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**SERIES ON HARMONISATION OF REGULATORY OVERSIGHT IN BIOTECHNOLOGY  
Number 42**

**CONSENSUS DOCUMENT ON SAFETY INFORMATION ON TRANSGENIC PLANTS EXPRESSING  
BACILLUS THURINGIENSIS - DERIVED INSECT CONTROL PROTEINS**

*This document has been cancelled and replaced to correct mistakes in formatting due to technical problems.*

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**OECD Environment, Health and Safety Publications**

**Series on Harmonisation of Regulatory Oversight in Biotechnology**

**No. 42**

**Consensus Document on Safety Information on Transgenic Plants  
Expressing *Bacillus thuringiensis* - Derived Insect Control Protein**

**Environment Directorate**

**Organisation for Economic Co-operation and Development**

**Paris 2007**

## ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 30 industrialised countries in North America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and the Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (<http://www.oecd.org/ehs/>).

**This publication is available electronically, at no charge.**

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## FOREWORD

The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of consensus documents that are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product. In the area of plant biosafety, consensus documents are being developed on the biology of certain plants species, on specific genes and resulting proteins that when introduced into a plant result in the expression of specific traits and on issues arising from the use of general trait types in plants.

This document addresses the general information concerning the  $\delta$ -endotoxin genes and their protein toxin products that confer insect protection to plants. The United States served as the lead country in the preparation of this document. The draft was revised on a number of occasions based on the inputs from other member countries. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology has since recommended that this document be made available to the public.

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## PREAMBLE

The environmental safety/risks of transgenic organisms are normally based on the information on the characteristics of the host organism, the introduced traits, the environment into which the organism is introduced, the interaction between these, and the intended application. The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on identifying parts of this information, which could be commonly used in countries for environmental safety/risk assessment to encourage information sharing and prevent duplication of effort among countries. Biosafety Consensus Documents are one of the major outputs of its work.

Biosafety Consensus Documents are intended to be a "snapshot" of current information on a specific host organism or trait, for use during regulatory assessments. They are not intended to be a comprehensive source of information on everything that is known about a specific host or trait; but they do address the key or core set of issues that member countries believe are relevant to risk/safety assessment. This information is said to be mutually acceptable among member countries. To date, 28 Biosafety Consensus Documents have been published. They include documents which address the biology of crops, trees and micro-organisms as well as those which address specific traits which are used in transgenic crops.

In reading the Consensus Documents, it is useful to consult two additional texts. The first, entitled *An Introduction to the Biosafety Consensus Document of OECD's Working Group for Harmonisation in Biotechnology* explains the purpose of the Consensus Documents and how they are relevant to risk/safety assessment. It also describes the process by which the documents are drafted using a "lead country" approach. The second text is *Points to Consider for Consensus Documents on the Biology of Cultivated Plants*. This is a structured checklist of "points to consider" for authors when drafting or for those evaluating a Consensus Document. Amongst other things, this text describes how each point is relevant to risk/safety assessment.

The Consensus Documents are of value to applicants for commercial uses of transgenic organisms, regulators in national authorities as well as the wider scientific community. As each of the documents may be updated in the future as new knowledge becomes available, users of Consensus Documents are encouraged to provide any information or opinions regarding the contents of this document or indeed, OECD's other harmonisation activities. If needed, a short pre-addressed questionnaire is attached at the end of this document that can be used to provide such comments.

The published Consensus Documents are also available individually from OECD's website (<http://www.oecd.org/biotrack>) at no cost.

## SUMMARY NOTE

This document summarises the information available on the source of *Bacillus thuringiensis*  $\delta$ -endotoxin genes, the structure and properties of the toxins they encode, unique mechanisms of action, use in plants, toxicity and exposure data, and assessment methods. Some information on *Bacillus thuringiensis*, the bacterial source of these traits, is included as background and where relevant to the risk assessment of the  $\delta$ -endotoxins in plants, however this document does not attempt to address the vast amount of information available on the micro-organism. In addition to the scientific literature, which grew substantially over the last few years, this document also contains data submitted by registration applicants for the US-registered plant pesticide products (called plant-incorporated protectants in US pesticide regulations). These studies are required to be performed according to good laboratory practices regulations (US Code of Federal Regulations 40 CFR 160) and have been peer reviewed by USEPA scientists for acceptability for use in an environmental assessment. In the US, data from these studies may be released to the public and are available from the companies on request by other regulatory bodies. Some of these data were submitted for products that are no longer registered; however, the data are still valid to illustrate  $\delta$ -endotoxin properties. Where it is necessary to illustrate assessments unique to these toxin genes, plant expression data are discussed. However, the intent of this document is not to address gene transfer or other issues unique to specific plants that have been transformed to express these toxins. Such information is outside the scope of this document. It is intended that this document should be used in conjunction with specific plant species biology consensus documents when a biosafety assessment is made of plants with *Bacillus thuringiensis*  $\delta$ -endotoxin-mediated insect protection. It was also agreed that this document would not address the issue of insect resistance management, designed to prevent or delay the onset of resistance to specific  $\delta$ -endotoxins in insects exposed to these transgenic crops.

## SECTION I - GENERAL INTRODUCTION

1. Advances in genetic engineering in recent years have led to the development of plants that are resistant to some insects through incorporation and expression of genes encoding delta-endotoxins ( $\delta$ -endotoxins) from the bacterium *Bacillus thuringiensis* (*B. thuringiensis*). Throughout this paper, the microbial pesticide will be referred to as *Bacillus thuringiensis* whereas the toxins incorporated into the plants will be referred to as  $\delta$ -endotoxins. Various subspecies of the bacterium, *B. thuringiensis*, are registered as pesticides and are highly regarded as being environmentally-friendly due to their species-specificity (primarily affecting only the pest insects) and their lack of environmental persistence. In addition,  $\delta$ -endotoxin genes have been inserted into bacteria such as *Pseudomonas fluorescens* (Stone *et al.*, 1989) and *Bacillus pumilus* (Selinger *et al.*, 1998) for soil insect control, *Clavibacter xyli* for European Corn Borer (ECB) control (Dimock *et al.*, 1988), and *Bacillus sphaericus* for mosquito control (Poncet *et al.*, 1997), although these have only been used for experimental purposes in their living form. A Mycogen Corporation product expressing a *B. thuringiensis*  $\delta$ -endotoxin in *Pseudomonas* was rendered non-viable to address environmental concerns. Four of these products, expressing different  $\delta$ -endotoxins were registered in 1995 in the United States. A number of plant species, particularly crops such as cotton, corn, potatoes, tobacco, tomato, and sugarcane have been modified to produce  $\delta$ -endotoxin proteins from *B. thuringiensis* (Prieto-Samsonov *et al.*, 1997; Mendelsohn *et al.*, 2003; Romeis *et al.*, 2006b).

2. There are advantages and disadvantages to using transgenic plants containing the  $\delta$ -endotoxins as compared to the conventional use of microbial *B. thuringiensis* preparations. The control of insects through the expression of  $\delta$ -endotoxins in the transgenic plant can provide for protection throughout the growing season of the plant. The insecticidal activity need not be short-term, as with conventional Bt preparations which are more rapidly degraded in the environment. Transgenic plants overcome the problem of traditional microbial preparations that may not reach insects that burrow through the soil or those that bore into and remain inside the plant stem or tissue, *e.g.*, the European Corn Borer (ECB) larvae damages the corn stalk from within. Also, microbial preparations have not been as effective as the transgenic cotton/ $\delta$ -endotoxins product against the Cotton Bollworm (CBW) because the CBW spends most of its time feeding inside the squares (flowers) and bolls (fruit) (Beegle and Yamamoto, 1992). The extended exposure, and relative higher amounts of  $\delta$ -endotoxins as compared to microbial foliar sprays (Szekacs *et al.*, 2005), may lead to the selection of insects that are resistant to one or more of the *B. thuringiensis*  $\delta$ -endotoxins, thus potentially reducing the usefulness of these *B. thuringiensis* pesticides (Tabashnik *et al.*, 1990; Bauer, 1995; Van Rie, 1990b). Tolerant insects have been produced in laboratory studies with purified forms of  $\delta$ -endotoxins. Various strategies may be employed if deemed necessary to prevent the development of insect resistance in the field (Williams *et al.*, 1992; Rajamohan *et al.*, 1998; Matten, 1998; Pittendrigh *et al.*, 2004; Bates *et al.*, 2005).

3. A major environmental advantage of genetically engineered insect-resistant plants expressing genes encoding  $\delta$ -endotoxins and of microbial Bt preparations, compared with use of many synthetic chemical insecticides, is the greater specificity of  $\delta$ -endotoxins to target species. Adverse impacts on non-target insects and other organisms are reduced significantly. In spite of the more targeted specificity, there may still be insects and other non-target organisms potentially affected by the  $\delta$ -endotoxins, and extended exposure might affect their populations. Another possible disadvantage of genetically engineered insect-resistant plants is a potential for increase in weediness due to  $\delta$ -endotoxin transgene transfer to populations of wild sexually compatible species. However it should be noted that multiple factors determine the

potential for an increase in weediness in wild plant populations, the most important of which is whether the transgene can introgress into related plants. For example, an assessment found that introgression into Australia's 17 native cotton species from the tetraploid cotton crop would not be significant because their native cotton is diploid (AOGTR, 2002). The potential for  $\delta$ -endotoxin transgene transfer to increase weediness in wild crop relatives has also been studied for sunflower (Snow *et al.*, 2003) and for oilseed rape (Halfhill *et al.*, 2002, Vacher *et al.*, 2004) and is further discussed in paragraph 115 of this document.

#### A. *Bacillus thuringiensis* and its Uses

4. *Bacillus thuringiensis* is a common bacterium capable of survival in the environment for long periods of time because it produces endospores that are extremely resistant to adverse environmental conditions. Once the spores are in the soil, they do not germinate into vegetative cells unless they are in the presence of a rich nutrient source (Petras and Casida, 1985), *e.g.* nutrients in the soil or available within organisms that ingest the spores. For example, one tested strain, *B. thuringiensis* subsp. *kurstaki* DMU67R has been shown to persist in the field with no significant reduction in numbers for seven years (Hendriksen and Hansen, 2002), see paragraph 8, below for more details. All members of the genus *Bacillus* are rod-shaped, Gram positive cells that produce not more than one endospore per cell. Cells have peritrichous flagella surrounding them and are aerobic or facultatively anaerobic. Sporulation is not repressed by exposure to air (Claus and Berkeley, 1986). The species *B. thuringiensis* is characterised by the production of one or more protein parasporal crystals in parallel with spore formation. The parasporal crystals consist mainly of insecticidal  $\delta$ -endotoxins with some scaffolding proteins and Cyt toxins. The  $\delta$ -endotoxins in the crystals are usually inactive protoxins, which are converted by enzymatic action within the environment of the larval gut to active toxins (Claus and Berkeley, 1986). These toxins, in addition to other toxins produced by some isolates of *B. thuringiensis*, account for the insecticidal activity of the commercialised products to lepidopteran, dipteran, and coleopteran insects. The microbial products often show some additional activity, compared to  $\delta$ -endotoxins alone, by expressing other factors while reproducing within the insects.

5. Naturally-occurring isolates of *B. thuringiensis* have been used for insect control for decades. The first description of a *Bacillus thuringiensis* bacterium was in 1901 by the Japanese microbiologist S. Ishiwata who isolated it from diseased silkworm larvae (Ishiwata, 1901). Ishiwata named the bacillus Sottokin. A decade later, a German microbiologist, E. Berliner, isolated a similar organism from a diseased granary population of *Ephestia kuehniella* larvae from Thuringia, Germany (Berliner, 1911, 1915; also cited in Beegle and Yamamoto, 1992). Berliner named the bacterium *Bacillus thuringiensis*, and because Ishiwata did not formally describe the organism he found, Berliner is credited with naming it. The first commercial *B. thuringiensis* product was produced in France in 1938 (Kumar *et al.*, 1996). An isolate was first registered as an insecticide in the United States in 1961. Microbial preparations of various isolates of *B. thuringiensis* are used on a wide variety of grain, forage, fruit, vegetable, tuber, and fibre crops, and tobacco. In addition, they are used for control of forest pests, particularly gypsy and tussock moth species, and also for control of mosquitoes and blackflies.

6. When applied as a microbial insecticide, *B. thuringiensis* toxins have a relatively short persistence of 1 - 4 days on plants due to degradation from UV light exposure, however, a study of a Bt forest spray showed continued toxicity toward lepidopterans for at least 30 days following the spray (Johnson *et al.*, 1995). The *B. thuringiensis* spores persist in the environment for extended periods, and have been isolated world-wide from soil (Martin and Travers, 1989; Bernhard *et al.*, 1997; Ejiofor and Johnson, 2002), and from plant surfaces (Smith and Couche, 1991). Typically, *B. thuringiensis* is not naturally found in high numbers, except in previously treated soils, but it is not rare. Significant numbers of various strains of *B. thuringiensis* have been found in many different kinds of soils in Denmark including areas where commercial products have not been used (Hendriksen and Hansen, 2004). Delucca *et al.* (1981) found it in 17% of the soils they tested from 12 US states and reported it was found in a wide

variety of soils: cultivated soils, a rocky soil, and in virgin (not previously treated) forests. Spores of *B. thuringiensis* can maintain their presence in the environment by germinating and replicating to high numbers in suitable hosts which are not harmed by their presence. Many animals have been shown to excrete *B. thuringiensis* in their faeces. These include voles (Swiecicka and De Vos, 2003), Japanese deer (Ohba and Lee, 2003), 14 species of wild mammals in Korea (Lee *et al.*, 2003) and 11% of rodents and 17% of insectivore mammals examined (Swiecicka *et al.*, 2002). These mammals may also include humans since *B. thuringiensis* was found to be a common part of the microbial flora in sewage plant sludge (Mizuki *et al.*, 2001). The same process has also been observed in soil inhabiting invertebrates since *B. thuringiensis* was shown to germinate in three species of earthworm and one tipulid larvae without harming them (Hendriksen and Hansen, 2002). Thus, *B. thuringiensis* spores and toxins are an integral part of the environment.

7. The microbial *B. thuringiensis* products, which contain differing numbers of  $\delta$ -endotoxins, may be toxic to a number of different species of insects from different genera, see Section 1C for more detail and the tables in appendixes 2.2 and 3.2 of Glare and O'Callaghan (2000) for extensive lists of insects resistant to different strains of *B. thuringiensis*. Many pest insects are resistant to the *B. thuringiensis*  $\delta$ -endotoxins. For example, the European cockchafers are significant pest insects but have developed protective proteolytic midgut enzymes that protect them against Cry8C which kills closely-related scarab insects (Wagner *et al.*, 2002). The results of various studies on the susceptibility of pest insects to *Bt kurstaki* spray were analysed by Schmitz *et al.* (2003). Among those groups for which sufficient data were available, the Geometridae appeared to be the most susceptible family. In contrast, the Noctuidae are relatively resistant to Bt spray. Overall the literature confirms the lepidopteran-specific toxicity of commercial *Bt kurstaki* toxins (Schmitz *et al.*, 2003).

### **B. *Bacillus thuringiensis* Toxins**

8. Most of the  $\delta$ -endotoxins from *B. thuringiensis* are contained in the parasporal crystal inclusions that are synthesised adjacent to the endospore during sporulation. The parasporal crystal inclusions consist of different insecticidal crystal proteins, each of which is coded for by a single gene. Depending on the composition of the insecticidal proteins, the crystals can occur in a number of shapes, such as bipyramidal, cuboidal, flat rhomboid, or a composite with two crystal types. The genes that code for the insecticidal crystal proteins are usually located on plasmids, which are autonomously replicating circular pieces of extrachromosomal DNA that may be transferred by conjugation between various serovars of *B. thuringiensis* and related bacterial species such as *Bacillus cereus* and *Bacillus subtilis* (Klier *et al.*, 1983, Battisti *et al.*, 1985, and Ruhfel *et al.*, 1984.) The *B. thuringiensis* plasmids are relatively large and may contain one quarter of the genetic coding capacity of the bacterial chromosome (Carlton and Gonzalez, 1985). Schnepf *et al.* (1998) noted that there is considerable evidence that *B. thuringiensis* and *B. cereus* should be considered a single species. A genetic analysis of many isolates of *B. thuringiensis*, *B. cereus*, and *Bacillus anthracis* has found extensive genetic diversity among *B. thuringiensis*, and *B. cereus* environmental isolates with no clear distinction between the two species. However, the *B. anthracis* strains were more closely related to each other (Ticknor *et al.*, 2001). Strains of *B. anthracis* also exhibit much less diversity (Keim *et al.*, 1997). The *B. thuringiensis* strains used for insecticides cluster separately from the closely grouped *B. anthracis* strains, except that the one H34 strain previously identified as producing a  $\delta$ -endotoxin, and had been reported to be pathogenic to mice, (Hernandez *et al.*, 2000) occurred in the branch that contained all the *B. anthracis* strains (Hill *et al.*, 2004).

9. The  $\delta$ -endotoxins include another *B. thuringiensis* toxin type, which has cytolytic activity against a number of invertebrate and vertebrate cells *in vitro*. These "Cyt" toxins have been shown to have specific activity on dipteran insects via a mode of action similar to the Cry toxins. The interaction of Cyt toxins with Cry toxins is complex because in some cases the toxicity of a given Cry/Cyt toxin combination is

synergistic and in others antagonism is found, *e.g.* between Cry1Ac1 and Cyt1A1 both *in vitro* and *in vivo* toxicity to *Trichoplusia ni* (Del Rincon-Castro *et al.*, 1999).

10. Recently binary toxins from Bt have been assigned a Cry designation, though they have little detectable homology to traditional Cry toxins. Dow AgroSciences LLC and Pioneer Hi-Bred International recently registered a binary toxin, Cry34Ab1/Cry35Ab1, from the Mycogen Corporation *B. thuringiensis* toxin library (USEPA, 2005). Monsanto also holds a patent on a binary toxin.

11. In addition to the  $\delta$ -endotoxins, other toxins may be produced by various isolates of *B. thuringiensis*. One such proteinaceous toxin class from *Bacillus* isolates is Vegetative insecticidal protein (Vip) 3A (Estruch *et al.*, 1996) which has broad toxicity against lepidopteran species (C.Yu *et al.*, 1997). Genetically engineered products expressing Vip3A are being evaluated in cotton and maize plants. Although it has similar properties to the  $\delta$ -endotoxins, the Vip3A toxin has not been classified as a  $\delta$ -endotoxin and will not be addressed in this document. Some isolates of *B. thuringiensis* produce a class of closely related adenine-nucleotide analogue insecticidal molecules called beta-exotoxin, (Hernandez *et al.*, 2001). The common name for the beta-exotoxins is thuringiensin. These heat-labile toxins may be responsible for the toxicity of some isolates of *B. thuringiensis* to non-target organisms including mice, some aquatic insects, and fish (Beegle and Yamamoto, 1992). Beta-exotoxin and the other *Bacillus* toxins may contribute to the insecticidal toxicity of the bacterium to lepidopteran, dipteran, and coleopteran insects (Crickmore *et al.*, 2005). Beta-exotoxin is known to be toxic to humans and almost all other forms of life and its presence is prohibited in *B. thuringiensis* microbial products. Engineering of plants to contain and express only the genes for  $\delta$ -endotoxins avoids the problem of assessing the risks posed by these other toxins that may be produced in microbial preparations.

### C. Susceptible Insects

12. Various isolates of *B. thuringiensis* have been reported to have pesticidal activity against insect species, primarily lepidopteran, coleopteran and dipteran species, and some non-insect species, for example nematodes, flatworms, protozoa (Feitelson *et al.*, 1992; Griffiths *et al.*, 2001; Kondo *et al.*, 1992), and also mites (Arachnida, Acarinae) (Feitelson *et al.*, 1992). However, some of this activity observed for bacterial isolates may be due to *Bacillus* toxins other than  $\delta$ -endotoxins. There are many different  $\delta$ -endotoxins and they have vastly different specificities against different insects. This great diversity is likely to have developed through sequence divergence and subsequent swapping of domains within the toxin molecules (de Maagd *et al.*, 2001). Many different  $\delta$ -endotoxins have been tested for activity against various insects both at the strain and individual toxin level. Most of the insects reported as susceptible to  $\delta$ -endotoxins are Lepidoptera, but many  $\delta$ -endotoxins are active against Diptera (*e.g.*, Cry4, 10, 11, 19, and 25) and Coleoptera (*e.g.*, Cry3 and 8). Among the Lepidoptera, there is variation in susceptibility to various  $\delta$ -endotoxins. In a survey of 42 species of non-target lepidopterans, 13 species were rated as very sensitive to a commercial product, Foray 48B (containing genes encoding, but not necessarily expressing, the Cry1Aa1, Cry1Ab1, Cry1Ac1, Cry2Aa1, and Cry2Ab1 proteins), and 11 species were rated as insensitive (Peacock *et al.*, 1998). Some  $\delta$ -endotoxins do not appear to directly affect any tested pest species. Lambert *et al.* (1992) reported that a Cry7Aa did not affect various lepidopteran larvae and was only weakly active against Coleoptera although *in vitro* pre-treatment with trypsin increased its activity against Coleoptera. Some representative  $\delta$ -endotoxins have been analyzed to the extent that host range and toxicity functions have been attributed to different domains of the protein molecule. The natural *B. thuringiensis* isolates generally produce more than one  $\delta$ -endotoxin. Many experiments, both *in vitro* and *in vivo*, suggest synergistic interactions between two or more  $\delta$ -endotoxins (Schnepf *et al.*, 1998). On the other hand, antagonism was also observed between Cry1Aa and Cry1Ab in the gypsy moth (Tabashnik, 1992).

