary studies presented at the Monarch Butterfly Research Symposium, 2 Nov 1999, Chicago, IL showed that monarch adults prefer not to oviposit on milkweeds surrounded by corn plants. In addition, because adult monarchs feed on nectar (which is not produced by corn), they also would not be present in a corn field to feed. In *cage* studies (USDA, 2000), corn plants were randomly assigned to four treatments: Bt corn pollen, isogenic pollen, gravel dust, and a no pollen control. In this study, there were no significant differences between any of these treatments. Based on this experiment, the authors conclude there is no preference for monarch oviposition on the basis of corn pollen or dust occurrence on milkweed leaves. In *flight chambers* designed to simulate a realistic field scenario, monarch oviposition behavior was observed by measuring the numbers of eggs laid on "patches" of potted milkweed plants which were either dusted with corn pollen or misted with de-ionized water (as a control). In this study, there was pronounced and significant avoidance of milkweed surrounded by corn plants. There was no evidence for discrimination between milkweed plants dusted with Bt-corn pollen and plants dusted with untransformed pollen or between two Bt hybrids. However, in this flight chamber study, monarch females exhibited a significant preference for ovipositing on milkweed plants that were not dusted with corn pollen vs those dusted with pollen, although the effect was less pronounced than in the case of avoidance of corn plants.

Studies to address the exposure of monarchs to corn pollen in corn fields, and the ensuing effects, are being conducted during the 2000 growing season. Results from these studies, when available, will shed more light on the effects of monarch larval exposure to B.t. pollen in fields where there is an overlap of pollen shed and monarch breeding.

Urquhart (1960), reported the observations of several researchers on the oviposition habits of monarch females. Consistent with data published by Borkin (1982), monarchs selectively deposited their eggs on only healthy plants and preferred the small young leaves of small plants. Of 1000 milkweed plants observed by one researcher, 40% of the plants observed possessed

flowers and seed pods, 20% of the plants were of medium height without flowers or seed pods and 40% of the plants were less than one foot in height. Of the 76 eggs counted, 74 or 97% of the eggs were located on the plants less than one foot in height. (In mid-July to August when pollen shed takes place most milkweed are much taller).

Urquhart (1960) also reported the observations of several researchers regarding whether eggs were laid on the under side or upper surface of leaves. These results were very consistent. In the first study, of 250 eggs, 38 (15%) were found on the upper surface and 212 (85%) were found on the lower leaf surface. In the second study, of 48 eggs collected, 8 (17%) were located on the upper surface and 40 (83%) on the lower surface.

Reports of more recent studies at the Monarch Workshop (USDA, 2000) confirmed the observations made by Borkin (1982) in monarch oviposition studies performed in glass house experiments. The data show that monarch adults preferred to lay eggs on the underside of the topmost leaves, which also represent the youngest leaves. Neonate larvae will begin feeding on the foliage immediately adjacent to the egg before moving to other areas of the plant.

✳ Conclusions

Common milkweed starts to grow sooner than corn and is taller than corn during the period of monarch's first generation in the Corn Belt. Since monarchs prefer to oviposit on milkweed plants that are taller than corn plants, at the time of the first monarch generation, milkweed in corn fields is more suitable to monarchs than at pollen shed when corn plants are tall. Once the corn has grown to pollination stage, there is very little probability of monarchs encountering toxic levels of pollen at any distance beyond 4 m away from a field of Bt corn. 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. These products show relatively low toxicity to monarch larvae. Consideration of other factors, such as the reports that there is no pollen shed and monarch breeding overlap in most of the corn belt, the distribution of milkweed plants within corn fields (or the near edge) compared to other milkweed habitats beyond the near edge of fields, the egg laying and feeding activity of monarch larvae, and the low toxicity of the Bt corn products covered by this assessment indicate a low probability for adverse effects of Bt corn on monarch larvae.

Considering the gains obviously achieved in the level of survival of populations of Monarch butterflies and other insects by eliminating a large proportion of the pesticides applied to corn, cotton and potatoes, some authors are predicting that the widespread cultivation of Bt Crops may have huge benefits for Monarch butterfly survival (Pimentel& Raven, 2000).

The weight of evidence of the published and preliminary data reviewed here indicate that

milkweeds in corn fields to within 1 meter of cornfields are unlikely to be dusted with toxic levels of Bt pollen from two of the most widely planted corn varieties, Monsanto's MON 810 and Novartis Seeds, Inc Bt11, as well as Aventis Cry9C. Based on the review of the submitted DCI data, the Agency concludes that the published preliminary monarch toxicity information is not sufficient to cause undue concern of widespread risks to monarch butterflies at this time. EPA will continue to closely monitor the results from the monarch butterfly research as a part of its regulatory oversight of Bt products.