13. Caution should be used when attempting to extrapolate the susceptible species to an individual  $\delta$ -endotoxin as expressed in plants from tests using microbial forms of *B. thuringiensis*. As noted before, most strains of *B. thuringiensis* express multiple toxins, which often may interact with each other. In some cases,  $\delta$ -endotoxins combined with spores can exhibit toxicity even though neither the spores nor the toxins, by themselves, are especially toxic to the test insect (Liu *et al.*, 1998). Frequently the  $\delta$ -endotoxin may be expressed in a plant in a truncated form, and it has been hypothesised this situation could increase the host range of the  $\delta$ -endotoxin to additional insects. It should be noted, however, that other factors contribute to the selectivity (see paragraph 30 of this document). Clearly, protocols screening for insect susceptibility need to ensure that test insects are exposed to Cry toxins that are the same or equivalent to Cry toxins that are expressed in the insect-resistant plants in question; for example use of Cry toxins isolated from *E.coli* strains, grown under contained conditions, that have been genetically engineered to express the relevant  $\delta$ -endotoxin.



## SECTION II - NOMENCLATURE/CLASSIFICATION OF TOXINS AND GENES

14. Unlike some systems where genes were named without knowledge of the gene products,  $\delta$ -endotoxins and the genes which produce them rely on the same nomenclature. However, standard nomenclature for genes requires that the gene name be italicised in lower case (e.g., *cryIA*), whereas, the  $\delta$ -endotoxin protein product produced by that gene is designated in regular font with an initial capital letter (e.g., CryIA).

15. Hofte and Whiteley (1989) proposed a classification scheme that related similar toxin gene sequences to the activity against susceptible insects. From a total of 52 genes, Hofte and Whiteley designated 14 distinct gene types and sorted them into four major classes based on their insect specificity. The four major gene classes consisted of *cryI* - Lepidoptera; *cryII* - Lepidoptera and Diptera; *cryIII* - Coleoptera; and *cryIV* - Diptera. More recently, two new classes were proposed, *cryV* and *cryVI*, (Feitelson *et al.*, 1992) based on additional analysis of the toxin domains of 29 distinct toxin proteins. This classification scheme, however, is no longer adequate to identify the many new varieties of  $\delta$ -endotoxin genes, in that some of the newly-analyzed genes show a high degree of DNA homology to known genes, but possess different insecticidal activities. Therefore, in 1993, a  $\delta$ -endotoxin nomenclature committee was established to revise the classification of  $\delta$ -endotoxins.

16. Crickmore *et al.* (1998) introduced a systematic nomenclature based on the similarity between amino acid sequences of full-length gene products, rather than their biological properties. The scheme, which arranges the genes according to possible evolutionary relationships, is based on a phylogenetic tree calculated with computer programs that are in the public domain. The *cry* genes designated by Hofte and Whiteley (1989) have been retained, although the Roman numerals have been replaced by Arabic numbers (*cryII* is now *cry2*), still followed by uppercase and lower case letters. This new nomenclature scheme defines the degree of homology that needs to be shared to have the same Arabic number ( $\geq 45\%$ ), the same uppercase letter ( $\geq 75\%$ ), and the same lowercase letter without parentheses ( $\geq 95\%$ ). A fourth ("quaternary") ranking is also given for gene products that differ in amino acid sequence but whose genes have more than 95% homology. For example, the gene designated by the Hofte and Whiteley (1989) classification scheme as *cryIA(c)* by Von Tersch *et al.* (1991) is currently designated as *cryIac2*. Note that a different quaternary ranking is assigned for each new Cry toxin submission, so that some of the toxins that have identical nomenclature except for different quaternary rankings may actually be identical. Although it may be argued that this system does not exactly meet some of the standards set for protein nomenclature, it has the advantage that the genes classified under the earlier system, for the most part, do not need a major change in their name, and there is no need to make changes in the vast existing literature. A few *cry* genes have been reassigned under the new system (Table 1). A *Bacillus thuringiensis cry* Gene Nomenclature Committee is now part of the Bacillus Genetic Stock Center. A current list of  $\delta$ -endotoxin genes can be found on the Internet at [http://www.lifesci.sussex.ac.uk/Home/Neil\\_Crickmore/Bt/](http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/) (Crickmore *et al.*, 2005).

17. Under the new classification system, there are 49 major classes of different *cry* genes, *cry1* through *cry49*, and two major classes of *cyt* genes, *cyt1* and *cyt2*. At the end of the year 2005, there were a total of 314 different *cry* genes (including 26 binary *cry* genes) and 24 *cyt* genes. In recent years, about 20 newly classified genes are generally added each year. Use of this nomenclature will greatly facilitate international harmonisation of  $\delta$ -endotoxin regulatory assessment. Wherever possible, this nomenclature

has been used in this document, however, in some cases, the older nomenclature is used when it was referenced as such in citations.

Some of the renamed  $\delta$ -endotoxin genes (Old vs. new nomenclature)

Old	<i>cryIG</i>	<i>cryIIIC</i>	<i>cryIIID</i>	<i>cryIVC</i>	<i>cryIVD</i>	<i>cytA</i>	<i>cytB</i>
New	<i>cry9A</i>	<i>cry7Aa</i>	<i>cry3C</i>	<i>cry10A</i>	<i>cry11A</i>	<i>cyt1A</i>	<i>cyt2A</i>

Source : Crickmore *et al.*, 1998

### SECTION III - CHARACTERISATION OF THE $\delta$ -ENDOTOXINS

#### A. Protoxins

18. The parasporal crystalline inclusion bodies formed within the *B. thuringiensis* cells adjacent to the endospore during sporulation are protoxins which are composed of precursors of the active  $\delta$ -endotoxins and DNA (Clarimont *et al.*, 1998). For the three conventional three-domain toxins, *e.g.* Cry1, Cry2 and Cry3, the C-terminal half of these inactive protoxins are enzymatically cleaved within the midgut of susceptible insect larvae by trypsin-like proteases to the active toxin, which consists of the N-terminal portion of the molecule (Federici, 1993; Rukmini *et al.*, 1999).

19. The lepidopteran-specific Cry1 protoxins are 130 – 140 kDa in size and except for Cry1I accumulate as bipyramidal crystalline inclusion bodies. There are a large number of different *cryI* gene sequences (Hofte and Whitely, 1989; Crickmore *et al.*, 1998; Crickmore *et al.*, 2005). The Cry1A through Cry1G protoxins are in the range of 1100-1200 amino acids and the active portion of the protoxin molecule is a 60 - 70 kDa fragment localised in the N-terminal half of the protoxin for the Cry1A and Cry1C proteins. For many of the Cry toxins the proteolytic cleavage site is not known experimentally, however it can be inferred from homology modelling approaches. The bipyramidal Cry4A and Cry4B protoxins, which are approximately 130 kDa, also consist of approximately 1100-1200 amino acids (Knowles, 1994; Kumar *et al.*, 1996).

20. The Cry2, Cry3, and Cry11A protoxins are smaller molecules of approximately 70 kDa which are similar to the N-terminal portion of the larger protoxins (as reviewed by Bauer, 1995). Crystals formed from the Cry2 proteins are cuboid, the Cry3 are rhomboid, and the Cry10A and Cry11A are bar-shaped (Knowles, 1994). These smaller protoxins still require enzymatic processing involving removal of amino acids from the N terminus to form the active toxins (Bauer, 1995). Cry11A can be processed differently than the other Cry toxins in some insect species. It is cleaved into two fragments of 30 and 35 kDa. The Cyt protoxins are smaller molecules of 29 kDa and have an amorphous crystal shape in the absence of Cry toxins (Knowles, 1994; Li, 1996). After solubilisation the toxin is present as a dimer and can be cleaved by proteinase K to uncover the active sites *in vitro* (Koni and Ellar, 1994; Li, 1996).

21. The variation in specificity of  $\delta$ -endotoxins to different species of insects may be, in some cases, due to the presence of different proteolytic enzymes. Cry1Ac is very active against *Mamestra brassicae* (Cabbage moth), but has little effect on *Pieris brassicae* (Cabbage white butterfly); two insects that are not closely related, being from different families. Extended proteolysis using proteases from each insect resulted in insoluble products, but with different molecular sizes resulting from differential processing (Lightwood *et al.*, 2000).

#### B. Truncated Active Toxins

22. Truncated active toxins are the N-terminal portion of the protoxin molecules obtained after enzymatic cleavage within the insect midgut of the larger Cry toxins. It is this portion of the protoxin which then binds to receptors and ultimately results in lethality due to membrane disruption. The smaller 70 kDa Cry2, Cry3, and Cry11 are sometimes considered truncated forms of the N-terminal portion of the larger Cry toxins, although some processing still occurs on these smaller molecules (Knowles, 1994). The 29 kDa Cyt protoxins are dimers that are cleaved into active monomers (Koni and Ellar, 1994; Li, 1996).

### C. Structure of Toxins

23. The three-dimensional structures of the coleopteran specific Cry3A (Li *et al.*, 1991) and the mosquitocidal Cyt2A  $\delta$ -endotoxins (Li *et al.*, 1996) have been published. The structure of the lepidopteran specific Cry1Aa has also been determined and is similar to the Cry3A structure (Grochulski *et al.*, 1995). In addition, the structures for Cry2Aa (Morse, *et al.*, 2001) and Cry3Bb1 (Galitsky *et al.*, 2001) have recently been published. Based on their sequence similarity, many other Cry  $\delta$ -endotoxins are thought to have a similar three-dimensional structure. The first 285 amino acids are a bundle of seven amphipathic helices. Six of these helices occur in a circle surrounding helix five in the centre of the Cry3A molecule. These helices are known as domain I. Domain II consists of amino acid residues 286-500 which form three antiparallel  $\beta$ -sheets. Domain III is the rest of the amino acids in  $\beta$ -sheets arranged like a sandwich (as reviewed by Kumar *et al.*, 1996).

24. The Cyt protoxins have just a single domain in which two outer layers of  $\alpha$ -helices surround a five stranded  $\beta$ -sheet. The protoxin is actually a dimer of two of these molecular domains associated at the N-terminus strands (Li, 1996).

### D. Prevalence of the $\delta$ -Endotoxins in Microorganisms

25. It is common for microbial *B. thuringiensis* strains to express more than one  $\delta$ -endotoxin, yet the same  $\delta$ -endotoxins may appear in many different isolates. Two published studies, in particular, examined the prevalence of certain  $\delta$ -endotoxins. The distribution of  $\delta$ -endotoxin genes in 58 new isolates showed 57% had *cry1C*, 45% had *cry1A(b)*, and 34% had *cry2A* genes (Kim *et al.*, 1998). Another study of 223 isolates looked at three families of  $\delta$ -endotoxin genes (Ferrandis *et al.*, 1999). They found *cry5*  $\delta$ -endotoxin genes in 66%, *cry1* in 54% and *cry2* in 42% of the isolates. A specific analysis of the isolates possessing *cry1* genes showed 62% had *cry1A(c)*, 49% had *cry1A(a)*, 43% had *cry1D*, 35% had *cry1C*, and 34% had *cry1A(b)*  $\delta$ -endotoxin genes (Ferrandis *et al.*, 1999).

26. The microbial *B. thuringiensis* strains are generally divided taxonomically into serovars based on differences in the antigens in their flagella, *e.g.* *B. thuringiensis* ser. *kurstaki*, or *B. thuringiensis* ser. *israelensis*. In some cases biochemical and morphological criteria are used to further distinguish the serovars. However, since the  $\delta$ -endotoxin genes are primarily carried on large plasmids with some mobility, the subspecies designations do not definitively allow predictions of their specific *cry* gene content. One analysis of Bt isolates reported that there is no apparent relationship between  $\delta$ -endotoxin gene content and serotype of the micro-organism (Ferrandis, 1999). Bacterial species other than *B. thuringiensis* have been shown to produce  $\delta$ -endotoxins. In 1990, crystalline  $\delta$ -endotoxin-like proteins were first reported in *Clostridium bifermentans*, serovar *malaysia* (de Barjac *et al.*, 1990) and subsequently found in 80% of 12 *C. bifermentans* subspecies and 8% of 13 other *Clostridium* strains and 13 *B. thuringiensis* isolates tested (Barloy *et al.*, 1998). A Cry2Aa  $\delta$ -like endotoxin, termed Cry18Aa, has been detected in *Bacillus popilliae*, which is a US registered microbial pesticide product for control of the Japanese beetle (Zhang *et al.*, 1997).

## SECTION IV - MECHANISM OF ACTION

27. An immense amount of research on representative  $\delta$ -endotoxins has been devoted to understanding the mode of action on susceptible insects. In general, following ingestion, the crystalline inclusions are dissolved and then converted to active toxins by insect proteases. The active toxins bind to specific receptor sites and produce pores in the insect gut which results in loss of homeostasis and septicemia, which are lethal to the insect (Broderick *et al.*, 2006). In addition, there may be other less-characterised insect control functions of these toxins such as avoidance of the toxins and feeding paralysis prior to completion of the full lethal pore-formation process (Aronson and Shai, 2001). In many cases, larvae become less susceptible to  $\delta$ -endotoxins as they age due to fewer binding sites in the older larvae (Gilliland *et al.*, 2002).

### A. Ingestion and Solubilisation

28. When the non-toxic *B. thuringiensis* crystalline inclusions are ingested by a susceptible lepidopteran insect larva, they dissolve in the high pH (>9.5) environment of the larval midgut, releasing one or more  $\delta$ -endotoxins. However, many coleopterans have a neutral pH midgut, yet solubilisation of the coleopteran-specific toxins occurs (Koller *et al.*, 1992). Solubilisation may occur due to initial proteolysis of Cry3A, which renders the toxin soluble at neutral pH allowing it to impart activity against coleopterans (Carroll *et al.*, 1997). Combinations of Cry proteins in inclusion bodies may also facilitate the solubility over that of crystals with only one Cry protein (Aronson, 1995). These proteins are protoxins that are converted enzymatically in the insect midgut by proteases into smaller active toxins which are resistant to further protease digestion. These active toxins bind to unique receptor sites on the epithelium cells in the midgut of susceptible insects. The proteolytic susceptibility of various Cry1A protoxins appears to be sensitive to DNA associated with the N-terminal end of the protoxin in the crystals (Clairmont *et al.*, 1998).

29. The ingestion of plant material containing the  $\delta$ -endotoxin, in the form of the protoxin or as an active truncated toxin, has been shown by many studies to control the same target hosts as does ingestion of *B. thuringiensis* crystalline inclusions containing the  $\delta$ -endotoxins (Mycogen and Novartis, 1995a, 1995b; Plant Genetic Systems, 1998c; Mycogen and Pioneer, 2001a, 2001b, 2001c, 2005a; Monsanto, 2002b, 2002f). It has been proposed that the solubilisation and proteolysis phase can contribute to the selectivity of action towards susceptible insects, and that if the  $\delta$ -endotoxin expressed in the plant is the truncated active form, a wider range of hosts may be affected (Stotzky, 2002, Hilbeck, 2002). However, there is no evidence to support the hypothesis that protease activated or truncated toxins alter the host range of non-target insects (Mycogen and Novartis, 1995a, 1995c; Plant Genetic Systems, 1998c; Mycogen and Pioneer, 2001a, 2001b, 2001c, 2005a; Monsanto, 2002b, 2002d; Evans, 2002). Additional studies will be required to shed more light on these issues (Evans 2002).

### B. Binding to Receptors

30. For the classic three-domain Cry toxins, *e.g.*, Cry1A and Cry2A, binding of the active Cry toxin molecules to a receptor in the brush border membrane of the epithelial cells in the midgut microvillae of the target insect is an essential process in achieving toxicity (Hoffman *et al.*, 1988), although additional post-binding processes, including membrane insertion and septicaemia, are required for insect lethality (Broderick *et al.*, 2006). The specificity of the Bt  $\delta$ -endotoxins is a primary result of their ability to bind to

specific receptor sites in the membrane. However, it has been shown that many toxins are capable of binding to more than one receptor (Van Rie *et al.*, 1989; Van Rie *et al.*, 1990a; Denolf *et al.*, 1993; Estada and Ferré, 1994; Escriche *et al.*, 1994), one receptor can bind more than one toxin (Escriche *et al.*, 1997), and different Cry toxins can compete for the same binding sites (Hua *et al.*, 2001; Estela *et al.*, 2004; Li *et al.*, 2004). The amino acid sequence of the receptor binding domain of the  $\delta$ -endotoxin molecule is thought to be a predictor of host specificity (as reviewed by Bauer, 1995). The binding domain of the  $\delta$ -endotoxin molecule is apparently the most variable portion of the active toxin molecule (Hofte and Whitely, 1989). In some cases binding of the active toxin molecule correlates directly with toxicity (Luo *et al.*, 1997; Jurat-Fuentes *et al.*, 2000), but binding has not always exhibited a correlation with toxicity *in vivo* (Van Rie *et al.*, 1990a; Gould *et al.*, 1992; Escriche *et al.*, 1994). The association of binding with potency is further supported in that some Cry1 toxins showed correlation with potency in some but not all cases studied (Gilliland *et al.*, 2002). Lee *et al.* (1999) hypothesised that cases where there is no correlation may be due to non-functional receptors, or alternatively, to initial binding to more than one receptor.

31. The high affinity binding of the active toxin molecule to the specific receptor within the insect midgut is thought to occur by interaction of the loops of domain II, the portion of the active toxin with three antiparallel  $\beta$ -sheets (as reviewed by Knowles, 1994). A revised model for the mechanism of action has been proposed based on studies conducted using site-directed mutagenesis of the Cry toxins. Dean *et al.* (1996) and Aronson and Shai (2001) suggest that domain III is also involved in the binding of the active toxin molecules to the receptors for many insects. For example, de Maagd *et al.* (2000) showed that domain III was essential for toxicity to *Spodoptera exigua* by producing *Spodoptera* activity in several Cry1 toxins by transferring an active domain III from Cry1Ca into them. However, in the specific case of the diamondback moth (*Plutella xylostella*), domain III was found to have a minimal effect on toxicity and binding (Ballester *et al.*, 1999).

32. Many putative receptors have been identified for various  $\delta$ -endotoxins in a number of different insects. The emerging picture is complex. The same toxin may bind to different receptors and different toxins may bind to the same receptors. For example, Cry1C is reported to bind to both a 40 kDa protein (Kwa *et al.*, 1998) and a 106 kDa aminopeptidase-N glycoprotein (Luo *et al.*, 1996). Wang and McCarthy (1997) identified seven Cry1C binding proteins (137, 120, 115, 68, 63, and 45 kDa). Cry1Ab and Cry1Ac compete for the same binding site in the striped stem borer (*Chilo suppressalis*) and the yellow stem borer (*Scirpophagus incertulis*) (Alcantara *et al.*, 2004). Cry1Ac domain III mutants could no longer bind to aminopeptidase-N, however, some toxicity remained to *Manduca sexta* indicating the presence of alternative receptors (Jenkins *et al.*, 1999).

33. Aminopeptidase-N proteins, belonging to a family of zinc-dependent metalloproteases, have been shown to function as receptors for many  $\delta$ -endotoxin proteins. Cry1Aa was shown to bind to a highly conserved region of the aminopeptidase-N family of proteins (Nakanishi *et al.*, 1999). A detailed analysis of the *Tenebrio molitor* midgut aminopeptidase revealed some common features with mammalian aminopeptidase-N, but it differed in details of substrate binding and in catalytic residues (Cristofolletti and Terra, 2000). Luo *et al.* (1997) have found that a specific 170 kDa aminopeptidase-N from *Heliothis virescens* would bind Cry1Aa, Cry1Ab, and Cry1Ac, but not Cry1C or Cry1E. A 120 kDa aminopeptidase-N from brush border membrane vesicles of a tortricid moth was shown to bind both Cry1Ac and Cry1Ba (Simpson and Newcomb, 2000). Two amino acid differences in aminopeptidase-like proteins were sufficient to make an Indianmeal moth (*Plodia interpunctella*) resistant to Cry1Aa (Zhu *et al.*, 2000). The receptor for the Cry1A(c) toxin in the lepidopteran *Manduca sexta* was identified as a 120 kDa aminopeptidase-N (Knight *et al.*, 1994). Two  $\delta$ -endotoxins, Cry1Ac and Cry1Fa, bind to several aminopeptidase-N's (110, 120, and 170 kDa.) from *Heliothis virescens* (Banks *et al.*, 2001). A thorough investigation of one system, Cry1Ac binding to *Lymantria dispar* aminopeptidase-N, suggested that an initial recognition of the aminopeptidase occurs by a region in domain III of Cry1Ac and a subsequent tighter binding occurs via a region in domain II (Jenkins *et al.*, 2000). However, the study using Cry1C,

Cry1E, and Cry1Ab in *Plutella xylostella* showed that binding specificity was due to domain II with no detectable involvement with domain III (Ballester *et al.*, 1999).

34. The aminopeptidase-N family of neutral zinc-dependent metallopeptidases has been well studied. They are classified in an M1 family, which, in turn, is part of a superfamily of 36 families. Currently there are two unique recognised classes, bacterial aminopeptidase-N and mammalian aminopeptidase-N. There have been a number of research efforts focusing on cloning and sequencing the genes coding for specific insect aminopeptidase-N  $\delta$ -endotoxin receptors (Knight *et al.*, 1995; Denolf *et al.*, 1997; Hua *et al.*, 1998; Garner *et al.*, 1999; Yaoi *et al.*, 1999; Emmerling *et al.*, 2001). These aminopeptidases play an important part in insect digestion of proteins, cleaving single amino acids from the N-terminus end (Ortega *et al.*, 1996). The sequencing information now available has allowed for the conclusion that the insect aminopeptidase-N's are a unique, distinct, group among the aminopeptidases (differing from the bacterial and mammalian aminopeptidases) and, among themselves, are quite diverse, falling into at least three distinct groups of midgut aminopeptidases (Gardner *et al.*, 1999; Emmerling *et al.*, 2001).

35. Carbohydrates may be involved in aminopeptidase-N binding, resulting in further specificity of  $\delta$ -endotoxins binding to insect midgut cells. A Cry1Ac domain III mutant  $\delta$ -endotoxin was developed which caused reduced binding to *Manduca sexta*. This mutant Cry1Ac  $\delta$ -endotoxin was not inhibited by the carbohydrate, N-acetylgalactosamine, which did inhibit binding of the wild-type Cry1Ac, indicating that this carbohydrate in the aminopeptidase-N from *Manduca sexta* is involved in the mechanism of toxin binding (Burton *et al.*, 1999). This is not always the case because a 100 kDa aminopeptidase-N from *Heliothis virescens*, which was bound by Cry1Ac and Cry1Fa, did not contain the N-acetylgalactosamine carbohydrate (Banks *et al.*, 2001). Further carbohydrate involvement with binding was shown in a system using plant-pathogenic nematodes that were susceptible to Cry5B and Cry14A (Griffitts *et al.*, 2001). Nematodes with mutation-inactivated  $\beta$ -1,3 galactosyltransferase genes were resistant to the  $\delta$ -endotoxins. The enzyme galactosyltransferase catalyzes the transfer of galactose to glycoproteins and glycolipids. Further research strongly suggests that the binding receptor for Cry5B in susceptible nematodes is a carbohydrate (Huffman *et al.*, 2004). Recent studies suggest that nematocidal and insecticidal three-domain Bt toxins use invertebrate glycolipids as host cell receptors (Griffitts *et al.*, 2005).

36. Another class of  $\delta$ -endotoxin receptors has been shown to be related to the superfamily of cadherin proteins. Cadherins are calcium-dependent proteins that are generally known for their cell to cell adhesion properties. A cadherin-like 175 kDa glycoprotein (BtR175) from *Bombyx mori* was shown to be a receptor for Cry1Aa (Nagamatsu *et al.*, 1998). Addition of the gene for BtR175 to Cry1Aa-resistant *Spodoptera fugiperda* Sf9 cells *in vitro* rendered them susceptible to the toxin (Nagamatsu *et al.*, 1999). Another cadherin-like 210 kDa glycoprotein was found in *Manduca sexta* that binds to the Cry1Ab  $\delta$ -endotoxin (Vadlamudi *et al.*, 1995; Francis and Bulla, 1997). In addition, *Heliothis virescens* was shown to have a cadherin-like receptor protein for Cry1Ac (Gahan *et al.*, 2001).

37. The cadherin superfamily consists of at least six subfamilies. The invertebrate cadherins occupy an isolated position in the superfamily (Nollet *et al.*, 2000). The uniqueness of these insect binding proteins gives further insight into the observed lack of mammalian toxicity for these Cry toxins.

38. Knowledge of the receptor binding process of the Cyt  $\delta$ -endotoxins toxins is not as extensive as with the Cry toxins. Based on *in vitro* experiments with artificial membranes, it was originally thought that the cytolytic toxins, which are capable of lysing a wide range of invertebrate and vertebrate cells including mammalian erythrocytes (Hofte and Whiteley, 1989), inserted directly into the insect midgut membrane without binding to a specific receptor. However, more recent data on mosquitoes has suggested that the Cyt toxins, particularly the toxin Cyt1A, bind to a specific region in the midgut (Ravoahangimalala *et al.*, 1993; Ravoahangimalala and Charles, 1995). The binding process appears to be more closely associated

with the membrane disruption process than for the Cry toxins (Li *et al.*, 1996; Luo *et al.*, 1997; Du *et al.*, 1999).

### C. Pore Formation and Bacterial Septicemia

39. Both binding and pore formation are necessary for optimum activity against insects. It has been shown that binding of the toxin alone is not enough to cause toxicity. Two proteins in the gut membranes of *Tenebrio molitor* larvae (137 and 107 kDa, respectively) were shown to bind Cry1Aa, however the insect is resistant to that toxin (Nagamatsu *et al.*, 1998). Escriche *et al.* (1998) showed that Cry1Ab would bind to *Spodoptera littoralis* midgut receptors, but would not produce pores and *in vivo* assays show that Cry1Ab is only marginally active against *S. littoralis*. Following the binding of active  $\delta$ -endotoxins to specific receptors on the brush border membrane in the insect midgut, the toxins insert into the membrane. The toxins, both Cry and Cyt, intercalate irreversibly into the membrane.

40. After insertion of the Cry  $\delta$ -endotoxin, several receptor-toxin complexes then form aggregates that form pores in the membrane (Walters *et al.*, 1993; Knowles, 1994; Soberon *et al.*, 2000). The pores formed in the plasma membrane disrupt the osmotic balance within the cells which causes the cells to swell and burst. At this point, the insects stop feeding. Domain I in Cry proteins was shown to be a pore forming domain (Walters *et al.*, 1993; VonTersch *et al.*, 1994). The left-handed supercoil of domain I, made up of  $\alpha$ -helices, is "clearly equipped for membrane insertion" (Li, 1996). Furthermore, the  $\alpha$ -helices of this domain, while sharing no amino acid similarity, resemble domains in diphtheria toxin and colicin A that also form pores in membranes. Research by Schwartz *et al.* (1997) suggests an interaction with domain III may also have some effect on the pore formation in membranes. Investigations are proceeding on even more specific details, *e.g.*, Masson *et al.* (1999) have shown that charged amino acids on one side of  $\alpha$ -helix four in domain I of Cry1Aa are involved with passage of ions through the pore. Non-conservative point mutations of Cry1Ab  $\alpha$ -helix seven resulted in proteins that were not readily degraded while more conservative alterations affected the ion channel activity (Alcantara *et al.*, 2001). Gerber and Shai (2000) showed that the hairpin loop of  $\alpha$ -helix four and  $\alpha$ -helix five inserted into the membrane and lined the channel and that  $\alpha$ -helix five participated in the oligomerisation of Cry1Ac. However, mutations of residues within  $\alpha$ -helix five of Cry1Ab showed that this helix was involved in pore formation and the stability of the toxin but not in oligomer formation (Nunez-Valdez *et al.*, 2001).

41. The Cyt  $\delta$ -endotoxins have a different mechanism of membrane insertion. Instead of forming small pores/channels in the midgut membranes as do the Cry proteins, *in vitro* data suggest that Cyt1A induces permeability via a detergent-like perturbation of the membrane (Butko *et al.*, 1997; Manceva *et al.*, 2005). Structural analysis of Cyt2A showed it is composed of two outer layers of  $\alpha$ -helices around a  $\beta$ -sheet structure, and further studies using mutants with reduced toxicity show that the molecular components in the  $\beta$ -sheet are responsible for both membrane binding and pore formation (membrane disruption) (Li *et al.*, 1996; Luo *et al.*, 1997; Du *et al.*, 1999). The  $\beta$ -strands are long enough to span the hydrophobic region of a membrane (Li, 1996). Studies with the similar Cyt1A indicate that the toxin disrupts the membrane through assembly of several monomers within the membrane mediated by two of the  $\alpha$ -helices (Gazit *et al.*, 1997). An analysis of the structure of Cyt  $\delta$ -endotoxins suggests that the surface helix hairpins first peel away, exposing the beta-strands, which can then disrupt the membrane (Li, *et al.*, 2001). Recent research suggests that a monomer of Cyt2Aa1 binds and inserts into the membrane. Then the monomers that are close to each other bind together into oligomers and form large pores (Promdonkoy and Ellar, 2003).

42. Tests on binding and pore formation in insect midgut membranes *in vitro* have suggested that receptor binding and pore formation are predictive of *in vivo* toxicity to susceptible insects. However, some very active  $\delta$ -endotoxins *in vitro* had less activity *in vivo* suggesting that other mechanisms may contribute to the full toxic activity (Peyronnet *et al.*, 1997). This may be due to differences in toxin solubility or



proteolysis, or experimental limitations. The pH of the insect midgut, which varies among insect species, can affect pore formation as shown with experiments with *Manduca sexta* midgut membranes *in vitro* (Tran *et al.*, 2001; Carroll *et al.*, 1997). Cry1C-induced permeability was much less at high pH than for Cry1Ac which correlates with *in vivo* studies demonstrating this insect is less susceptible to Cry1C than to Cry1Ac. Another possible mechanism resulting in the observed variation in insect susceptibility to  $\delta$ -endotoxins is that many insects have mechanisms to repair some damage caused by cell lysis. Moreover, the amount of repair an insect is capable of is dependent on a number of factors such as genetics of the insect host, host age, dosage and potency of the toxins ingested, and various environmental factors (Bauer, 1995). If repair cannot be accomplished, the target insect dies within 2 - 3 days, usually due to bacterial septicaemia (Broderick *et al.*, 2006). The insect hemolymph provides a rich nutrient source for various invading bacteria. Microbial Bt insecticides have shown a synergistic effect of combining *B. thuringiensis* spores along with the  $\delta$ -endotoxin proteins, presumably leading to more rapid bacterial septicemia, although in some cases, spores do not appear to affect toxicity levels (Liu *et al.*, 1998).

## SECTION V - EXPRESSION OF $\delta$ -ENDOTOXIN GENES IN PLANTS

43. Several papers over the past two decades have examined the introduction and expression of *B. thuringiensis*  $\delta$ -endotoxin genes in various plants, e.g. Mendelsohn *et al.* (2003). The first regulatory review of a plant pesticide product field release by EPA was in 1986 of a transgenic tobacco plant producing a  $\delta$ -endotoxin from a *B. thuringiensis* isolate identified at that time as subsp. *berliner*. There are a number of limitations to obtaining high levels of expression of the prokaryotic  $\delta$ -endotoxin genes in the eukaryotic plant cells basically due to the differences in the transcription and translation systems between eukaryotes and prokaryotes. Differences in transcriptional regulation, mRNA stability, preferential codon usage, and translation efficiency have led to the frequent use of modified *B. thuringiensis cry* genes for insertion in plants (as reviewed by De la Riva and Adang, 1996). Recent work has found that insertion of genes into plant chloroplasts may result in much higher expression levels. One experiment demonstrated that Bt *cry2Aa2* operon in chloroplasts resulted in toxin being expressed at a level of 45.3% of soluble protein in the leaves. They noted formation of insecticidal crystals (De Cosa *et al.*, 2001).

### A. Methods of Gene Insertion

44. A number of recombinant DNA technologies are available for engineering plants for insect resistance through insertion of the *B. thuringiensis*  $\delta$ -endotoxin genes (De la Riva and Adang, 1996). The most commonly used method has been bacterial mediated transformation using the plant pathogenic bacterium, *Agrobacterium tumefaciens* that causes crown gall disease in plants (Gasser and Fraley, 1989; Cheng *et al.*, 2004). Another commonly used plant transformation method consists of direct gene transfer via microprojectile bombardment.

### B. Promoters

45. Initial attempts at producing *B. thuringiensis* transgenic plants were conducted using full-length protoxins or truncated versions of the *cry* genes under the control of constitutive promoters. Although truncated versions of *cry* genes containing the N-terminal fragment proved more successful than the full-length protoxins, expression of the toxin protein was quite low. Currently, a number of different promoters are available to drive expression of Bt genes in both a spatial and temporal manner (Potenza *et al.*, 2004). The most commonly used promoter is the cauliflower mosaic virus 35S promoter (CaMV 35S). This constitutive promoter has better activity in dicotyledonous plants than in monocotyledons, although it was used in commercial maize constructs. Other strong or constitutive promoters for monocotyledons include the rice actin 1 promoter, the synthetic maize *Emu* promoter, and the maize polyubiquitin 1 promoter (De la Riva and Adang, 1996).

### C. Expression Levels in Plants

46. Detailed publications on transgenic plants containing the  $\delta$ -endotoxins from *Bacillus thuringiensis* first appeared in the late 1980's (Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987). Early attempts of engineering the plants containing the full-length protoxin led to very low levels of expression of the  $\delta$ -endotoxins that was inadequate for proper pest control. Truncated  $\delta$ -endotoxin consisting only of the active N-terminal portion rather than the full-length protoxin without further modification are still not efficiently expressed, although higher levels of activity against insect pests are seen (Vaeck *et al.*, 1987; Koziel *et al.*, 1993). Levels of  $\delta$ -endotoxin up to 0.02% of the total leaf-soluble

protein from tobacco and tomato have been reported with the use of truncated toxins (Kumar *et al.*, 1996). Shivakumar *et al.* (1986), as reported by De la Riva and Adang (1996), obtained an expression level of only 0.0001% using the full-length protoxin, but a level of 0.07% of the total leaf protein in tobacco leaves with a recombinant truncated version of *cryIAb*. (*cryIAb*) Generally, maximum levels of the Cry proteins for most U.S. registered plant pesticide products were in the 0.001% (dry weight) range (see Table 2).

47. Expression levels of the  $\delta$ -endotoxins in plants, even with the use of truncated toxins, can be quite variable and are dependent on a large number of factors. Expression levels can be enhanced through modification of the *cry* gene sequences to make them more compatible with plant transcription and translation systems (Perlak *et al.*, 1991). In a review article, De la Riva and Adang (1996) suggest the following modifications of the *cry* gene sequences for increasing expression of the  $\delta$ -endotoxins in transgenic plants: (1) change to preferential codon usage of the plant by eliminating CG and TA dinucleotides at codon positions two and three, and conserving the AT base composition typical within the plant, (2) modify sequences that could lead to mRNA instability or degradation including polyadenylation signals, termination sequences, or splicing sites, (3) reduce regions of mRNA known to form hairpins and other secondary structures of mRNA known to reduce the speed of ribosome translocation, (4) optimise the ATG consensus flanking nucleotides for protein translation (and termination), and (5) introduction of plant viral untranslatable mRNA leader to improve translation initiation.

48. A major limitation to efficient gene expression in plants results from organisational differences between prokaryotic and eukaryotic genes. For example, coding regions of eukaryotic genes are separated by non-coding regions known as introns, which are generally not present in prokaryotes. In addition, the *B. thuringiensis cry* genes have been shown to be very AT- rich compared to plant coding genes. AT- rich regions in plants are often contained in the non-coding introns, or have a regulatory role in polyadenylation. The AT- rich regions in the *cry* genes may contribute to RNA instability in the plant either through acting as polyadenylation signals or as termination sequences for RNA-polymerase. In addition, poly ATTTA sequences in the *cry* genes can serve as mRNA degradation signals. Other AT sequences can signal incorrect mRNA splicing (De la Riva and Adang, 1996).

49. Besides the overall greater percentage of A and T bases in the  $\delta$ -endotoxin prokaryotic genes, there is also a difference in codon usage preference between prokaryotes and eukaryotes that leads to inefficient expression of the  $\delta$ -endotoxin genes in plants. Whereas the *B. thuringiensis cry* genes often have A or T as the third base of the codon triplets, plants tend to have G or C. Due to the lower number of AU recognizing tRNAs in plants, the speed of translation and synthesis of the  $\delta$ -endotoxin proteins is reduced if the high AT codon of the bacterial gene is inserted in plants (De la Riva and Adang, 1996).

50. In addition, there may be tissue and temporal variation in expression. For example, variable expression of Cry1Ac was found between eight cotton hybrids tested in India and the expression also declined consistently as the plant aged, raising concerns for efficacy (Kranthi *et al.*, 2005). Other experiments showed a strong decline, occurring as the plant ages, in Cry1Ac expression in cotton (Greenplate, 1999) and Cry1Ab expression in maize (Dutton *et al.*, 2004).

#### **D. Variable Expression in Plant Parts**

51. Current techniques in genetic engineering may allow specific control of the  $\delta$ -endotoxins expression in different parts of the plant. Most of the transgenic products developed to date, however, exhibit a wide range of expression in the various plant parts assessed (Table 2). Strong constitutive promoters such as CaMV 35S generally result in production of the gene product under their control (*e.g.*  $\delta$ -endotoxins) in all the tissues of the plant. However, the CaMV 35S promoter does not seem to express well in pollen as evidenced by low expression seen in maize pollen (Kozeil *et al.*, 1993) and relatively low expression in cotton pollen (Greenplate, 1997). Other constitutive promoters such as the CaMV 4AS1

promoter used in MON 863-maize (Cry3b), express well in pollen. Other transgenic plants have restricted expression of the  $\delta$ -endotoxin to specific plant tissues through the use of tissue-specific promoters. For example, in maize, a promoter derived from the phosphoenolpyruvate carboxylase gene was used to promote expression of *cryIAb* (*cryIAb*) in green tissue (Hudspeth and Grula, 1989). Tissue-specific transgenic tobacco has been developed by insertion of the  $\delta$ -endotoxin in the chloroplasts resulting in high levels of expression (McBride *et al.*, 1995). In addition, a promoter derived from a maize calcium-dependent protein kinase gene was used to get expression of the  $\delta$ -endotoxin only in pollen (Estruch *et al.*, 1994). These selective-expression technologies may turn out to be particularly useful to direct exposure toward the target insect and/or to restrict exposure to non-target insects.

**Table 1. An example of variation in expression levels of  $\delta$ -endotoxin in different maize constructs expressing five different  $\delta$ -endotoxins**

Cry Protein Tissue Expression\*

Active Ingredient/ OECD Unique ID	Leaf	Root	Pollen	Seed	Whole Plant
Cry1Ab SYN-BT11-1	3.3 ng/mg	2.2-37.0 ng/mg protein	< 90 ng Cry1Ab/ g dry wt. pollen	1.4 ng/mg (kernel)	–
Cry1Ab MON-00810-6	10.34 ng/mg	–	< 90 ng Cry1Ab/ g dry wt. pollen	0.19-0.39 ng/mg (grain)	4.65 ng/mg
Cry1F DAS-01507-1	56.6 - 148.9 ng/mg total protein	–	113.4 - 168.2 ng/mg total protein or 31 to 33 ng / mg pollen	71.2 - 114.8 ng/mg total protein	803.2 - 1572.7 ng/mg total protein
Cry3Bb1 MON-00863-5	30-93 ng/mg	3.2-66 ng/mg	30-93 ng/mg	–	13-54 ng/mg
Cry34Ab1 DAS-59122-7	5 – 302 ng/mg dry weight	24 – 102 ng/mg dry weight	63 – 88 ng/mg dry weight	29 – 85 ng/mg dry weight	9 – 88 ng/mg dry weight
Cry35Ab1 DAS-59122-7	2 – 113 ng/mg dry weight	1 – 16 ng/mg dry weight	0 – 0.2 ng/mg dry weight	1 – 2 ng/mg dry weight	1 – 16 ng/mg dry weight

\*Information in table was provided directly by companies at time of data submission in the absence of specific format requirements, resulting in the differences seen among rows. Data are provided only to show variance among tissues within plants (*i.e.*, in rows).

## SECTION VI - RISK ASSESSMENT OF $\delta$ -ENDOTOXINS IN PLANTS

### A. General Issues

52. The use of *Bacillus thuringiensis*  $\delta$ -endotoxins in transgenic plants poses some of the same kinds of risk concerns as the use of microbial Bt preparations containing the same  $\delta$ -endotoxins in terms of understanding the potential toxicity to non-target organisms. The US registered the first microorganism (*Bacillus popilliae*) for pesticidal use in 1948 and the first *Bacillus thuringiensis* microbial pesticide was registered in 1961. The regulatory system that evolved in the years since then was based on the assessment system for conventional chemical pesticides. The risk assessment framework that has been used for the Bt plants has been influenced by experiences with microbial and chemical pesticides, as well as the extensive experience in evaluating transgenic crops (Mendelsohn *et al.*, 2003; Romeis *et al.*, 2006a; Romeis *et al.*, 2006b). As in chemical risk assessment (described in Commission Regulation (EC) 1488/94), the assessment of Bt plants must consider both their potential for producing hazardous effects and the exposure to susceptible organisms resulting from the dissemination and persistence of the toxin.

53. The literature concerning potential hazards that might be caused by the microbial forms of *B. thuringiensis* is not necessarily relevant to predicting hazards from plant-produced  $\delta$ -endotoxins in plants because the microbial strains can produce other toxins. However, the registered microbial pesticidal strains of *Bacillus thuringiensis* are virtually non-toxic to mammals, and generally show low toxicity to non-target terrestrial and aquatic species (USEPA, 1998). In those instances in which toxicity to non-targets unrelated to the target species, *i.e.*, daphnia species, has been demonstrated, the toxicity has been attributed to other toxins produced by the micro-organism rather than to the  $\delta$ -endotoxins (USEPA, 1998). Furthermore, the  $\delta$ -endotoxins from the microbial spore/crystal preparations have historically appeared to be rapidly degraded in the environment. Therefore, the negligible toxicity to non-target organisms and the low persistence allowed for a conclusion of negligible risk for the registered *B. thuringiensis* microbial pesticides (USEPA, 1998). More recent studies on persistence of  $\delta$ -endotoxins from both plants and microorganisms indicate that they may bind to some substances in the soil, thus increasing the duration of their presence in the soil (Saxena and Stotzky, 2000; Stotzky, 2000; Crecchio and Stotzky, 2001; Saxena *et al.*, 2002a, 2002b), but no adverse effects have been observed in this increase in exposure. Engineering of the  $\delta$ -endotoxins into plants reduces one aspect of the risk over that of the naturally-occurring microbial forms of *B. thuringiensis* because it eliminates the potentially toxic exotoxins that are produced by some strains of *B. thuringiensis*. At the same time the use of engineered Bt crops may present a longer and higher level of exposure of  $\delta$ -endotoxins in a truncated active form as compared to the application of conventional Bt preparations. The current information available from studies on long-term cultivation of Bt toxin-expressing plants on residual toxin levels in soil indicate they are not present at detectable levels and do not appear to build up over time (Sanvido *et al.*, 2006).