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 endotoxin (Cry1Ac) showed no toxicity to honey bee larvae when exposed to concentrations 1700 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 endotoxin showed no toxicity to honey bee adults when fed a concentration for seven days 10,000 and 1700 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 endotoxin showed no toxicity to adults of the parasitic hymenopteran *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 endotoxin 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 endotoxin 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 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 endotoxin 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 Coleropteran–Lady Bird 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 *N. 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 endotoxin 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 on a 2 years 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 delta endotoxin 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 delta-

endotoxin-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

A Biological Opinion from the Department of the Interior Fish and Wildlife service was issued on December 18, 1986, concerning the possible effect of foliar spray of *Bacillus thuringiensis* subsp. *kurstaki* (Bt) on threatened and endangered species. Based on the difference in exposure scenarios between foliar spray and expression of Bt in cotton plants, EPA believes that the Biological Opinion is inapplicable, and that reinitiation of consultation is not required.

Although cotton pollen containing the Cry1Ac delta-endotoxin can drift out of fields, such pollen, at relatively very high dosages, was not toxic to the test species representative of organisms likely to be exposed to such pollen when cotton plants containing the *cry1Ac* gene are grown. The amount of pollen that would drift from these cotton plants onto plants fed upon by endangered/threatened species, would be very small compared to the levels fed to the test species. Therefore, EPA does not expect that any endangered/threatened species will be adversely affected

by pollen containing the Cry1Ac delta-endotoxin.

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 delta-endotoxin gene cannot escape into plants on which endangered/threatened species feed on in these areas.

Because EPA expects that in most cases, 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 B.t. Cry3A delta endotoxin. 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 B.t. Cry3A delta endotoxin produced in potato and BPPD believes that no additional avian studies should be needed.	429322-14; 429322-15
Cry3A Protein Comparison	To ensure that the truncated B.t. Cry3A delta endotoxin 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 B.t. Cry3A delta endotoxin 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 B.t. Cry3A delta endotoxin 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 B.t. in a sucrose and honey solution. The testing indicated that there was no significant loss of B.t. 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 this study was not required, it will not have to be repeated. The larval honeybee study produced useable results and indicated that B.t. Cry3A delta endotoxin in potato showed no toxicity to honeybee larvae. However, the study was not validated with a positive control. This validation may be submitted as a condition of the registration.	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
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 endotoxin. 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 delta-endotoxin was stable in this type of solution. Testing indicated that there was no significant loss of Cry3A delta-endotoxin 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 *B.t.t.* 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, 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 bird beetle (*Hippodamia convergens*).

An additional field study on the comparative impacts of foliar-applied microbial *B.t.t.*, 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 delta-endotoxin 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 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 a.i. 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 was 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 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 200micro 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 the use of potato Bt plant-pesticides will not affect a threatened or endangered species. The known host range for the Cry3A protein is restricted to Coleopteran species. Submitted data confirm that other species tested are not effected including lady bird beetles (Coleopteran). 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. There are a number of endangered and threatened species of coleopterans, but it is extremely improbable that they would be exposed to Cry3A found in potatoes because they are not likely to live near potato fields nor would they feed on potato plants.

5. Environmental Reassessment Summary

This reassessment finds no hazard to the environment at the present time from MON810, Bt11 and Cry9C transformation events in corn, Cry1Ac in cotton and Cry3A in potatoes as currently registered. The reassessment considered the following issues.

a) Gene Outcrossing and Weediness

The movement of transgenes from the host plant into weeds and other crops has been considered

for each of the *B.t.* plant-pesticides currently registered and EPA believes that these concerns have been satisfactorily addressed. The Agency has determined that as currently registered there is no significant risk of gene capture and expression of any *B.t.* Cry protein by wild or weedy relatives of corn, cotton, 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 for gene transfer in locations where wild or feral cotton relatives exist. Therefore, EPA requires stringent sales and distribution restrictions on *Bt* cotton within these areas to preclude outcrossing or hybridization from the crop to sexually compatible relatives. Based on data submitted by the registrant and a review of the scientific literature, the Agency concluded that there is no foreseeable risk of unplanned pesticide production through gene capture and expression of the Colorado potato beetle control protein gene (*cry3A*) in wild relatives of the transformed plant, *Solanum tuberosum* L in the U.S.