54. The scientific literature has some examples of tests for effects on non-target organisms including humans, but these tests were conducted with microbial preparations that may have contained multiple toxins. In recent years there have been many publications describing the effects of isolated *B. thuringiensis*  $\delta$ -endotoxins on both target and non-target insects (see the *Bacillus thuringiensis* toxin specificity database, van Frankenhuyze and Nystrom, 1999). These proteins have also been extensively studied *in planta* (Bhatti *et al.*, 2005a, 2005b; Bitzer *et al.*, 2006; Daly and Buntin, 2005; Dively, 2005; Head *et al.*, 2005; Lopez *et al.*, 2005; Naranjo, 2005a, 2005b; Naranjo *et al.*, 2005; Pilcher *et al.*, 2005; Prasifka *et al.*, 2005; Torres and Ruberson, 2005; Whitehouse *et al.*, 2005).

55. There are also numerous studies that are submitted by private companies or testing laboratories to support regulatory decisions for engineered products (Mendelsohn *et al.*, 2003; Romeis *et al.*, 2006b). While this information is less easily obtained than public literature, it is still useful to discuss in the current context. It should also be stressed that any information provided by companies to facilitate regulatory decisions must meet rigorous data quality standards. These standards are referred to as Good Laboratory Practices (GLP) and are codified in national regulations (US Code of Federal Regulations, 40 CFR 160; EU Directives 87/18/EEC and 88/320/EEC) and described by international organisations such as the OECD (GLP, 2006).

56. Annex I contains brief summaries of studies related to toxicity received by USEPA in support of Cry1Ab, Cry1Ac, Cry3A, Cry9C, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1, and Cry35Ab1  $\delta$ -endotoxins as used in plant products. The tables in the Annex are presented as examples of the kinds of specific data that are reviewed to support regulatory decisions and do not contain the detailed information available from the actual decision documents. Data on other  $\delta$ -endotoxins are periodically received and their reviews will be available to the public by means of the various national and international databases. Many, if not most of these studies in the tables, have also been submitted to other countries for evaluation and have been used in international review of these products. The opinions based (in part) on these studies by the EU scientific committees can be found on the internet at [http://www.efsa.eu.int/science/gmo/gmo\\_opinions/catindex\\_en.html](http://www.efsa.eu.int/science/gmo/gmo_opinions/catindex_en.html) and the Scientific Committee on Plants at [http://europa.eu.int/comm/food/fs/sc/scp/outcome\\_gmo\\_en.html](http://europa.eu.int/comm/food/fs/sc/scp/outcome_gmo_en.html), and [http://europa.eu.int/comm/food/fs/sc/scp/outcome\\_en.html#opinions](http://europa.eu.int/comm/food/fs/sc/scp/outcome_en.html#opinions). At least one country (the UK) has a web site, <http://www.defra.gov.uk/environment/gm/regulation/index.htm>, listing scientific regulatory opinions that have used some or all of these studies. In addition, the United States Regulatory Agencies Unified Biotechnology Website lists decisions about reviewed crop plants (<http://usbiotechreg.nbii.gov/>) and the International Biosafety Clearinghouse (<http://bch.biodiv.org/decisions/default.shtml>) keeps a database that gives access to decisions on transgenic plants in many countries.

57. The exposure to the  $\delta$ -endotoxin relates to the kind of plant containing the  $\delta$ -endotoxin and the potential for transfer of the  $\delta$ -endotoxin to other plants. Various  $\delta$ -endotoxins have been field tested in many crops including maize, potatoes, cotton, soybeans, peanuts, alfalfa, broccoli, cranberries, eggplants, rapeseed, rice, tomatoes, tobacco, and walnut, spruce, apple, and poplar trees (OECD BioTrack Database of Field Trials: <http://webdomino1.oecd.org/ehs/biotrack.nsf>; Biosafety Clearinghouse: <http://bch.biodiv.org/decisions/default.shtml>). Commercial uses include  $\delta$ -endotoxins in potatoes, maize, and cotton (<http://www.epa.gov/pesticides/biopesticides>).

58. Risk considerations for Bt crops are based on the same risk assessment process as for other transgenic crops. Potential adverse changes in the plant are considered in the context of increased weed potential and adverse environmental effects. The potential for increased weediness in the transgenic plant should be evaluated as well as the potential for the transgene to increase weediness of wild relatives by cross breeding between the transgenic plant and wild relatives. Outcrossing of  $\delta$ -endotoxin genes to wild relatives may also produce unintended exposure to susceptible insects that are not pests. In this case, as well as in the case of the cultivation of Bt plants in general the potential exposure to endangered species must also be considered. In addition, a continuous exposure to *B. thuringiensis*  $\delta$ -endotoxins presents the possibility for sublethal effects on insects and/or development of insects that are no longer susceptible to the lethal effects of the  $\delta$ -endotoxins.

## B. Human Health Assessment

### 1. Acute Toxicity

59. Throughout several decades of use of commercial microbial *B. thuringiensis* products, mammalian toxicity has been evaluated. The toxicological database on *B. thuringiensis* shows no mammalian health effects attributable to  $\delta$ -endotoxins. A review of numerous infectivity and pathogenicity studies indicates a pattern of clearance of the *B. thuringiensis* organisms from rodents after oral, pulmonary, or intravenous challenge (McClintock *et al.*, 1995a, 1995b). No significant adverse health effects attributable to the test microbe have been reported in these studies in either body weight gain or mortality by clinical observations, or through examination of the test animal's internal organs at necropsy. To support the Bt plant risk assessments, acute toxicity testing has been performed on rodents using Cry1Ab, Cry1Ac, Cry9C, Cry3A, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1, and Cry35Ab1 (DEKALB, 1997; Monsanto and Novartis, 1996a; Monsanto, 1995a, 1995b; 2001a, 2001b, 2001c; Mycogen and Novartis, 1995c, 1995d; Plant Genetic Systems, 1998c; Mycogen and Pioneer, 2001d, 2001e; 2005b). For all these studies, since maximum levels of toxin were needed for a feeding assay, the toxin was produced in an engineered microbial culture since sufficient amounts of pure toxin could not be extracted from the plants to supply a standard limit dose. This approach required an analysis of the microbially-produced toxin to show that the toxin was sufficiently similar to that produced in the plant using a range of techniques including SDS-PAGE, Western blot, ELISA, N-terminal amino acid sequencing, Glycosylation, and Bioactivity (host range). In all cases, these  $\delta$ -endotoxins showed no adverse effects at high doses in the range of 3760 to 5220 mg/kg in mice via an oral route of exposure. The European Food Safety Authority (EFSA) evaluated the food safety of  $\delta$ -endotoxin expressed in maize plants such as Cry1Ab in Bt11 (EFSA, 2005a), Cry1F in maize 1507 (EFSA, 2005b), Cry3Bb1 in MON 863 (EFSA, 2004), and hybrids derived from Cry1Ab and Cry3Bb1 in MON 810xMON 863 (EFSA, 2005c). Short term feeding/toxicity studies on poultry, pigs, calves and cattle also provided additional information on the behaviour of Cry1Ab protein in the gastro intestinal tract (Jennings *et al.*, 2003; Chowdhury *et al.*, 2003; Einspanier *et al.*, 2004; Lutz *et al.*, 2005). Cry1Ab was not completely degraded in the gastro-intestinal tract and fragments of the gene and/or immunoreactive protein fragments were still present in the intestinal content and in the faeces, but no residual DNA/protein could be found in animal tissues nor in the peripheral blood, nor was any risk associated with these findings.

60. The mode of action of *B. thuringiensis*  $\delta$ -endotoxins in susceptible insects is well-known and was discussed previously in Section IV of this document. Within the insect midgut, the  $\delta$ -endotoxins bind to unique receptor sites on the cell membrane, causing development of pores, disruption of osmotic balance, and ultimately septicaemia (Gill and Ellar, 2002; Broderick *et al.*, 2006). There are no known equivalent receptor sites in mammalian species which could be affected (Noteborn *et al.*, 1995). Additional factors that support an insect specific mode of action are the reliance of the lepidopteran midgut on unique ATPases for potassium influx regulation and insect midgut's unique susceptibility to ionic stress (Knowles, 1994), plus the observations that even when Cry toxin binding site proteins are expressed in mammalian cells, the mammalian cells are unable to express the proteins in a form that allows the toxins to bind to the cells (Keeton and Bulla, 1997). The more acidic environment of the mammalian gut also leads to degradation of Cry proteins.

61. Several recent studies have found some toxicity from several strains of microbial *B. thuringiensis* in immunocompromised mice and severely-stressed mice infected with influenza virus. However, the authors attribute the effects to *Bacillus* toxins other than the  $\delta$ -endotoxins (Hernandez *et al.*, 1998; Hernandez *et al.*, 1999; Hernandez *et al.*, 2000). This is further supported by the close mapping of the strain that was associated with a human tissue infection to strains of *Bacillus anthracis* indicating that this particular bacillus, in addition to having a plasmid that expressed the characteristic  $\delta$ -endotoxin crystal, also expressed a mammalian toxin similar to the very potent tripartite *B. anthracis* toxin (Hill *et al.*, 2004).

Another *B. thuringiensis*-like strain lacking  $\delta$ -endotoxins showed mammalian toxicity that appeared to be due to haemolytic toxins (Salamitou *et al.*, 2000).

## 2. Food Allergenicity

62. In the absence of a suitable animal model to predict food allergenicity, a screening model was recommended by a conference entitled “Scientific Issues Related to Potential Allergenicity in Transgenic Food Crops” (FDA Docket 94 N-0053, document TR-1) held April 18-19, 1994, in Annapolis, Maryland. The participants, who were expert food allergy researchers, recommended that new proteins be evaluated by determining their similarity to characteristics of known food allergens. Specifically, the questions to be considered were, does it have a similar amino acid sequence, is it resistant to enzymatic and acid degradation, is it heat stable, is it found in high amounts in edible plant parts, and is it of the appropriate molecular size? Of these criteria, the  $\delta$ -endotoxins tested to date do not share similar amino acid sequences with known proteinaceous food allergens. Cry1Ac, Cry1Ab, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1, and Cry35Ab1 were shown to be heat labile (Monsanto, 1995b; 2001a, 2001b; 2002c; Monsanto and Novartis, 1996b; Mycogen and Pioneer, 2001f, 2001g; 2005c, 2005d, 2005e, 2005f). The resistance to enzymatic and acid degradation of each of the  $\delta$ -endotoxins in commercial products has been analysed with a protein digestibility study on the pure gene product (DEKALB, 1997; Herman *et al.*, 2003; Monsanto and Novartis, 1996b; Monsanto, 1995a, 1995b; 2001a, 2001b; 2002a, 2002c, 2002d, 2002e; Mycogen and Novartis, 1995c; Mycogen and Pioneer, 2001h; 2005b; Plant Genetic Systems, 1998c). These studies are performed using simulated gastric (acid and pepsin) and intestinal fluids (trypsin at neutral pH) as described in the United States Pharmacopeia (USP, 1995). The degradation process may be tracked by disappearance of a band on SDS-PAGE or assayed using susceptible insects. The active form of the  $\delta$ -endotoxin, of course, is resistant to trypsin digestion, but of the  $\delta$ -endotoxins commercialised to date, all (Cry1Ac, Cry1Ab, Cry1F, Cry3A, Cry2Ab2, Cry3Bb1, Cry34Ab1, and Cry35Ab1), except Cry9C, have been readily inactivated by the digestibility studies. The Cry9C  $\delta$ -endotoxin passed part of the allergenicity screen because it shows no homology to known toxins or allergens, however it was resistant to degradation by proteases, pepsin at pH 2.0 and to heat at 90°C for 10 minutes (Plant Genetic Systems, 1998c). As a result, Cry9C products were removed from the market, but no instances of human allergenicity have been found although some Cry9C had entered the food supply (see paragraph 67, below). The European Food Safety Authority (EFSA) evaluated the potential allergenicity risk of  $\delta$ -endotoxin expressed in maize plants such as Cry1Ab in Bt11 (EFSA, 2005a), Cry1F in maize 1507 (EFSA, 2005b), Cry3Bb1 in MON 863 (EFSA, 2004), and hybrids derived from Cry1Ab and Cry3Bb1 in MON 810xMON 863 (EFSA, 2005c). The allergy risk evaluation of Cry proteins has been completed using different approaches, which led to indirect evidence for an allergenicity risk being very low. This included the absence of known allergenicity of the source, absence of significant sequence homology with known allergens and rapid and extensive degradation by pepsin. To date, despite extensive scientific scrutiny no methodology has been found to conclusively assess the potential for dietary allergenicity if a substance does not pass the screening tests.

63. Bernstein *et al.* (1999) is sometimes cited as support for the potential for  $\delta$ -endotoxins to be food allergens. However, this research was designed to examine the potential for farm workers to develop reactions and/or antibodies to microbial forms of *B. thuringiensis* products following inhalation exposure, not dietary exposure to  $\delta$ -endotoxins. Furthermore, the authors did not find a significant reaction to a preparation of  $\delta$ -endotoxins in the group of workers that exhibited an immune response to whole microbial *B. thuringiensis* extracts (as shown by skin prick testing, no allergy or clinical symptoms were ever seen). The protoxin preparation for this test was derived from the commercial strain of Javelin to which the workers were exposed. The authors concluded that “... it is unlikely that consumers would develop allergic sensitivity after oral exposure to transgenic foods (e.g. tomatoes, potatoes) that currently contain the gene encoding this protein.” A similar conclusion can be drawn from Siegel (2001).



### **3. Human Exposure**

64. The primary significant human exposure to  $\delta$ -endotoxin in plants is by the oral route for food crops. Exposure to the aerosols produced during the processing of material (*e.g.* seed) of Bt plants is an additional, although small, route of human exposure. Many countries require pesticide residue studies to determine the maximum levels of chemical pesticides in or on raw agricultural commodities. Due to the lack of mammalian toxicity for the  $\delta$ -endotoxins tested at very high doses, these traditional pesticide residue studies are not necessary. Microbial *B. thuringiensis* pesticides that are registered in countries that require tolerances (a.k.a Maximum Residue Levels) have been given an exemption from the requirement for setting a numerical tolerance (MRL). However, an analysis of  $\delta$ -endotoxin expression levels in various parts of the plant is useful for analysis of non-target organism effects as well as issues related to insect resistance management. These data show that the transgenic plant pesticides in current commercial use have relatively low levels of  $\delta$ -endotoxins in edible plant parts.

### **4. Human Risk Assessment**

65. The acute oral toxicity data on Cry1Ab, Cry1Ac, Cry9C, Cry3A, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1, and Cry35Ab1 supports the prediction that the Cry proteins would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose level (Sjoblad *et al.*, 1992). Therefore, since no effects were seen in the acute tests, even at relatively high dose levels, these  $\delta$ -endotoxin proteins are not considered toxic to humans. Both the long history of safe use of *B. thuringiensis* and the acute oral toxicity data allow for a conclusion that these and other  $\delta$ -endotoxins pose negligible toxicity risk to humans. The one aspect of human health concern identified in their assessments was the potential for the Cry9C protein to be a food allergen. Cry9C was conditionally registered in the U.S. for animal feed uses only, with restrictions on cultivation to provide containment. However some unintentional mixing occurred probably either in the field through pollination or after harvest at grain handling facilities and resulted in low levels of the toxin appearing in a few processed maize products. The registration was subsequently withdrawn at the company's request. Studies by the U.S. Food and Drug Administration and the Centers for Disease Control and Prevention did not reveal any cases of human allergenicity attributable to exposure to Cry9C. One individual who showed possible allergenicity to the Cry9C protein by self-administered oral doses and one skin test volunteered for a fully controlled, double-blind, test in a medical centre which proved that he was not allergic to Cry9C protein (Sutton *et al.*, 2003). The overall safety record for Bt has been established in laboratory and field studies, which have looked at both formulated Bt sprays and specific Bt genes *in planta* (Betz *et al.*, 2000; Siegel, 2001; Federici, 2002).

## **C. Non-target Species**

### **1. Effects on Non-target Organisms**

66. Effects studies for non-target organisms are designed to determine the actual hazard to a test species, usually using high doses to ensure a margin of safety and certainty and to give a maximum hazard result (Rose, 2006; Romeis *et al.*, 2006a). Exposure and assessment studies (including field studies that incorporate both exposure and effects in the same study) will be found in the subsequent non-target species sections of this document. A substantial number of lab-based effects studies have been submitted in support of commercial products. Tests include acute, sub-acute, and reproductive dietary tests for  $\delta$ -endotoxins in plants on non-target species, preferably those with a history of survival under laboratory conditions. The test substance was the  $\delta$ -endotoxin expressed in the particular plant tissue expected to be involved in the non-target exposure, or, if it could not be incorporated as such in the diet of the test organism, it was the pure  $\delta$ -endotoxin. In the US non-target species were generally an avian species (bobwhite quail), a rodent (mouse and/or rat) and a wide range of unrelated non-target insects (honeybees and predacious beneficial insects such as parasitic wasps, ladybird beetles, and green lacewings) selected

as representative species that fill some functional or surrogate role and have been demonstrated to survive under laboratory conditions. Laboratory tests on other non-target insects are developed as needed. There have also been numerous six week feeding studies done with broiler chickens. Effects on non-target mammals can partly be assessed by using the acute dietary studies that were performed for human health effects analysis. Aquatic species (fish, *e.g.* rainbow trout, and aquatic invertebrates, *e.g.* daphnia) testing may be useful if they are likely to be exposed, but often, there may be no significant aquatic exposure from substances produced in transgenic plants with the exception of transgenic Bt rice. Studies have also been performed on soil organisms, *e.g.* collembola, which are involved with detritus degradation, and earthworms. The number of soil organisms tested however is limited.

#### ***a. Effects on Non-target Mammals***

67. Data available on laboratory rodents on microbial forms of *B. thuringiensis* do not indicate that there are adverse effects of *B. thuringiensis* preparations on the test animals (*e.g.* USEPA, 1998). Tests with cattle or swine, representing mammals with different digestive systems, are rare and not focused on long-term effects. However, due to the previous mentioned specific mode of action of  $\delta$ -endotoxins, effects on non-target mammals can be considered quite unlikely. This conclusion is supported by the lack of effects observed for purified  $\delta$ -endotoxins tested on rodents in support of commercial use of transgenic plants (Annex I). Furthermore, as previously mentioned, there are no known equivalent receptor sites for binding of the  $\delta$ -endotoxins in mammals (Noteborn *et al.*, 1995; Gill and Ellara, 2002; Broderick *et al.* 2006). The mode of action also appears to be insect specific due to the reliance of the lepidopteran midgut on unique ATPases for potassium influx regulation and the insect midgut's unique susceptibility to ionic stress (Knowles, 1994), plus the observations that even when Cry toxin binding site proteins are expressed in mammalian cells, the mammalian cells are unable to express them in a form that allows the toxins to bind to them (Keeton and Bulla, 1997; Gill and Ellar, 2002; Broderick *et al.*, 2006).

#### ***b. Effects on Avian Species***

68. Acute and subchronic testing of northern bobwhite quail and mallard duck has demonstrated that microbial products using *B. thuringiensis* are not toxic or pathogenic (*e.g.* USEPA, 1998). Acute avian oral studies on the actual  $\delta$ -endotoxins have been submitted in support of commercial use. No effects have been seen from a dietary exposure to bobwhite quail for crops containing Cry1Ab, Cry1Ac, Cry3A, Cry9C, Cry1F, Cry2Ab2, and Cry3Bb1 (DEKALB, 1997; Monsanto and Novartis, 1996c; Monsanto, 1995a, 1995b; 2001c; 2002a; Mycogen and Novartis, 1995c; Plant Genetic Systems, 1998c; Mycogen and Pioneer, 2001e). Additionally, no effects have been observed from a dietary exposure to poultry for maize expressing the binary toxin Cry34Ab1 and Cry35Ab1 (Mycogen and Pioneer, 2005c).

#### ***c. Effects on Freshwater Fish***

69. Microbial products using *B. thuringiensis* have not demonstrated toxicity or pathogenicity to bluegill sunfish or rainbow trout, both freshwater fish. Aqueous LC<sub>50</sub>'s ranged from  $8.7 \times 10^9$  to  $4.6 \times 10^{10}$  cfu/L (USEPA, 1998). Cry1Ab was tested in corn meal as 100% of the diet in a catfish assay with no effects seen at the maximum dose tested (>200 ppm) (Monsanto and Novartis, 1996d). Cry2Ab2 and Cry3Bb1 were tested in catfish at dietary levels of 20% w/w cottonseed meal and 35% w/w corn meal respectively with no effects seen (Monsanto, 2001c, 2001d; 2002a). Cry34Ab1 and Cry35Ab1 proteins were tested on rainbow trout for eight consecutive days as a standard fish diet containing a mixture of 100 mg/kg of a mixture of the two Bt proteins with no adverse effects (Mycogen and Pioneer, 2005g).

#### ***d. Effects on Freshwater Invertebrates***

70. Toxicity testing of registered microbial products identified as *B. thuringiensis* subspecies *kurstaki* and *israelensis* demonstrated moderate toxicity to the freshwater invertebrate *Daphnia magna*. Reported LC<sub>50</sub>'s were in the range of 5 to 50 ppm. A high level of toxicity was shown by *B. thuringiensis* to daphnia with EC<sub>50</sub>'s in the range of 0.8 to 3 ppm (USEPA, 1998). The toxicity, however, was shown to be unrelated to  $\delta$ -endotoxins, but rather was a result of heat-labile soluble substances in supernatant fluids. The toxicity is also not attributable to the heat-stable  $\beta$ -exotoxin. The expression of well-characterised  $\delta$ -endotoxin proteins alone in plants mitigates concerns about toxicity caused by exotoxins or other metabolites produced by various subspecies of *B. thuringiensis* during fermentation for traditional Bt products. Tests using Cry1Ab (Mycogen and Novartis, 1995c), Cry9C (Plant Genetic systems, 1998c), Cry1F (Mycogen and Pioneer, 2001e), and Cry3Bb1 (Monsanto, 2002a) expressed in pollen showed no effects on daphnia. Although corn pollen is assumed to be too large for ingestion by daphnia, tests have reported daphnids becoming yellow in colour internally when exposed to corn pollen (Monsanto, 2002a). Despite the uncertainty of ingestion, these studies are still useful since the major aquatic exposure from most plants expressing  $\delta$ -endotoxins would be by pollen deposition and these studies serve to rule out these effects on daphnia. In addition, no adverse effect were observed when daphnia were exposed to the Cry34Ab1/Cry35Ab1 proteins at a target concentration of 100 mg protein L<sup>-1</sup> water (Mycogen and Pioneer, 2005g)

#### ***e. Effects on Estuarine and Marine Animals***

71. Toxicity studies of several subspecies of the microbial form of *B. thuringiensis* demonstrated that *B. thuringiensis* subspecies *kurstaki*, *israelensis*, and *aizawai* are not toxic or pathogenic to grass shrimp, sheepshead minnows, or copepods (e.g. USEPA, 1998). Similar tests using  $\delta$ -endotoxins expressed in plants have not been required by regulatory agencies because there would be no significant exposure from the plants that have been assessed to date.

#### ***f. Effects on Earthworms***

72. Studies using the  $\delta$ -endotoxins found in commercial plant products observed no effects for earthworms dosed with Cry1Ab (Monsanto and Novartis, 1996d; Mycogen and Novartis, 1995a), Cry1Ac (DEKALB, 1997), Cry3A (Monsanto, 1995b), Cry9C (Plant Genetic Systems, 1998c), Cry1F (Mycogen and Pioneer, 2001e, 2001c), Cry2Ab2 (Monsanto, 2001c), Cry3Bb1 (Monsanto, 2002a, 2002f), or Cry34Ab1/Cry35Ab1 (Mycogen and Pioneer, 2005h). Adult *Lumbricus terrestris* fed with Bt corn litter (Cry1Ab, Bt11) showed no significant difference to earthworms fed with non-Bt litter during the first 160 days of the experiment in a single worst-case laboratory study. However, after 200 days Bt-fed earthworms had a significant 18% weight loss compared to a weight gain of 4% of the control. There was no difference in mortality between the Bt and the non-Bt treatment (Zwahlen *et al.*, 2003a). The authors concluded that "Further studies are necessary to see whether or not this difference in relative weight was due to the Bt toxin or other factors discussed in the study". Vercesi *et al.* (2006) found no negative impact of Bt maize on important life-history traits of *Aporrectodea caliginosa*, an earthworm species abundant in agricultural soils. Considering all available studies the predominant weight of evidence gives no indication for harmful effects of Bt maize on earthworms. Field studies have been performed and are referenced in the risk section since they incorporate both hazard (effects) and exposure (see paragraph 113).

#### ***g. Effects on Non-target Insects***

73. Because the  $\delta$ -endotoxins are used as an insecticide, extensive testing has been performed on pest insects related to those known to be affected by the toxins. In order to be susceptible, non-target insects must have specific receptor sites to which  $\delta$ -endotoxins can attach and must have the proper midgut pH

and enzymatic conditions so that pores are formed in the midgut membranes. Insects are the primary targets of Bt-toxins, and therefore the main non-target organisms that need to be considered for the risk assessment of well-characterised Cry  $\delta$ -endotoxins and proteins closely related to them. The studies for effects testing in this section (1) will consider only those studies that are designed to report hazard and don't reflect field exposures. Full risk studies, primarily field testing, will be reported in Section 3 Risk Assessment.

74. As expected from the selective mode of action, registered microbial products incorporating *B. thuringiensis* subspecies *kurstaki*, *israelensis*, and *tenebrionis* which contain different Cry and Cyt toxins that affect various lepidopteran and/or Diptera insects, were shown to have little to no toxicity to the non-target indicator species for insects, e.g. neuropterans, hymenopterans, and coleopterans. These same *B. thuringiensis* subspecies were also minimally toxic to honey bees. However, U.S. registered microbial products identified as *B. thuringiensis* subspecies *aizawai* have been shown to be highly toxic to honey bees ( $LE_{50} = 15$  ppm) (e.g. USEPA, 1998). This toxicity was attributed to a heat labile exotoxin, not the  $\delta$ -endotoxins. A table in appendix 4 of Glare and O'Callaghan (2000) has a list of 92 studies of the effects of various strains of *B. thuringiensis* on 24 Families in nine Orders of beneficial insects (predators of insects, and parasitoids). Only about eight of the effects reported could be judged as harmful to the predator, and that activity might well be attributable to toxins other than  $\delta$ -endotoxins. The vast majority of studies reported no adverse effects.

75. Generally, as previously described in this document, the  $\delta$ -endotoxins have a relatively high specificity. Bt toxins for this reason can be assumed to affect fewer non-target organisms than conventional chemical pesticides, where it is assumed that insecticides will kill most non-target insects. A review by Dutton *et al.* (2003) on risk assessment for entomophagous arthropods acknowledges that the comparison to the effects of a conventional chemical pesticide can be used as an argument for not requiring testing on non-target herbivores, but suggests that it would be useful to have information on the effects on some non-target herbivores because of the season-long expression of the  $\delta$ -endotoxins in the plants. Most of the research on the host range of the  $\delta$ -endotoxins has been conducted on potential pest insects in order to see if those toxins can control them. For example, the Cry2A toxins seem to be highly species-specific, exhibiting insecticidal activity toward lepidopteran and some dipteran species only (van Frankenhuyzen and Nystrom, 1999 database, version 24 January 2002). A considerable number of tests have been performed using Cry2Aa toxins on insect species from the Orders Lepidoptera, Diptera, Coleoptera, Orthoptera, Hymenoptera, Homoptera, Neuroptera, Hemiptera, Isoptera, the insect relatives Collembola and the Crustacean order Isopoda (Crickmore *et al.*, 1998).

76. On the other hand, species more closely related to the target pest species may well be affected by Bt toxins. For genetically engineered crops effects often depend on the specific event. Forest spray uses may affect some species of lepidopterans related to the pest species (Miller, 1990; Johnson *et al.*, 1995; Wagner *et al.*, 1996) which is why the US Forest Service does not use *B. thuringiensis* spray products where endangered lepidopterans may be present and some lepidopterans have been reported to be affected in and immediately adjacent to Bt maize fields (Zangerl *et al.*, 2001). Larvae of the butterfly species, *Danaus plexippus*, *Papilio polyxenes* and *Pseudaeschnia maha* were affected negatively when feeding on pollen of the Bt maize event 176 (Cry1Ab) which may be deposited on their host plants if they are growing in close association with the maize plants (Losey *et al.*, 1999; Hansen Jesse and Obrycki, 2000; Hellmich *et al.*, 2001; Wraight *et al.*, 2000; Zangerl *et al.*, 2001; Shirai and Takahashi, 2005). Also, Felke and coworkers showed in laboratory studies that the consumption of pollen of Bt176 maize (Cry1Ab) has adverse effects on the larvae of several European non-target lepidopteran species, although some could be considered pests, e.g. *Plutella xylostella* (diamondback moth), while others such as *Nymphalis io* are protected in certain European regions (Felke and Langenbruch, 2001, 2003; Felke *et al.*, 2002). In general, pollen consumption of Bt176 pollen had a negative effect on survival of larvae, their consumption rate, body weight and development time. The LD50 values were 61 – 80 applied pollen grains of Bt176 maize

for *Nymphalis io*, 19 pollen grains for *Pieris rapae* and 139 pollen grains for *Pieris brassicae*, but the actual LD50 value is lower as the larvae did not consume all of the applied pollen (Felke and Langenbruch, 2001, 2003; Felke *et al.*, 2002).

77. Many of the above tests were conducted with young larvae (often neonate), however, older larval stages are less susceptible to Bt maize pollen consumption (Felke and Langenbruch, 2001, 2003; Hellmich *et al.*, 2001; Felke *et al.*, 2002). The toxicity of Bt maize pollen depends on the specific event. Bt176 expresses a higher concentration of Cry1Ab in pollen than other events such as MON 810 and Bt11 (Sears *et al.*, 2001). In laboratory assays pollen consumption of MON 810 and Bt11 maize had no acute impact on butterfly larvae of *Danaus plexippus*, *Papilio polyxenes*, or *Antheraea pernyi* (Hansen Jesse and Obrycki, 2000, 2002, Wraight *et al.*, 2000, Hellmich *et al.*, 2001, Li *et al.*, 2005), and impact of these events was considered to be non-existent or negligible. However, in a recent publication Dively *et al.* (2005) demonstrated that a prolonged and natural exposure to MON 810 and Bt11 pollen had adverse effects on larvae of the Monarch butterfly, *D. plexippus*. In laboratory and greenhouse experiments (and field studies, see paragraph 111), larvae exposed to Bt maize pollen had a significantly longer development duration and reduced survival. Also, the resulting pupae and adults showed a lower weight (Dively *et al.*, 2004).

78. In addition to pollen feeding, consumption of maize anthers can also have adverse effects on monarch butterfly larvae causing a lower survival, lower consumption rate, reduced body weight and a longer development time (Hellmich *et al.*, 2001; Anderson *et al.*, 2004), but later instars were again less affected (Anderson *et al.*, 2004). Anderson and coworkers suggested the anther effects may also have been caused by avoidance behaviour of larvae (Anderson *et al.*, 2004, 2005) and concluded that there is no risk for the monarch butterfly regarding anther exposure alone due to a low exposure probability in the field (see paragraph 111 in exposure section). However, simultaneous exposure to both Bt anthers and pollen had an additive effect and resulted in a lower survival and consumption rate of monarch larvae (Anderson *et al.*, 2005). Some studies exist on the effects of Bt maize endotoxins other than Cry1Ab on butterfly larvae. Testing the toxicity of Cry1Ac and Cry1F on neonate larvae of the monarch butterfly, Hellmich *et al.* (2001) demonstrated that both Bt toxins were less toxic as compared to Cry1Ab. Mattila *et al.* (2005) tested the effect of pollen consumption from a stacked maize hybrid (Cry1Ab x Cry2Ab2) and of a Cry3Bb1 event on first-instar larvae of the monarch butterfly. Cry3Bb1 had no adverse effects at all, whereas pollen from a Cry1Ab x Cry2Ab2 stack produced both lethal and sublethal effects (Mattila *et al.*, 2005). Additional stressors can act in an additive or synergistic way with Bt toxins, thus enhancing the efficacy of  $\delta$ -endotoxins. For instance, larvae of the European cornborer (Lepidoptera: Crambidae) which had been treated with *B. thuringiensis* formulations and Cry1Ab were less tolerant to protozoan *Nosema* infections than the controls resulting in a higher mortality and stronger negative sublethal effects (*e.g.*, Pierce *et al.*, 2001; Reardon *et al.*, 2004). For a binary toxin, Cry34Ab1/Cry35Ab1, an insecticidal activity spectrum study which tested the proteins on insects from three orders (Lepidoptera, Homoptera and Coleoptera) and four families (Pyralidae, Chrysomelidae, Aphididae and Noctuidae) demonstrated that only larvae of *Diabrotica* spp. were affected by the Cry34Ab1 and Cry35Ab1 proteins (Mycogen and Pioneer, 2005b).

79. Toxicity testing of non-target pest insects to determine host range of  $\delta$ -endotoxins has also been conducted and reviewed (Mendelsohn *et al.*, 2003; Rose, 2006; Romeis *et al.*, 2006a, 2006b). Dankocsik *et al.* (1990), who reported the isolation of the gene for Cry2Ab, also reported toxicity to Lepidopteran species (*Lymantria dispar*, *Heliothis virescens*, *Trichoplusia ni*, *Helicoverpa zea*, *Ostrinia nubilalis*), but, unlike Cry2Aa, not to a Dipteran species (*Aedes aegypti*), even at a very high dosage. Two studies on a very closely related toxin, Cry2Ab1, showed that it was not active towards Diptera (*Aedes aegypti*) and confirmed its activity against Lepidoptera (*Manduca sexta*) (Widner and Whiteley, 1989, 1990). Laboratory experiments showed that Bt potatoes expressing Cry3Aa had no effect on larval development, longevity and fecundity of the aphid *Myzus persicae* (Kalushkov and Nedved, 2005). The performance of the aphid *Rhopalosiphum padi* feeding on Bt maize (Cry1Ab) was studied in the laboratory by Lumbierres

*et al.* (2004). No differences were found on aphid mortality, developmental and pre-reproductive times, fecundity and intrinsic rate of natural increase between the offspring of apterous aphids maintained on Bt or non-Bt maize for several generations. However, the offspring of the first generation of apterous mothers had lower mortality, shorter development and pre-reproductive times, a higher effective fecundity rate and greater intrinsic rate of increase when fed on Bt maize. In contrast the offspring of the first generation of alatae performed better on Bt maize and had a shorter developmental and pre-reproductive time and a higher intrinsic rate of increase on Bt maize (Lumbierres *et al.*, 2004). The authors conclude that given these findings, economic effects on maize crops should not be expected (Lumbierres *et al.*, 2004).

80. In contrast with the above reports, the van Frankenhuyzen and Nystrom (1999) database lists a paper by Ahmad *et al.* (1989) reporting that Cry2Ab from *B. thuringiensis* var. *galleriae* is toxic to *Aedes aegypti*, suggesting that the Cry2Ab toxin in Bollgard II could be toxic to Diptera. In contrast, studies by Widner and Whiteley (1989, 1990) showed no toxicity by Cry2Ab2 to Diptera. The apparently contrasting results regarding dipteran toxicity of Cry2Ab could be related to the testing procedures. Dankocsik *et al.* (1990) dissolved the toxin in water and immersed mosquito larvae in the solution while Ahmad *et al.* (1989) immersed larvae in water containing *Bacillus megaterium* expressing the toxin. It is not clear that either of these exposure pathways is appropriate, and there should be a feed-based test of this toxin for dipteran activity if needed to assess the risk to a dipteran species that might be exposed to plant material expressing this particular toxin.

81. The aphid *Aphis gossypii* showed a shorter reproductive duration and maximum lifespan, lower survival rates and lower potential maximum fecundity on Bt cotton (Cry1A) in the first or second generation (Liu *et al.*, 2005a). However, the aphid population soon overcame the negative effects in the second or third generation, and aphids on Bt cotton had longer reproductive durations in the first generation, higher survival rates in the third generation, and longer potential maximum fecundity. Still, fluctuating asymmetry in three morphological parameters suggests that the stress of cotton on the aphids may have been higher on Bt cotton (Liu *et al.*, 2005a). These studies demonstrate that in evaluating Bt effects on non-target herbivores it is important to apply a crop-specific approach, test for lethal and sublethal parameters within, to test several generations and developmental stages of the focus organisms, and to test exposure to the whole Bt plant.

82. Non-target insect studies have been submitted to support registration of the Bt plants using various  $\delta$ -endotoxins in assays against several species of representative beneficial insects. These species were chosen in some cases because, as common predators or parasites, they were used for integrated pest management or biocontrol. In other cases, the species have a long history of use in evaluating pesticides. Moreover, they were laboratory-adapted and available for testing using standardised and validated protocols that have been used by many professional laboratories for many years (Rose, 2006; Romeis *et al.*, 2006a).

83. Honey bees (*Apis mellifera*) have probably been the most studied non-target insect for the detection of conventional pesticide effects, thus commercial wildlife testing laboratories are very experienced in performing laboratory tests with them, although the new emphasis on detecting effects on honeybee larvae (since  $\delta$ -endotoxins primarily affect larvae of the target insects) has required some new protocols to be developed. No effects on honeybees have been observed in these new studies submitted in support of the Bt plant registrations. No effects were seen for Cry1Ab and Cry1Ac against honey bees (adult and larvae dosed with toxin and toxin-containing pollen) (Monsanto and Novartis, 1996b; Mycogen and Novartis, 1995c; Monsanto, 1995b). Cry9C in maize pollen showed no effect on adult honeybees or ladybird beetles (Plant Genetic Systems, 1998a, 1998c). Cry3A demonstrated no effects in two honeybee larval studies (Monsanto, 1995a, 1995e). Cry1F showed no effect on honey bees during larval development to adults when exposed to both toxin and toxin-containing pollen (Mycogen and Pioneer, 2001a, 2001c, 2001e.). Similar developmental studies on honey bees from larval stage to adult demonstrated no toxicity

from Cry2Ab2 and Cry3Bb1 (Monsanto, 2001c, 2001d; 2002a). The Cry34Ab1 and Cry35Ab1 proteins have no observed adverse effects on honeybee larvae development. No adverse effects were observed on three to five day old larval honeybees when fed with either (i) a single dose of 2 mg of maize pollen expressing the Cry34Ab1 and Cry35Ab1 proteins, (ii) a single dose of 5.6 µg of a mixture of the Cry34Ab1 and the Cry35Ab1 proteins, (iii) a single dose of 3.4 µg of the Cry34Ab1 protein, or (iv) a single dose of 2.8 µg of the Cry35Ab1 protein (Mycogen and Pioneer, 2005i).

84. The difficulties of developing study protocols using new methodologies can be illustrated by a series of studies that have suggested that Cry1A toxins may have a toxic effect on *Chrysoperla carnea* (lacewing) larvae. Hilbeck *et al.* (1998b) conducted bioassays of purified Cry1Ab toxin on *C. carnea* larvae using two different no-choice feeding strategies. Using direct diet incorporation of 100 µg toxin per ml diet, they observed 57% mortality compared to 30% mortality of the diet only control. Using the other feeding strategy, where toxin free eggs are supplied as the first food source, then larvae are placed onto the diet medium with and without the toxin, the results were 29% and 17% respectively. In another study, Hilbeck *et al.* (1998a) reported that *C. carnea* larvae fed on prey that had been fed on Bt maize (Cry1Ab) had increased mortality rates and slightly increased developmental times. Prey species were target lepidopteran *Ostrinia nubilalis* (European Corn Borer) and non-target lepidopteran *Spodoptera littoralis* (Egyptian Cotton Leafworm) larvae. Averaged across these two prey species (the difference between prey species was not significant), 'Bt-prey' fed *C. carnea* larvae exhibited 62% mortality whereas 'non-Bt-prey' fed *C. carnea* larvae exhibited 37% mortality. In their next study, Hilbeck *et al.* (1999) extend their analysis of prey-mediated effects of the Cry1Ab toxin on the lacewing *C. carnea* by including multiple concentrations of Bt in the prey's food and by comparing the effects of the Cry1Ab toxin, protoxin and the Cry2A protoxin using their bioassay system. They report that *C. carnea* fed on *S. littoralis* reared on the highest concentration of Cry1Ab, 100µg/g of diet, had a mortality rate of 78% compared to the control mortality rate of 26%.

85. Review of the studies by Hilbeck *et al.* (1998a; 1998b) by the US National Research Council (NRC, 2000) concluded that the effects reported may be due to differences in feeding strategy and amount of toxin supplied. They recommended that field studies be done to assess the effects of Bt crops on natural predators and cited an example of such a study: a two year, relatively small scale field test, that found no differences in natural enemies on Bt and non-Bt corn (maize) crops (Pilcher *et al.*, 1997). There has been subsequent field testing at larger scale (see part 3 of this subsection on risk to non-target organisms). In the Hilbeck *et al.* work (1998b; 1999) high levels of toxin were used in no-choice feeding situations for both the lacewing itself (Hilbeck *et al.*, 1998b) and the prey species (Hilbeck *et al.*, 1999). However, from the studies it is not possible to differentiate between effects mediated via the ingestion of the toxin itself or effects mediated via a decreased host quality. A translation of the Hilbeck studies into the field is also difficult because behavioural mechanisms such as prey avoidance and alternative prey will need to be considered. A more recent study did not observe direct toxicity of high doses of Cry1Ab on the green lacewing larvae (Romeis *et al.*, 2004). Romeis *et al.* (2004) point out that effects on *C. carnea* due to Cry1Ab may be rather due to diet quality effects than due to direct toxic effects. However, difference in experimental design makes direct comparisons of the results between the two studies open to interpretation.

86. Despite the differences seen in the above studies, their results are valuable in showing the need for research in developing laboratory testing protocols using more representative exposure techniques that better reflect field exposures and involve representative non-target insect species that often are difficult to rear under laboratory conditions. For example, a review by Dutton *et al.* (2003) on risk assessment for entomophagous arthropods recommends an assessment for these predators combining laboratory testing and exposure assessment based on knowledge of their feeding habits, plus field studies, if necessary. Andow and Hilbeck (2004) proposed an integrated ecological whole plant assessment strategy. A USEPA Scientific Advisory Panel (August 7, 2002) concluded that the green lacewing (*Chrysoperla carnea*)

dietary testing was complicated by the difficulty of getting an adequate exposure and of laboratory testing with this species. Therefore, the USEPA is now following the recommendation from its advisory panel (SAP) and asks for dietary testing on the minute pirate bug (*Orius insidiosus*) as a more appropriate test species than the green lacewing. *Orius* spp. typically occur in US maize fields as egg predators and typically feed on pollen.

87. Data for Cry1Ab, Cry1Ac, and Cry3A showed no effect on adult ladybird beetles, green lacewing larvae (direct exposure), and parasitic wasps (Monsanto, 1995a, 1995b; Monsanto and Novartis, 1996a). Cry9C in maize pollen showed no effect on ladybird beetles (Plant Genetic Systems, 1998c). Cry1F fed in toxin form to green lacewing larvae, parasitic wasps, and adult ladybird beetles showed no effects (Mycogen and Pioneer, 2001c, 2001e). When Cry1F was fed to Monarch larvae, no mortality was seen, although there was some growth inhibition seen at the high dose, 30,000 ng/ml diet (Hellmich *et al.*, 2001). Cry2Ab2 and Cry3Bb1 toxin studies showed no effect on adult ladybird beetles and green lacewing larvae (Monsanto, 2001c; 2002a). In addition, no effects were seen in a developmental Cry3Bb1 pollen feed study on ladybird beetles from larvae to adults (Monsanto, 2002d), and two Cry3Bb1 pollen feeding studies on two different species of ladybird beetles (Monsanto, 2002b; Duan *et al.*, 2002). Similarly Cry34Ab1 and Cry35Ab1 proteins did not show toxic effects on green lacewing larvae (Mycogen and Pioneer, 2005g), parasitic wasps (Mycogen and Pioneer, 2005g) or adult ladybird beetles (Mycogen and Pioneer, 2005b). No effects were seen when ladybird larvae were fed a mixture of 50% corn earworm eggs and 50% maize pollen expressing the Cry34Ab1 and Cry35Ab1 proteins (Mycogen and Pioneer, 2005a).