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. 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. No differences were found in the types and numbers of microorganisms and enzymes in Bt soil. Bt soils show no effect on total biomass, bacteria, actinomyces, fungi, protozoa, nematodes, springtails or earthworms. The C/N ratio is also not changed in the Bt Cry protein containing soil. 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 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 and chronic toxicity with Bt corn, potatoes and cotton seed to avian species. Therefore, no avian hazard is expected from the continued uses of Bt crops.

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 delta endotoxin 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 B.t. 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 B.t. crops than non-B.t. 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.

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 corn likewise was 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 is being reevaluated and much research effort is being devoted to this issue.

The weight of evidence of the published and preliminary 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 most widely planted corn varieties, Monsanto's MON810 and Novartis Seeds, Inc. Bt11 as well as Aventis CBH 351. 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, a lack of 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. Actual studies of the effects of corn pollen on monarch butterflies in the field are underway in the summer of 2000 and should provide data for a more definitive hazard assessment.

Based on the review of the submitted DCI data, the Agency concludes that the published preliminary 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 butterfly species 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 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. Moreover, the time of the year when corn

pollen is shed does not coincide with the times of the year when Karner blue larvae are likely to be present.

7) Conclusions

In general, the reviewed publications, preliminary 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 B.t. 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 B.t. crop registrations in the event that unexpected long range population, community or ecosystem effects are detected.

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

a) Studies Needed to Complete the Database of Registered Plant-Pesticides

Common Name and Cry Protein	OPP Chemical Code	Study Types
CBH351, Cry9C Corn	6445	1)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.
Cry1Ac *Bt* Cotton	6445	1) Cry1Ac expression levels in the root.
*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 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) 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-pesticide is required. The Agency notes that the registrant has only been recently appraised of this finding.
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.

Cry3A Potato	6432	Data on the degradation of Cry3A protein in soil has not been supplied by the registrant. Recent published data suggests that at lower soil pH, approaching pH 5.0, degradation of Cry protein may be substantially reduced. Since potatoes may be grown in soil between pH 5.0 and 6.0, a soil degradation study of Cry3A in potato tissue must be conducted at soil pH 5.0.

b) Future Ongoing Research

While the available data do not show a hazard at the present time, the long term soil accumulation of *Bt* Cry proteins and the effects on non-target organisms from the use of *Bt* crops are being examined by monitoring and research and will be closely followed by the Agency.

The USDA is coordinating an on-going research program concerning the potential effects of *Bt* corn on non-target insects, particularly Lepidoptera species. A significant amount of research has been undertaken, and is ongoing, to elucidate the potential impact of *Bt* corn pollen on non-target lepidopterans, other invertebrates and long term ecosystem effects. The industry is actively participating by working with USDA and independent academic researchers to address key questions. The USDA and the *Bt* corn industry are providing funds to continue research and to develop a more comprehensive database on several key topics related to monarch butterflies, other non-target invertebrates and *Bt* crops.

The amount of hazard that constitutes an unreasonable effect on monarch butterflies and other non-target Lepidoptera will have to be completed as the new USDA coordinated research, and product-specific data generated by the Monarch Task Force member companies, and independent university and government research programs becomes available in the next few years. Actual studies of the effects of corn pollen on both black swallowtails and Monarch butterflies in the field, among other issues, are being conducted in 2000, and will be reviewed by EPA prior to completing the final risk assessment. The Agency is confident that this approach will provide data to more fully demonstrate a real impact, if any, to monarchs, other Lepidoptera and non-target species.

Among the issues being looked by the Monarch Task Force are the following:

-How important are cornfields to monarch population production?
-Long term population monitoring
-Field verification of models that predict co-occurrence and dose mortality response
-Effect of buffer strips on biodiversity
-Effects of milkweed management on monarchs
-Can habitat restoration mitigate or offset other mortality factors?
-Effects of sublethal toxicity

ECOSYSTEM - Level Analysis

-How do we measure impacts of new technology at ecosystem level?
- Identify key ecosystems health indicators
- Impacts on natural enemies
- Effects of traditional management practices on species at risk to transgenic corn
- Fate and degradation of transgene products
- Impact on soil invertebrates and soil processes
- Impact on vertebrates feeding on insects affected by transgenic corn
- Effects on non-target plant communities
- Long term changes in pest population dynamics - including resistance and biodiversity
- Test toxicity of transgene products on taxa in or near corn
- Develop indicator species/surrogates

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