88. Short term laboratory studies showed that four lepidopteran species were sensitive to Cry1Ac, but six species of non-target insects and four species of beneficial insects showed no toxic effects after being fed purified Cry1Ac at concentrations 100 times higher than found in the field in pollen and nectar of transgenic cotton (Sims, 1995). In the laboratory, the majority of beneficial natural enemies tested so far showed no adverse effects due to consumption of Cry1A toxin or of transgenic Cry1A plant material, *e.g.*, *Orius* spp., *Geocoris* spp., *Cyrtorhinus* spp., *Nabis* spp. and *Zelus* spp. (Heteroptera), and *Coleomegilla* spp. and *Propylea* sp. (Coccinellidae) (*e.g.*, Pilcher *et al.*, 1997; Zwahlen *et al.*, 2000; Al-Deeb *et al.*, 2001; Bernal *et al.*, 2002a; Bai *et al.*, 2005). Likewise, no adverse effects were detected for Cry3A and Cry3B toxins for *Orius* sp. and *Lygus* spp. (Heteroptera) and *Coleomegilla* sp. (Coccinellidae) (Riddick and Barbosa, 1998; Armer *et al.*, 2000; Duan *et al.*, 2002; Lundgren and Wiedenmann, 2002; Kalushkov and Nedved, 2005).

89. Romeis *et al.* (2006b) reported no effects on natural enemies fed directly with Bt plant material, but confirmed that tritrophic effects do occur, *i.e.*, predators and parasitoids may be adversely affected when feeding on Bt-fed prey. Romeis *et al.* (2004; 2006b) attribute the tritrophic effect to inferior nutritional quality of the prey. Ponsard *et al.* (2002) examined the effect of Bt cotton and of lepidopteran prey that had ingested Bt cotton on the survivorship of four important heteropteran predators of cotton pests. Longevity significantly decreased by 27-28% for *Orius tristicolor* and *Geocoris punctipes*, whereas no effect was found for *Nabis* sp. and *Zelus renardii* (Ponsard *et al.*, 2002). Consumption of pollen of transgenic Bt rice caused a lower survival in *Propylea japonica* (Coccinellidae) (Bai *et al.*, 2005).

90. Hymenopteran parasitoids often show adverse effects when parasitizing host reared on Bt plants or diets, which is mostly attributed to a reduced quality of the host (cf. Lövei and Arpaia, 2005; Romeis *et al.*, 2006b). A laboratory study on soybean loopers (*Pseudoplusia includens*) parasitised with hymenopteran parasites and raised on Cry1Ac cotton showed retarded development that was attributed to possible sublethal effects on the host (Baur and Boethel, 2003). Likewise, *Microplitis mediator*, an important endoparasitoid of the cotton bollworm in China, suffered from reduced survival and growth inhibition when parasitizing *Helicoverpa armigera* raised on Bt cotton leaf powder (Cry1Ac) (Liu *et al.*, 2005). *Cotesia marginiventris* (Hymenoptera: Braconidae) survival, development times and cocoon weights were significantly negatively affected if their *Spodoptera littoralis* host larva (Lepidoptera:



Noctuidae) had been fed Cry1Ab Bt maize (Vojtech *et al.*, 2005). Prütz and coworkers studied the effect of hosts, *Chilo partellus* (Lepidoptera: Crambidae), raised on Bt corn leaf material (Cry1Ab) on the parasitoid *Cotesia flavipes* (Hymenoptera: Braconidae), and parasitoids on Bt-fed hosts suffered under reduced weight and a lower probability to complete their development (Prütz and Dettner, 2004; Prütz *et al.*, 2004). The adverse effects on the parasitoid *C. flavipes* had a secondary effect on the fourth trophic level. Female hyperparasitoids *Tetrastichus howardi* (Hymenoptera: Eulophidae) parasitizing *C. flavipes* developing in Bt-fed *C. partellus* had lower body weight and offspring (Prütz *et al.*, 2004). Survival and adult size of the parasitoid *Aphidius nigripes* (Hymenoptera: Braconidae) was reduced when developing on non-target aphids fed Bt potato (Cry3A) (Ashouri *et al.*, 2001). Bt maize (Cry9C) fed hosts led to adverse effects regarding development time, longevity and mortality of the parasitoid *Parallorhogas pyralophagus* (Hymenoptera: Braconidae), but did not affect sex ratio, egg load, or adult size (Bernal *et al.*, 2002b). In conclusion, sublethal impacts on target and non-target herbivores can affect parasitoids and may translate into impacts on the degree of biological control provided by parasitoids by altering parasitoid-host population dynamics, and secondary effects can also include secondary pests or pests in subsequent or neighbouring crops (Bernal *et al.*, 2002b). However, sublethal effects also need to be assessed in the context of the role of beneficial insects in the pest-controlled crop and the population dynamics of the respective insects (Mendelsohn *et al.*, 2003; Romeis *et al.*, 2006b).

91. Potential effects to ladybird beetles and aphids have been examined with Bt (Cry3Aa) potatoes. In a study by Dogan *et al.* (1996), aphids fed on potato leaves expressing a gene for *Bacillus thuringiensis* var. *tenebrionis*  $\delta$ -endotoxin (Cry3 toxins) were force-fed to lady beetle larvae and adults (*Hippodamia convergens*). Since lady beetles are in the insect Order Coleoptera and are thus potential targets of Cry3 toxins, this study was aimed at determining whether these beneficial predatory insects would be affected by feeding on Bt transgenic potato-fed aphids. Results showed no aphid prey-mediated effect on lady beetles. The exact mechanisms for the lack of effect observed in this study are not clear, however, it is known that aphids hardly ingest Bt toxins when sucking on Bt plants (*e.g.*, Head *et al.*, 2001; Raps *et al.*, 2001; Dutton *et al.*, 2002); consequently, prey-mediated effects by aphids are unlikely. Riddick and Barbosa (1998) detected no adverse effects mediated by the prey *Leptinotarsa decemlineata* feeding on Bt potato (Cry3A) onto the predatory coccinellid *Coleomegilla maculata*.

92. Two soil arthropods, a collembolan, *Folsomia candida* Willem, and an oribatid mite, *Oppia nitens* Koch, were tested with Cry1Ab and Cry1Ac in cotton and with Cry3A in potato. No adverse effects were seen (L.Yu *et al.*, 1997). Collembola studies have been submitted in support of the registrations for all the commercial constructs, Cry1Ab, Cry1Ac, Cry1F, Cry3A, Cry9C, Cry2Ab2, Cry3Bb1, Cry34Ab1, and Cry35Ab1 (see references). No effects were seen for separate studies using plant-produced and microbial-produced Cry1Ac and Cry9C  $\delta$ -endotoxins (DEKALB, 1997; Novartis and Monsanto, 1996; Plant Genetic Systems, 1998c). There were two apparently contradictory studies that were submitted for registrations of Cry1Ab producing maize products. One study using pure 200 ppm Cry1Ab toxin derived from recombinant *Escherichia coli* had no observable effects on two collembola species (*Folsomia candida* and *Xenylla grisea*) (Novartis and Monsanto, 1996). The other study using lyophilised leaf extract reported mortality to *Folsomia candida* at a level of 125 mg Cry1Ab - maize leaf protein/kg of soil (Mycogen and Novartis, 1995d). It has not been established if the toxicity observed in this study is due to the Cry1Ab  $\delta$ -endotoxin or to some other protein interactions of the leaf extract. A worst-case assessment can be performed using these hazard data as discussed in paragraph 112. A study using 200 ppm Cry3A microbial-produced toxin showed no effect on *Folsomia candida* and *Xenylla grisea* (Novartis and Monsanto, 1996). No effects were seen in a chronic 28 day study of Cry1F, and dietary studies of Cry2Ab2 and Cry3Bb1 (Mycogen and Pioneer, 2001e; Monsanto, 2001c, 2002a). The woodlouse, *Porcellio scaber* (Crustacea: Isopoda), performed better when fed with Cry1Ab maize as compared to the non-transgenic isolate, which was attributed to a better nutritional quality of the Bt corn (Escher *et al.*, 2000). A laboratory study of 16 species of Carabidae ground beetles fed Cry3Bb1 and Cry1Ab in maize pollen found no effects from the Bt toxins (Mullin *et al.*, 2005). A maximum hazard dosing laboratory study with an artificial diet

containing 930 µg/g of diet of Cry3Bb1 protein showed no adverse effects on the survival, development and growth of the ground beetle, *Poecilus chalcites* (Duan *et al.*, 2006). Larvae of *Poecilus cupreus* (Carabidae) fed with prey raised on Bt maize showed a higher mortality than larvae fed with non-Bt prey. These effects may be prey-mediated, however, direct effects cannot be excluded as the carabid larvae did ingest Bt toxin (Meissle *et al.*, 2005). A study designed to test the effects of Cry3Bb1 and Cry1Ab maize toward 16 species of Carabidae ground beetles found no effects from the Bt toxins (exposure to pollen) whereas nearly complete mortality was found for seeds treated with neonicotinoid insecticides (Mullin *et al.*, 2005).

## 2. Exposure to Non-target Organisms

93. If adverse effects are seen for an organism in the acute hazard testing, exposure analysis will enable a risk assessment to be performed. Several routes of exposure exist which can be either linked to the exposure from the toxin produced in the crop or the exposure from toxin produced in wild relatives if outcrossing can take place. However, the potential for outcrossing is crop and region specific and is best addressed in the consensus documents for the respective crops. Exposure to non-target organisms depends on the habitat and feeding ecology of the organism and its life stages. Exposure can be either direct via the uptake of Bt plant material and  $\delta$ -endotoxin bound to soil or indirect via the food chain. A worst case direct exposure can be estimated from the maximum levels of  $\delta$ -endotoxin that may be present in the different plant parts. The data submitted in support of the U.S. EPA registration applications showed great variation in toxin concentration for different constructs, tissues, and different ages of the plant. As an example of variation among constructs, Cry1Ab  $\delta$ -endotoxin protein expression levels were reported for several commercial constructs in maize. One construct showed maximum levels of 10.34 µg/g leaves, 4.65 µg/g whole plant, and <0.09 µg/g pollen (dry weight) (Monsanto, 1995c; 1995d). Another Cry1Ab maize construct showed maximum levels of 4.4 µg/g leaves, 0.6 µg/g whole plants, and 7.1 µg/g pollen (Mycogen and Novartis, 1995d). Cry3Bb1 expression in another construct showed maximum levels of 450 µg/g leaves, 390 µg/g roots, and 42 µg/g pollen (dry weight).

94. Some of the highest expression levels were seen for a Cry9C construct in maize (Plant Genetic Systems, 1998c). The highest amounts (on a dry weight basis) seen in the various plant parts (for the vegetative growth stage) were 250.0 µg/g whole plant, 175.0 µg/g tassel, 44.0 µg/g leaves, 25.87 µg/g root, 18.6 µg/g kernel, 2.8 µg/g stalk, and 0.24 µg/g pollen. The amounts of  $\delta$ -endotoxin declined rapidly as the plant aged and no new protein was produced to replace the protein being degraded. The whole plant  $\delta$ -endotoxin analysis on a dry weight basis showed 250 µg/g for the vegetative growth stage, 230 µg/g at pollen shed, 96 µg/g at silage, and 22 µg/g at harvest. These exposure numbers could be used directly for organisms that feed on the plants. However, with the exception of pollen feeding insects, those organisms can be considered target pest organisms and are not intended to be protected from the toxin. There are some organisms that feed on other insects as well as plants, *e.g.* heteropteran predators, which could be considered to be both beneficial and potential plant pests. In addition, soil detritivores feeding on decaying transgenic Bt plant material and predators consuming herbivores and detritivores feeding on Bt plants can be also exposed to  $\delta$ -endotoxins.

95. Pollen is a potential source for exposure to non-target insects. As described in the effects section, pollen consumption from deposition on plants can affect non-target susceptible insects as well as pest insects (Felke and Langenbruch, 2001, 2003; Felke *et al.*, 2002). The majority of the maize fields in Europe shed pollen during July (Zscheischler *et al.*, 1990; Lang *et al.*, 2004). Usually pollen anthesis continues for 5-8 days, however, under favourable conditions the vast majority of pollen shedding may occur within a 2-day period (Treu and Emberlin, 2000; Wolt *et al.*, 2003), but maize fields can shed pollen also up to 10-14 days after the onset of anthesis (Treu and Emberlin, 2000, Oberhauser *et al.*, 2001). Considerable amounts of pollen can be shed by maize, and Emberlin *et al.* (1999) estimated maize pollen output to be approximately 70 kg per acre (= 0.4 ha) within a maize field. Maize pollen may be dispersed

by wind as far as 800 m (Treu and Emberlin, 2000) or even several kilometre (Brunet *et al.*, 2003), but due to their large size and weight only less than 1% of maize pollen grains are deposited further than 60 m away from the “source” field (Raynor *et al.*, 1972). In general, the majority of the maize pollen is deposited within 10 meters of the maize field edge as there is an exponential decline of pollen numbers with growing distance from the maize field (Hansen *et al.*, 2000; Wraight *et al.*, 2000; Stanley-Horn *et al.*, 2001; Zangerl *et al.*, 2001; Lang *et al.*, 2004; Li *et al.*, 2005; Shirai and Takahashi, 2005). On average, one third of the maize pollen, which drifted into field margins, was found on the surfaces of butterfly host plants (Pleasants *et al.*, 2001; Lang *et al.*, 2004). Pollen on butterfly host plants within the range recorded can cause adverse effects on some butterfly larvae if the pollen contains Bt protein(s) active against lepidopteran species and the densities exceed a toxic threshold (Felke and Langenbruch, 2003; Zangerl *et al.*, 2001; Dively *et al.*, 2004). Knowledge of naturally occurring maize pollen densities on food plants is indispensable for assessing the expected effects of Bt maize on butterfly larvae along field edges, together with the concentration of toxin in the Bt maize pollen, and its toxic effect on butterfly larvae (Lang *et al.*, 2004).

96. Predators consuming prey (or insects consuming honeydew secreted by some insects) feeding on Bt plants are potentially exposed to Bt toxins if the prey (or honeydew) contains the  $\delta$ -endotoxin. Different prey organisms will differ in the amount of toxin they incorporate. For instance, aphids seem to not (or barely) ingest Cry proteins when sucking on Bt plants such as Cry1Ab corn, presumably because maize phloem sap contains no Bt (Head *et al.*, 2001; Raps *et al.*, 2001; Dutton *et al.*, 2002). In contrast, lepidopteran larvae feeding on Bt maize incorporate Cry1Ab proteins in varying concentrations depending on the species (Head *et al.*, 2001; Raps *et al.*, 2001; Dutton *et al.*, 2002; Vojtech *et al.*, 2005). Also, other herbivores feeding on Bt plants contained  $\delta$ -endotoxins (Cry1Ab, Cry1Ac), e.g., a mite, a thrip, a hymenopteran species and a slug (Dutton *et al.*, 2002; Howald *et al.*, 2003; Obrist *et al.*, 2005; Harwood and Obrycki, 2006). In the detritivore *Porcellio scaber* (Isopoda), feeding on decaying Bt maize Cry1Ab toxins could be detected (Wandeler *et al.*, 2002). In a laboratory study, a ground beetle feeding on Bt-contaminated prey incorporated the Bt toxins and exhibited a higher mortality than controls (Meissle *et al.*, 2005). Field data demonstrate that non-target herbivores occurring in Bt maize field can take up Bt endotoxins. Harwood *et al.* (2005) showed that Araneae, Coccinellidae, and Nabidae contained on average between 0.42 to 2.53  $\mu\text{g}$  Bt toxin/g fresh weight, while Zwahlen and Andow (2005) were able to measure Bt toxin levels between 6.4 to 117.3  $\mu\text{g/g}$  fresh weight in some Carabidae 6.4 to 117.3  $\mu\text{g/g}$ .

97. As Bt plants express endotoxins during the whole season, potential tritrophic exposure of predators via prey feeding on Bt plants may be increased in comparison to Bt sprays if the Bt sprays are not applied throughout the growing season. The implications of exposure on the performance of these non-target organisms are still not clear; however, the above data show that long-term exposure to Bt toxins can occur in the field. Behavioural characteristics of predators, in particular prey choice, can affect their exposure to Bt endotoxins. In prey choice experiments adult *P. cupreus* (Carabidae) did not avoid Bt containing prey, and even selected Bt-fed *Spodoptera littoralis* (Meissle *et al.*, 2005). Another ground beetle, *Lebia grandis*, consumed prey fed with Bt potato leaves (Cry3A) as much as prey fed with non-Bt potato (Riddick and Barbosa, 2000). Larvae of *Chrysoperla carnea* showed a preference for prey fed non-transgenic corn as compared to prey fed Bt corn (Cry1Ab), which would potentially reduce the exposure of this predator (Meier and Hilbeck, 2001). Rovenska *et al.* (2005) showed in laboratory experiments that eggplant expressing the Cry3Bb toxin are preferred by the herbivorous spider mites, *Tetranychus urticae* (Acari). At the same time the predator, *Phytoseiulus persimilis* (Acari), consumed significantly less Bt-fed spider mites.

98. Bt plant residues remain in the field after harvest. Cry1Ab was still detectable in Bt maize leaves or in the soil of Bt maize fields after the growing seasons, though mostly in low concentrations (Hopkins and Gregorich, 2005; Zwahlen *et al.*, 2003b; Baumgarte and Tebbe, 2005). The worst-case exposure to soil organisms can be estimated from the whole plant  $\delta$ -endotoxin expression levels at harvest. For the Cry9C

construct that expressed high levels of toxin (Plant Genetic Systems, 1998c), the amount of  $\delta$ -endotoxin at harvest is 99 g/acre (assuming that an acre contains 25,000 maize plants) and the expected environmental concentration (EEC) is 0.11 mg/kg in 15cm deep soil. A laboratory bioassay submitted in support of a commercial product showed, using a susceptible insect (*Heliothis virescens*), that plant-produced Cry9C  $\delta$ -endotoxins in test soils biodegraded over 42 days and had a half-life of 4.5 days (Plant Genetic Systems, 1998b). These results are consistent with the half-life of 2 to 46 days reported for Cry1Ac in cotton in a microcosm study (Palm *et al.*, 1996). Similarly, for the second Cry1Ab construct described above, if senescent post-harvest maize plants were tilled into the top six inches of soil, there would be a maximum of  $4.2 \times 10^{-4}$  mg Cry1Ab/kg soil ( $190 \text{ mg Cry1Ab/acre} \times 1/0.5 \text{ extraction efficiency} \times 1 \text{ acre (6" deep)}/9.08 \times 10^5 \text{ kg soil} = 4.185 \times 10^{-4} \text{ mg Cry1Ab/kg soil}$ ). However, soil  $\delta$ -endotoxins from *B. thuringiensis* can bind to humic acids, clays, and the organomineral complex found in soil which may give some protection from degradation (see below paragraph 102). Moreover, the distribution of the Bt toxin in the soil may be unevenly distributed as a result of decaying plant material (Baumgarte and Tebbe, 2005; Hopkins and Gregorich, 2005).

99. Vettori *et al.* (2003) studied the persistence and activity of Bt in soil following application of a commercial Bt spray (FORAY 48B®) against the gypsy moth in oak forests in Sardinia, Italy. The results indicated that *Bacillus thuringiensis kurstaki* and its toxin introduced into soils in sprays can persist for long periods (at least 88 months for *Btk* and at least 28 months for its toxin). One laboratory study of six non-transgenic maize lines and two Cry1Ab lines showed that due to feeding avoidance by a decomposer not affected by the Bt toxin, one of the Bt lines was not degraded as fast as any of the other lines, although there was considerable difference among the non-transgenic lines too (Wandeler *et al.*, 2002). One publication (Zwahlen *et al.*, 2003a) reported slower degradation for Cry1Ab protein in corn litter in the field as compared to the laboratory and another publication (Zwahlen *et al.*, 2003b) reported detection of Cry1Ab in maize leaves buried in bags in the soil and in plant material on the surface for up to 200 to 240 days suggesting that the Cry protein persists in the plants as long as the plants have not yet been degraded. Recently, the decomposition of different plant species expressing Bt toxins was analysed in laboratory experiments and results were discussed in relation to lignin content and potential environmental consequences. Generally, Bt plants showed lower decomposition rates than non-Bt plants. However, this effect was not clearly related to lignification or reduced microbial activity in soil (Flores *et al.*, 2005).

100. Recent research has suggested that Cry1Ab toxin from Bt corn (Bt maize) is released in root exudates in soil and liquid growth situations (Saxena *et al.*, 1999; Saxena and Stotzky, 2000). In the first study, Saxena *et al.* (1999) show that the Cry1Ab in a transgenic corn (Bt maize) crop, truncated to an active form of toxin, is released into the liquid growth medium after seven days and that after 25 days was absent probably due to microbial and/or plant mediated degradation. In this and their subsequent study (Saxena and Stotzky, 2000), they also show that the toxin is released from roots of transgenic maize grown in different soil types. In both cases, Stotzky and co-workers used ELISA and tobacco hornworm larval bioassays to detect the toxin, and in the first study they also used SDS PAGE (protein gel electrophoresis). They suggest that, because these maize plants are expressing a truncated form of the Cry1Ab toxin, thus eliminating the solubilisation and proteolytic processing aspects of toxin specificity, and because there are few field data on the levels of toxin in soils, there may be unintended non-target effects on soil organisms. Soil  $\delta$ -endotoxins from *B. thuringiensis* microbial cells, as well as those produced from plants, can bind to humic acids, clays, and an organomineral complex found in soil thereby giving some protection from degradation by soil micro-organisms (Saxena and Stotzky, 2000; Stotzky, 2000; Crecchio and Stotzky, 2001; Saxena *et al.*, 2002a, 2002b). The toxins can be detected in soil for several months (Tapp and Stotzky, 1995a, 1997), and maintain bioactivity in the laboratory when bound to soil particles (Tapp and Stotzky, 1995b). However, additional laboratory bioassays of plant-produced  $\delta$ -endotoxin incorporated into natural soil showed a decrease in activity equivalent to the decrease in non-bound toxin that is biodegraded by soil microbial flora (Palm *et al.*, 1996; Pratt *et al.*, 1993). For example, the rapid degradation of Cry1F protein in soil has been confirmed using insect bioassays (*Heliothis virescens*) as the analytical

quantification method, resulting in a half-life of 0.6 days (Herman *et al.*, 2001; 2002b). In a similar way, soil degradation of Cry34Ab1 and Cry35Ab1 was analysed with insect bioassays using southern corn rootworm (*Diabrotica undecimpunctata howardi*), resulting in a half-life of less than four days for this binary toxin (Herman *et al.*, 2002a).

101. If the toxin is actively "exuded" by roots, *i.e.* secreted via the cell secretory apparatus, it would likely be present in greater concentrations in the soil than if it were released from 'leaky' cells or from normal plant dynamics such as the shedding ('sloughing') of root tip cells or degradation of some root during overall root growth. This is an important consideration in analysing any risk that arises from these types of transgenic crops (USEPA, 2000). Since the Cry1Ab toxin does not have a signal peptide, a short N-terminal sequence required for secretion in eukaryotic cells, it is not expected to be secreted by plant cells (Vitale and Denecke, 1999). It seems more likely that the source of the 'exudate' is shedding ('sloughing') of root tip cells or degradation of some root during overall root growth. This effect may be unique to maize since a multiple year study did not find any Cry1Ac protein in the soil from Bt cotton (Head *et al.*, 2002). This phenomenon and the above mentioned experiments do not appear to predict the amount of Bt protein remaining in the soil during active cultivation as evidenced by the fact that multiple field studies did not find any Cry1Ac (Head *et al.*, 2002) or Cry1Ab protein in the soil from Bt cotton or corn fields (Dubelman *et al.*, 2005). In addition, soils collected during monitoring studies in fields planted with MON810 or Bt11 corn for three or more consecutive years, in five corn-growing areas of the USA, were analyzed using a statistically validated insect bioassay. The Cry1Ab protein was found in soil at only one site, at pollination time, and at levels very near the detection limit (LOD = 0.03 µg/g). This transient residue dissipated soon after harvest. There was no Cry1Ab protein detected in any of the other four sites, or at any other time during or after the corn growing season (Dubelman *et al.*, 2005). Soils collected from multi-year field studies of Cry3Bb1 protein in MON863 (YieldGard Rootworm) field plots in Kansas were analyzed using Cry3Bb1 ELISA kits. Only one sample showed a trace residue of Cry3Bb1 protein (<0.007 µg/g), which dissipated rapidly. There was no soil persistence of the Cry3Bb1 protein and no detectable effects on surface or sub-surface soil arthropods (Ahmad *et al.*, 2005). Multi-year monitoring studies of the Cry1Ac protein in several Bollgard fields using insect bioassay (LOD = 0.008 µg/g) and ELISA analysis (LOD = 0.004 µg/g) also showed no detection of the Cry1Ac protein in soil specimens collected three months after tillage (Head *et al.*, 2002).

### **3. Risk to Non-target Organisms**

102. Because of the selectivity of the Bt  $\delta$ -endotoxins, non-target organisms belonging to a similar taxonomic group as the target organisms are those most likely to be affected. Predatory insects can be exposed to the  $\delta$ -endotoxin in plant parts if their prey feed on the transgenic plant. Their prey, however, may be susceptible to the  $\delta$ -endotoxin and, in consequence, be of inferior quality or not be available as a diet for the predatory insect. Generally, control of herbivorous crop pests by any sort of pesticide will negatively affect predatory insects by removing their food, even if the pesticide does not directly affect them. Information about predatory insect species and the effect of Bt plants on their populations would also be useful for the purpose of planning integrated pest management if releases of biocontrol insects are to be conducted simultaneously with the use of Bt crops.

103. Field surveys can be good indicators of overall effects against non-target insects, but are generally difficult to design and control and are expensive to conduct and analyze. The ability to detect changes in the abundance of species or taxa depends much on the experimental design and the statistical power (Lang, 2004). EPA considers field testing for effects on non-target arthropods as a higher tier evaluation that could be required depending on the conclusions from laboratory testing. A Scientific Advisory Panel (USEPA, 2003) concluded that "appropriately chosen single species Tier I laboratory tests showing no detrimental effects are sufficient to make a short term hazard assessment and that field studies be conducted when these tests show toxicity (as higher Tier testing described in the OPPTS Microbial

Testing Guidelines) but that proper multi-year commercial field studies with appropriate statistical power are needed to determine long term ecological effects.” This allows, for example, for testing on appropriate field plots which avoids the potential sampling errors caused by arthropod movement to and from small plots (Prasifka *et al.*, 2005).

104. Many field tests have now been conducted and most have been published. A field survey of beneficial arthropods (including lady beetles, predacious Carabids, brown lacewings, green lacewings, minute pirate bugs, assassin bugs, damsel bugs, parasitic wasps, damselflies, dragonflies, and spiders) revealed no significant differences in insect numbers between two transgenic Cry1F maize lines and their equivalent non-engineered maize lines, except for some slight variations that had no consistent pattern (Mycogen and Pioneer, 2001e). A two year field study on Cry3Bb1 maize collected a total of 156,572 organisms from 16 orders and 36 families. The invertebrates included pests, predators, parasitoids, detritivores and decomposers. The Bt maize showed no detectable overall effect on the abundance of non-target invertebrates (Monsanto, 2002e; 2002g). As part of a Spanish specific monitoring program for Bt maize (Bt176), a farm-scale study was initiated in the year 2000 to assess the potential impacts of Bt maize on predatory arthropods. The data indicate that Bt maize had no adverse effect on naturally occurring predators (De La Poza *et al.*, 2005) or on certain maize pests including aphids, leafhoppers, cutworms and wireworms (Pons *et al.*, 2005).

105. Reductions of population densities of specialist predators and parasitoids of *Ostrinia nubilalis* are to be expected as this is the target pest to be controlled in Bt maize fields (Bourget *et al.*, 2002). Siegfried *et al.* (2001) found that populations of specific natural enemies of *Ostrinia nubilalis* are less abundant in Bt maize fields than in non-Bt maize fields. In a field test in France, Bt maize had a negligible impact on non-target herbivores or beneficial arthropods collected on the plants throughout the growing season (Candolfi *et al.*, 2004). However, results of field studies comparing the effects of Bt maize with insecticide treatments against the target pest show that broad-spectrum insecticides, like pyrethroids, reduce abundance of a range of predator and parasitoid species not specific to *Ostrinia nubilalis* (Dively and Rose, 2003; Candolfi *et al.*, 2004). A three year field test with Cry1Ab and Vip3 maize showed that effects observed in the Bt maize plots were significantly lower than the community disturbances caused by insecticide applications and these changes did not carryover to the following growing season (Dively, 2005). A two year field test of Cry1Ab maize showed a slight decrease in a generalist predator species, *Nabis sp.* (Heteroptera), but no other non-target phytophagous or predaceous arthropod populations were decreased in the Bt maize plots. It appeared that the nabids, which are not very common in maize plots, were reacting to the reduced numbers of prey (Daly and Buntin, 2005).

106. No effect was seen on four generalist predators (two coleopterans, one heteropteran, and one neuropteran) of the European Corn Borer in three years of large scale field tests of Cry1Ab maize (lepidopteran-protected) at three sites in Iowa, but *Macrocentrus cingulum*, a European Corn Borer specialist hymenopteran parasitoid was seen at significantly reduced densities in Bt maize as compared to the non-Bt maize. This specialist was shown to be attracted to, and have increases in their population densities in, the non-Bt maize plots (Pilcher *et al.*, 2005). A three year field study in Illinois of Cry3Bb1 maize (rootworm-protected) surveyed foliage-dwelling arthropods and found no consistent adverse impact on the relative abundance of any non-target foliage-dwelling arthropod taxon, including predators and parasitoids (140,000 were captured and identified) (Bhatti *et al.*, 2005b). A companion three year study on Cry3Bb1 maize in Illinois found no consistent adverse impacts on the abundance of any non-target, ground-dwelling taxon compared with the non-Bt isoline. The taxa included Araneae (spiders), Carabidae (ground beetles), Staphylinidae (rove beetles), and detritivores (decomposers), such as Japygidae (diplurans), Lathridiidae (scavenger beetles), Formicidae (ants), Chilopoda (centipedes), and Oligochaeta (earthworms) (Bhatti *et al.*, 2005a).

107. Several field tests completed in Bt cotton fields found no significant effect of Bt cotton on secondary heteropteran pests, aphids and natural enemies (Wang and Xia, 1997; Fitt and Wilson, 2002; Liu *et al.*, 2002b; Wu and Guo, 2003; Torres and Ruberson, 2005; Head *et al.*, 2005). A six year large scale field study in Arizona on Cry1Ac cotton showed 19% reduction in five of 22 taxa of foliar-dwelling arthropod natural enemies compared with non-Bt cotton (Naranjo, 2005a). However a companion five year field study examined whether the Bt cotton had an effect on the natural enemy community's impact on three key pests and found that the potential predator impact was unaltered by Bt cotton but was depressed with insecticide applications, thus indicating that the effects observed in the six year study had little ecological impact (Naranjo, 2005b). In a field study conducted by Sisterson *et al.* (2004), arthropod abundance did not differ between Bt cotton and non-Bt cotton plots, but abundance was lower in pure Bt cotton plots as compared to a row mixture of Bt and non-Bt plants. In a three year field study in Australia, species richness of beneficial arthropod communities were reduced in pesticide sprayed cotton compared to Cry1Ac cotton and non-sprayed cotton. Slightly higher numbers of dipterans, damsel bugs, and jassids were found in conventional, non-sprayed cotton compared to Bt cotton (Whitehouse *et al.*, 2005). In a three year field study in China, ladybird beetle numbers were lower in Cry1Ac Bt cotton fields (attributed to reduced number of prey), whereas spider densities increased on Bt cotton. Acarids were not affected by Bt cotton, and the impact on aphids was observed to be inconsistent over years (Men *et al.*, 2004). The overall arthropod diversity and the diversity of pest sub-communities were increased, but diversity of natural enemy sub-communities were decreased in Bt cotton (Men *et al.*, 2003). Although insecticides were not applied against the main pest (Cotton Bollworm) on transgenic cotton, the total number of insecticide applications in three years was no less than on non-Bt cotton, because additional applications were necessary against piercing/sucking pests on Bt cotton (Men *et al.*, 2004). This is in contrast to the situation in Australia where pesticide reduction of 75-85% has been achieved over a ten year period (APVMA, 2003) and key pollution indicators have shown substantial declines in streams and rivers draining cotton growing areas (NSW Dept. Land & Water Conservation, 2001). In another field study in China, the densities of two secondary pest species (Hemiptera: Miridae) did not differ between Bt and non-Bt cotton, however, pest damage by mirids was significantly higher in unsprayed Bt cotton as compared to non-Bt sprayed cotton, indicating that these mirids have become key pests in transgenic cotton that may require additional control measures (Wu *et al.*, 2002). Chinese publications reported that a possible tritrophic adverse effect on natural enemies in the laboratory depended on the Bt cotton variety (Guo *et al.*, 2004), that natural enemies increased and phytophagous pests decreased in Bt cotton as compared to non-Bt IPM cotton fields (Liu *et al.*, 2002a), and that arthropod predators had generally higher population densities in transgenic Bt cotton field than in non-Bt cotton fields either with IPM or chemical control (Wan *et al.*, 2002). A review of field tests published to date concluded that the large-scale studies in commercial Bt cotton have not revealed any unexpected non-target effects other than subtle shifts in the arthropod community caused by the effective control of the target pests (Romeis *et al.*, 2006b).

108. As described above, in the majority of field studies there were no observed effects of Bt plants on invertebrate natural enemies. However, there are some exceptions, some with reduced and some with increased abundance of focus organisms in Bt treatments, of which the following are examples. Jumping spiders (Salticidae) were less abundant in Bt cotton (Cry1Ac, Cry2Aa) (Whitehouse *et al.*, 2005), but spiders as a whole were recorded to be more numerous in Bt maize (Cry3Bb), Bt cotton (Cry1Ac, Cry2Aa) and Bt potato (Cry3A) (Riddick *et al.*, 2000; Men *et al.*, 2004; Bhatti *et al.*, 2005b; Men *et al.*, 2004). Effects were often found with regards to predacious bugs (Heteroptera) with reduced numbers in Bt fields for Bt cotton (Cry1Ac, Cry2Aa), Bt corn (Cry1Ab) (Daley and Buntin, 2005; Naranjo, 2005a; Whitehouse *et al.*, 2005), increased numbers in Bt fields for Bt maize (Cry1Ab) (Musser and Shelton, 2003), and inconsistent varying results in other studies (Wold *et al.*, 2001; Reed *et al.*, 2001; De la Poza *et al.*, 2005). Lacewings (Neuroptera) were found to be less abundant in Cry1Ab × Vip3A cotton (Dively, 2005), and showed an inconsistent pattern in Cry1Ab maize (De la Poza *et al.*, 2005). The majority of field studies on Coccinellidae showed no or inconsistent Bt crop effects, the exceptions being higher numbers in Bt fields for Cry1Ab maize and Cry3Aa potato (Musser and Shelton, 2003; Pilcher *et al.*, 2005) and lower numbers

in Cry1Ac cotton and Cry3Bb1 maize (Men *et al.*, 2004; Bhatti *et al.*, 2005b). Abundance of some parasitoid Hymenoptera was lower in Cry1Ab maize (Dively, 2005; Pilcher *et al.*, 2005). However, Bt treatments were sometimes only compared to insecticide treated conventional crops (and not untreated controls), therefore the specific effect of the Bt construct was not studied but only compared to the application of chemical insecticides (*e.g.* Riddick *et al.*, 2000; Head *et al.*, 2005; Torres and Ruberson, 2005). The density shifts of natural enemies recorded above were often ascribed to prey dynamics or plant-mediated indirect causes. In the context of field tests it is important to be aware that the abundance of insects may be highly variable and influenced by multiple factors. As a consequence, experimental design and sample size are critical to obtain the necessary statistical power so that the probability to detect potential effects is reasonably high (Marvier, 2002).

109. The pollen of the Bt maize event 176 (Cry1Ab) was shown to cause negative effects in the field on two butterfly species, the Monarch and the black swallowtail (Stanley-Horn *et al.*, 2001; Zangerl *et al.*, 2001). Bt176 is no longer cultivated in the United States, but is registered in the European community, *e.g.*, with a cultivation area of 32,000 hectares in Spain in 2003 (Lumbierres *et al.*, 2004). In the United States an extensive series of research studies to analyse any potential harm to Monarch butterflies was begun following a research letter to *Nature* suggesting that they could be susceptible to pollen from Bt maize (Losey *et al.*, 1999). A series of workshops initiated many studies (*e.g.* Hellmich *et al.*, 2001; Oberhauser *et al.*, 2001; Pleasants *et al.*, 2001; Sears *et al.*, 2001; Stanley-Horn *et al.*, 2001; Zangerl *et al.*, 2001). Of these, the studies conducted in the field under normal cultivation practices found no adverse effect of pollen (and of maize anthers) of the events MON 810 and Bt11 on larvae of the Monarch butterfly, *Danaus plexippus*, and the black swallowtail, *Papilio polyxenes* (Wraight *et al.*, 2000; Stanley-Horn *et al.*, 2001; Jesse and Obrycki, 2003; Anderson *et al.*, 2004). In contrast to these results, Dively *et al.* (2004) could demonstrate that Monarch larvae and adults are negatively affected in survival, development, weight and size after continuous and natural exposure to MON 810 and Bt11 during anthesis in the field. Considering both insect sensitivity and exposure, it was concluded that cultivation of Bt maize expressing Cry1Ab poses no great risk to the Monarch butterfly, because only a minor part of the whole population would be exposed to pollen shedding maize fields in the United States (Mendelson *et al.*, 2001; Dively *et al.*, 2004). In other areas of the world, exposure of non-target lepidopterans may merit closer scrutiny where agricultural land and natural habitats are more closely integrated.

110. Only a few field studies on the effect of Bt plants on soil arthropods exist. A field study of the effects of microbial *B. thuringiensis* subsp. *kurstaki* (Dipel ES) on forest soil fauna showed no effect on earthworms, enchytraeids, oribatids, gamasids, and collembolans (Beck *et al.*, 2004). Dively (2005) showed no adverse effects of transgenic corn (Cry1Ab × Vip3A) on saproverous soil arthropods, including springtails and mites, in a three year study. Likewise, Bt maize expressing Cry3Bb1 against corn rootworm had no effects on Collembola, mites and nematodes and other soil-dwelling invertebrates (Al-Deeb *et al.*, 2003; Jasinski *et al.*, 2003; Ahmad *et al.*, 2005; Bhatti *et al.*, 2005a; Bitzer *et al.*, 2005). Only in the study of Bhatti *et al.* (2005a) were a few effects observed on 2 - 3 taxa out of 14 taxa tested: Chilopoda numbers were slightly lower in Bt corn plots during two years of the three year study, Staphylinidae abundance was lower in Bt plots in one year, and the Bt effect on Diplura varied among years. In a field test in France involving three field of Bt corn (event 176), no statistically significant treatment effects were observed for diversity indices and for behaviour of soil dwelling arthropod taxa throughout the season. (Candolfi *et al.*, 2004).

111. Because of the concern that  $\delta$ -endotoxins from both the naturally occurring *B. thuringiensis* microbial residency in the soil and from the Bt plants, might persist in the soil (see paragraphs 100, 102 and 103, above), experiments have been performed to assess the effects on soil non-target organisms, including both soil microorganisms and macroorganisms. The first of these (Saxena and Stotzky, 2001) reported that earthworms, nematodes, protozoa, fungi, and bacteria, including actinomyces were not affected by 40 days in soil planted with Cry1Ab maize or 45 days in soil with added Cry1Ab maize



biomass. The toxin was found in the earthworm guts, but was cleared in 2 to 3 days after moving them to non-Bt soil. The earthworm results agree with results of a seven year field trial with a strain of *B. thuringiensis* subsp. *kurstaki* where the microbial Bt was shown to germinate in three species of earthworm and one tipulid larvae with no harm noted to the organisms (Hendriksen and Hansen, 2002). The earthworms and other soil organisms seem to provide a soil niche for replication of the many subspecies of *B. thuringiensis* that can account for the widespread distribution of *B. thuringiensis* in soils worldwide. No mortality was observed in earthworms fed Cry1Ab maize litter in a 200 day study in the laboratory and the field, although there was some unexplained weight loss after 200 days for the adults, see also paragraph 74 73 (Zwahlen *et al.*, 2003a). A laboratory study with Dipel 176 in microbial microcosms concluded that it would be unlikely that Bt would have a significant impact on the non-target microflora under field conditions (Visser *et al.*, 1994). In addition to the 2001 Saxena and Stotzky publication, there have been a number of more recent publications that found no significant effects of Bt plants on soil microflora (Dunfield and Germida, 2004; Motavalli *et al.*, 2004; Blackwood and Buyer, 2004 (effects seen "are small"); and Devare *et al.*, 2004).

112. It should be noted that it is difficult, if not impossible, to adequately assess any risk associated with any changes in soil microflora. The soil microflora is extremely variable according to type of soil, temperature, moisture, plant growth, nutrients, pH, and many other factors which may vary between locations abut also within a single plot and over very small distances. The soil food web structure varies with climate and geography (Neher, 1999). Cultivation and planting monocultures of agricultural crops has a major impact on the composition of the soil microflora. Furthermore, the microbial populations are very resilient. Even after intentional chemical fumigation, as with methyl bromide, the soil microorganisms regrow rapidly. Measuring microbial mediated reactions is a more general way to assess soil population activity, but the effects of changes in these are also not fully understood. A recent review (Nannipieri *et al.*, 2003) of the state of knowledge of soil microbial diversity notes that generally a reduction in any group of microbes results in other microorganisms taking over the previous group's function because of the redundancy inherent in microbial activities. It also cautions against using the newer community analysis techniques without critically considering their limits. The question of whether some change is an adverse effect or a beneficial effect is likely to depend on the context of the question and may often not have an answer, which also cautions against generalisations of results.

#### **D. Other Ecological Issues**

##### ***1. Loss of Effectiveness of Biological Control of Weedy Species.***

113. Wild relatives of crop plants that have weedy characteristics may become protected from insects released as classical biological control agents if they acquire and express a  $\delta$ -endotoxin gene from the related crop. It is unlikely that a biological control insect would be intentionally used for this purpose since it would probably also be a pest of the crop plant. However, the naturally-occurring crop pest insects might also be contributing to reducing the impact of related weeds. The potential for increasing weediness has been studied in sunflower and rape plants. Wild varieties of sunflower (*Helianthus annuus*) can be a weed in agricultural settings. Cultivated sunflower is known to hybridize frequently with wild sunflower in the western and midwestern United States. Snow *et al.* (2003) studied a *cry1Ac* gene in backcrossed wild sunflower populations. Lepidopteran damage on transgenic plants was strongly reduced relative to control plants at their two study sites, while damage by several weevil and fly species was unaffected. The results suggest that reduced herbivory (by lepidopteran species but not other herbivores) caused transgenic plants to produce an average of 55% more seeds per plant relative to non-transgenic controls at the field site in Nebraska. A similar but non-significant trend was seen at the site in Colorado (14% more seeds per plant). In a greenhouse experiment the transgene had no effect on fecundity, suggesting that it was not associated with a fitness cost. If Bt sunflowers are released commercially, the authors expect that Bt genes will spread to wild and weedy populations, limit damage from susceptible herbivores on these plants, and increase

seed production when these herbivores are common. In other experiments, Bt oilseed rape has been shown capable of hybridising with wild relatives in the lab and in the field. Greenhouse experiments have suggested there may be a fitness advantage conveyed by the Cry1Ac but field studies have not yet been done to confirm this (Halfhill *et al.*, 2002; Vacher *et al.*, 2004).

114. The potential for outcrossing is a critical part of an assessment of this kind of risk. As previously mentioned outcrossing potential is very crop and region specific and is best addressed in the consensus documents for the crop. As a mitigation measure, various engineering or planting strategies could be used to reduce or eliminate the potential for out-crossing to wild relatives if they occur in proximity to areas in which the transgenic crops are grown.

### **2. Potential for Adverse Effects on Endangered or Threatened Species**

115. The risk to non-target species, especially endangered species, should be considered in a risk assessment. Any endangered species site restrictions on the use of conventional chemical insecticides would be an indication that the potential for adverse effects from the more specific  $\delta$ -endotoxins should be evaluated. Testing has shown that  $\delta$ -endotoxins are relatively specific, *i.e.*, they do not affect all the species within any given order. In the case of plants expressing Cry1 or Cry3 proteins effects on endangered Lepidoptera or Coleoptera therefore are the major concern and the risk assessment should consider if there is likely to be an exposure to rare or endangered species. Although potential effects will focus on agricultural habitats a transfer of the Bt toxin via pollen to adjacent habitats needs to be considered. This is especially the case in structured landscapes such as parts of Europe where agricultural land is in close proximity to, or part of, nature conservation sites or ecologically sensitive areas (Lang, 2004). In the United States larvae of 229 lepidopteran species feed on host plants associated with maize (Losey *et al.*, 2003). According to Schmitz *et al.* (2003) seven percent of the German Macrolepidopteran species mainly occur in arable land and are potentially exposed by Bt maize pollen. The study showed that over 39% of these 97 species are rare or endangered. The authors advised implementing a risk related monitoring plan for species of concern in the EU. Wolt *et al.* (2005) suggest a stepwise approach to monitoring where a thorough risk assessment is conducted based on the trait, the crop plant in which it is expressed including the spatial and temporal pattern of expression, factoring in the receiving environment to determine the need for monitoring or mitigation procedures.

116. Any potential for outcrossing also needs to be considered in the assessment of risks to rare or endangered species. The introgression of the Bt trait to wild relatives would considerably increase the exposure and may lead to the spread of the Bt trait into non-managed habitats (Snow *et al.*, 2003). Letourneau *et al.* (2003) listed 502 species of Lepidoptera worldwide that feed on cotton, rapeseed and rice or their wild relatives, and which would be exposed and potentially at risk if Bt plants would escape or outbreed.

### **3. Potential for Loss of Efficacy.**

117. Up to now, Bt resistant lepidopteran pest species like *Ostrinia nubilalis* or *Sesamia nonagrioides* have not been found in fields in Europe (Evans, 2002; Bourguet *et al.*, 2003; Farinós *et al.*, 2004). Although laboratory tests showed that maize borer populations are capable of developing some degree of tolerance to the Cry1Ab protein (Huang *et al.*, 2002), laboratory selection and F2 screening to generate highly resistant *O. nubilalis* strains have not been published so far (Bourguet, 2004). However, another lepidopteran pest (*Plutella xylostella*) has developed resistance to Bt toxins in the US (Tabashnik *et al.*, 2003). Large scale cultivation of Bt crops over several years could increase the selection pressure on pest species, which might result in the development of resistance (Fox, 2003). This could have several consequences including the use of alternative phytosanitary measures to control the pest including the use of insecticides other than Bt toxins. The likelihood of occurrence is low since, under field conditions and

several years of cultivation, no resistance has been reported. However, it is difficult to predict future responses of pest populations. Therefore, if long term efficacy is a concern, potential target pest resistance development could be monitored during Bt crop cultivation. In addition, or as an alternative, methods have been developed that may be used to prevent or delay the development of insect resistance in the field (Williams *et al.*, 1992; Rajamohan *et al.*, 1998; Matten, 1998; Pittendrigh *et al.*, 2004).

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\* Health and safety studies submitted to the US Environmental Protection Agency in support of pesticidal registration can be released to the general public (with a few restrictions) after the product is registered. These, as identified by a Master Record Identification (MRID) number, can be obtained through the Freedom of Information Office at: HQ FOIA Operations Staff, United States Environmental Protection Agency, 1200 Pennsylvania Avenue (1105A), Washington, DC 20460, (202) 564-7333, Email: [hq.foia@epa.gov](mailto:hq.foia@epa.gov), web page address: <http://www.epa.gov/foia/>.



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#### Additional References

During final review of the document, additional references were suggested by reviewing countries. Since all countries would not have the chance to review their content, these references are added as a supplemental list to serve as an additional resource for the document.

#### Section I – GENERAL INTRODUCTION

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*Section I. C. Susceptible Insects*

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**ANNEX I. EXAMPLES OF COMPANY SPONSORED STUDIES SUBMITTED IN SUPPORT OF THEIR PRODUCT.**

The following tables reference these studies by their US identification numbers since the identification numbers as used by other countries are not available at this time. Studies judged as inadequate by USEPA reviewers are not included in these tables. Some of these studies in this document were submitted for products that have since been withdrawn or are in the process of being withdrawn from registration. However, these studies are still useful as general information on  $\delta$ -endotoxins as a class. These studies are identified by a Master Record Identification (MRID) number which is used to locate them in the file system. They are available to the public. The best way to obtain the information (because of US legal restrictions) is through the Freedom of Information Office at: HQ FOIA Operations Staff, United States Environmental Protection Agency, 1200 Pennsylvania Avenue (1105A), Washington, DC 20460, (202) 564-7333, Email: [hq.foia@epa.gov](mailto:hq.foia@epa.gov), web page address: <http://www.epa.gov/foia/>.

**Table A. Studies submitted to and reviewed by USEPA in support of registration of Cry1Ab products.**

Assay	Toxin derived from Microbes (MT) or Plants (PT)	Results	USEPA - MRID Number
microbial (MT) and plant toxin (PT) equivalence (* + ELISA)	MT expressed in E.coli + PT	MT and PT are equivalent (6444)	433972-02
microbial and plant toxin equivalence (*)	MT from Dipel + PT	MT and PT are equivalent (6430)	435332-03
acute oral, mice		no effect>4000 mg/kg	434680-01
acute oral, mice	MT	no effect>3280 mg/kg	433236-08
acute oral, mice	PT	no effect>5050 mg/kg	434175-02
digestibility	MT and PT	degraded by pepsin	433236-06
digestibility + heat stability	MT	degraded by gastric fluid but not intestinal fluid - inactivated in processed maize and cottonseed meal	434392-01
acute oral, quail	PT in corn meal	no effect> 100,000 ppm maize grain	435332-05
acute oral, quail	PT	no effect>2000mg/kg	433236-09
adult honey bee	MT	no effect>20ppm	434392-03
honey bee larvae	MT	no effect>20ppm	434392-02
honey bee larvae	PT in pollen	no effect	434157-03
ladybird beetle	MT	no effect>20ppm	434680-05
ladybird beetle	PT in pollen	no effect	433396-02
green lacewing larvae	MT	no effect>20ppm	434680-03
parasitic wasp	MT	no effect>20ppm	434680-05
daphnia	MT	no effect>150mg/l	433236-10
collembola, 2 species	MT	no effect >200ppm	439416-01
collembola	PT	LD <sub>50</sub> 240mg/kg/soil NOEL 125mg/kg/soil	434635-01
collembola, chronic	PT	no effect, including reproduction>50% of diet	442715-01
catfish	PT in corn meal	no effect at 100%of diet	438879-01
earthworm	MT	non-toxic	433396-01
earthworm	MT	non-toxic>200ppm	438879-02

\* SDS-PAGE, Western blot, N-terminal amino acid sequencing, glycosylation, and bioactivity

**Table B. Studies submitted to and reviewed by USEPA in support of registration of Cry1Ac products.**

Assay	Toxin derived from Microbes (MT) or Plants (PT)	Results	USEPA MRID Number
microbial and plant toxin equivalence (*)	MT expressed in E.coli + PT	MT and PT are equivalent (6445)	431452-02
acute oral, mice	MT	no effect > 4200 mg/kg	431452-13
acute oral, mice	MT	no effect > 5000 mg/kg	439995-01
digestibility		degraded by pepsin	439995-03
digestibility + heat stability	PT in diet	degraded by gastric fluid inactive in processed cottonseed meal	431452-14
acute oral, quail	PT in pollen	no effect > 10,000ppm	431452-11
<i>Manduca sexta</i>	MT	no effect	439995-11
parasitic wasp	MT	no effect > 10,000x levels found in pollen and nectar	431452-08
adult honey bee	MT	no effect > 10,000x levels found in pollen and nectar	431452-07
honey bee larvae	MT	no effect > 10,000x levels found in pollen and nectar	431452-06
ladybird beetle	MT	no effect > 10,000x levels found in pollen and nectar	431452-09
green lacewing larvae	MT	no effect > 10,000x levels found in pollen and nectar	431452-10
green lacewing larvae	MT	no effect > 20ppm	434680-03
collembola	PT	no effect > 8.0 g/kg	439995-12, -63
collembola	MT	no effect > 0.1 mg/kg	439416-01

\* SDS-PAGE, Western blot, N-terminal amino acid sequencing, glycosylation, and bioactivity



Table C. Studies submitted to and reviewed by USEPA in support of registration of Cry3A products.

Assay	Toxin derived from Microbes (MT) or Plants (PT)	Results	USEPA MRID Number
microbial and plant toxin equivalence (*)	MT expressed in E.coli + PT	MT and PT are equivalent (6432)	429322-03, -04, -05, and -06
acute oral, mice	MT	no effect > 5220 mg/kg	429322-17
digestibility		degraded by gastric fluid but not intestinal fluid	429322-18
acute oral, quail	PT in diet	no effect > 50,000 ppm	429322-14 429322-15
parasitic wasp	PT	no effect	429322-11
honey bee larvae	MT	no effect > 100ppm	441247-02
honey bee larvae	PT	non-toxic	429322-09
ladybird beetle	PT	no effect	429322-12
green lacewing larvae	PT	no effect	429322-13
collembola, 2 species	MT	no effect at 200ppm	439416-01
earthworm	MT	no effect > 100mg/kg soil	441247-01

\* SDS-PAGE, Western blot, N-terminal amino acid sequencing, glycosylation, and bioactivity

**Table D. Studies submitted to and reviewed by USEPA in support of registration of Cry9C products.**

<b>Assay</b>	<b>Toxin derived from Microbes (MT) or Plants (PT)</b>	<b>Results</b>	<b>USEPA MRID Number</b>
microbial and plant toxin equivalence (*)	MT expressed in E.coli + PT	MT and PT are equivalent (6466)	443844-01
acute oral, mice	MT	no effect > 3760 mg/kg	442581-07
digestibility + heat stability	MT	not degraded by gastric fluid not degraded by heat (90°C-10min)	442581-08
homology		no homology found with allergenic protein sequences in SWISS database	442581-09 443844-04
acute oral, quail	PT	no effect > 58ug/l diet	442581-14
honey bee, adult	PT in pollen	no effect > 5.8ug/l diet	443843-02
ladybird beetle	PT in pollen	no effect > 0.36ug/l diet	442581-11
daphnia	PT in pollen	no effect > 0.36ug/l diet	442581-12
collembola	MT	no effect > 20gm/kg soil	442581-10
collembola	PT	no effect > 180mg/kg soil	442581-10
earthworm	PT	no effect > 1.84mg/kg soil	442581-13
non-target beneficial insect field study	PT	over 3 years, no differences in numbers and types of insects in Bt and non-Bt fields	442581-15
host range insect studies	MT, PT, PT in pollen	susceptible to Cry9C: European Corn Borer, tobacco budworm, and diamondback moth; non-susceptible: corn earworm	442581-06

\* SDS-PAGE, Western blot, N-terminal amino acid sequencing, glycosylation, and bioactivity

Table E. Studies submitted to and reviewed by USEPA in support of registration of Cry1F products.

Assay	Toxin derived from Microbes (MT) or Plants (PT)	Results	USEPA MRID Number
microbial and plant toxin equivalence (*)	MT expressed in E.coli + PT	MT and PT are equivalent	450201-03 447149-03
acute oral, mice	MT	no effect>5050 mg/kg	446911-01 450201-18
digestibility	MT	degraded by pepsin	447149-03
glycosylation	MT + PT	No glycosylation	447149-03
heat stability	MT	heat labile at and above 75 C	452748-01
amino acid sequence similarity to known allergens		no amino acid homology at a level of 8 contiguous amino acids exists for Cry1F and known allergens	449717-01
acute oral, quail	PT in corn meal	no effect>100,000ppm	450201-12
parasitic wasp	PT in pollen	no effect>320 ppm, 10x levels found in pollen	450201-11 453078-03
honey bee larvae	PT in pollen	LC50>640ng/larvae through development into adults	450415-03 453078-05
ladybird beetle	PT in pollen	no effect>480 ppm, 15x levels found in pollen	450201-10 453078-02
green lacewing larvae	PT in pollen	no effect>480 ppm, 15x levels found in pollen	450201-09 453078-01
collembola	MT	no effect>12.5 mg/kg soil	450201-07
<i>Daphnia magna</i>	PT in pollen	no effect>100mg/l	450201-08
earthworm	MT	no effect>2.26 mg/kg dry soil	450201-06 453078-04
monarch larvae	PT in pollen	LC50>10,000ng/ml no effect<10,000ng/ml, some growth inhibition seen at highest dose tested	451311-02
Field survey: ladybird beetles, predacious carabids, brown and green lacewings, minute pirate bugs, assassin bugs, damsel bugs, parasitic wasps, damselflies, dragonflies, and spiders.	PT	visual counts showed no significant differences except for greater numbers in Bt maize of lady beetles, pirate bugs and spiders than seen in non-transgenic lines sticky trap counts showed no significant differences except for greater numbers in Bt maize of parasitic wasps and pirate bugs	450201-13

\* SDS-PAGE, Western blot, ELISA, N-terminal amino acid sequencing, glycosylation, and bioactivity

**Table F. Studies submitted to and reviewed by USEPA in support of registration of Cry2Ab2 products.**

<b>Assay</b>	<b>Toxin derived from Microbes (MT) or Plants (PT)</b>	<b>Results</b>	<b>USEPA MRID Number</b>
microbial and plant toxin equivalence (*)	MT expressed in E.coli + PT	MT and PT are equivalent	449993-01 449394-03
acute oral, mice	MT	no effect > 1450 mg/kg	449666-02
digestibility	MT	degraded by simulated gastric acid	449666-03
amino acid sequence similarity to known allergens and heat stability		no amino acid homology at a level of 8 contiguous amino acids exists for Cry2Ab2 and known allergens; heat labile at and above 120 C	449666-04 449666-05 442353-04
acute oral, quail	PT	no effect > 100,000ppm	450863-16
freshwater fish	PT cottonseed meal	dietary LC50 of Bt cottonseed meal > 20% of diet	450863-18 453371-03
honey bee adult and larvae	MT	no effect > 100mg/ml larvae through development into adults	453371-02 450863-07 450863-08
ladybird beetle	MT	no effect > 4500 ppm	450863-11
green lacewing larvae	MT	no effect > 1100 ppm, 21.6x levels found in cotton	450863-09
collembola	PT cotton leaf tissue	no effect > 69.5 mg/g diet	450863-14
earthworm	MT	no effect > 330mg/kg dry soil	450863-13

\* SDS-PAGE, Western blot, glycosylation, and bioactivity

Table G. Studies submitted to and reviewed by USEPA in support of registration of Cry3Bb1 products.

Assay	Toxin derived from Microbes (MT) or Plants (PT)	Results	USEPA MRID Number
microbial and plant toxin equivalence (*)	MT expressed in E.coli + PT	MT and PT are equivalent	451568-03 454240-04 454240-05 454240-10 454240-11 455382-01
acute oral, mice	MT	no effect>2980 mg/kg	449043-06
acute oral, mice	MT	no effect>3200 mg/kg	455382-02
acute oral, mice	MT	no effect>3780 mg/kg	449043-05
gastric digestibility	MT + PT	degraded by simulated gastric fluid	449043-07 454240-06 455382-03
intestinal digestibility	MT	degraded by simulated intestinal fluid to a smaller substance which was not degraded further (Cry proteins are general resistant to trypsin)	455770-02
heat stability	PT	heat labile at and above 240 C	454240-07
amino acid sequence similarity to known allergens		no amino acid homology at a level of 8 contiguous amino acids exists for Cry2Ab2 and known allergens	449043-09 454240-08
amino acid sequence similarity to known protein toxins		no amino acid homology for Cry2Ab2 and known protein toxins	449043-08
acute oral, quail	PT maize grain	no effect>70,000ppm	449043-15
freshwater fish	PT maize grain	dietary LC50 of Bt maize>35% of diet	449043-19
<i>Daphnia magna</i>	PT in pollen	no effect>120mg pollen/l	449043-18
parasitic wasp larvae	MT	no effect>400 ppm	449043-13
honey bee larvae	MT	LC50>1,790 ppm - larvae through development into adults	449043-10
honey bee adults	MT	LC50>360ug/ml (20X concentration in pollen)	449043-11
green lacewing larvae	MT	LC50>8,000 ppm, 20x field exposure	449043-12
adult ladybird beetle	MT	LC50>8,000 ppm, 20x levels found in plants	449043-14
ladybird beetle larvae pollen feeding	PT in pollen	LC50>93ug/gm pollen, larvae through development into adults	455382-04
ladybird beetle adult pollen feeding	PT in pollen	no effect – 50% pollen feeding <i>C. maculata</i>	453613-01
ladybird beetle adult pollen feeding	PT in pollen	no effect – 50% pollen feeding <i>H. convergens</i>	453613-02
chronic dietary collembola	PT in leaf tissue	LC50>872.5ug (50% maize leaves in diet)	449043-17
earthworm	MT	LC50>570mg/kg dry soil	449043-16
earthworm	MT	LC50>166.6mg/kg dry soil	457571-01
monarch larvae pollen feeding	PT in pollen	no acute toxicity or developmental effects	455382-05
insecticidal activity spectrum bioassays	MT	of 6 Coleoptera Families and 2 Lepidoptera species, only 2 beetle species of one Coleoptera Family affected (corn rootworm and Colorado potato beetle)	455328-07
two year field survey		no overall differences in abundance of non-target invertebrates and less impact on beneficial insects than traditional insecticides	455382-06 457916-01

\* SDS-PAGE, Western blot, ELISA, N-terminal amino acid sequencing, MALDI-TOF analysis of protein digests, glycosylation, and bioactivity

Table H. Studies submitted to and reviewed by USEPA in support of registration of Cry34Ab1/Cry35Ab1 products.

Assay	Toxin derived from microbes or plants	Results	USEPA MRID Number
microbial & plant toxin equivalence (*)	MT expressed in <i>Pseudomonas fluorescens</i>	MT & PT are equivalent	461239-05 461239-06
acute oral, mice	MT, Cry34Ab1 alone	no effect > 2700 mg/kg pure protein	452422-07
acute oral, mice	MT, Cry35Ab1 alone	no effect > 1850 mg/kg pure protein	452422-08
acute oral, mice	MT, Cry34Ab1/Cry35Ab1 mixture	no effect > 482 and 1520 mg/kg of Cry34Ab1 and Cry35Ab1 pure proteins respectively	452422-09
gastric digestibility	MT	Cry34Ab1 and Cry35Ab1 degraded by simulated gastric fluid	452422-12 455845-02
heat stability	MT	mixture of Cry34Ab1 and Cry35Ab1 proteins is deactivated after exposure to 60°C, 75°C and 90°C for 30 minutes	453584-01 455845-01 458086-01 458602-01
amino acid sequence similarity to known allergens		no amino acid homology at a level of 8 contiguous amino acids exists for Cry34Ab1 and Cry35Ab1 with known allergens	452422-05
amino acid sequence similarity to known protein toxins		no amino acid homology for Cry34Ab1 and Cry35Ab1 with known protein toxins	465847-01
freshwater fish	MT, Cry34Ab1/Cry35Ab1 mixture(**)	8-d acute toxicity NOEC > 100 mg/kg diet	457904-03
<i>Daphnia magna</i>	MT, Cry34Ab1/Cry35Ab1 mixture(**)	48-h acute toxicity NOEC > 100 µg/mL	457904-04
parasitic wasp larvae	MT, Cry34Ab1/Cry35Ab1 mixture(**)	11-d acute toxicity NOEC > 280 µg/mL diet	457904-05
honey bee larvae	MT, Cry34Ab1/Cry35Ab1 mixture(**)	6-d acute toxicity NOEC > 5.6 µg/larva	453407-01
green lacewing larvae	MT, Cry34Ab1/Cry35Ab1 mixture(**)	10-d acute toxicity NOEC > 280 µg/g diet	457904-07
adult convergent ladybird beetle	MT, Cry34Ab1/Cry35Ab1 mixture(**)	11-d acute toxicity NOEC > 280 µg/mL diet	452422-10
twelve-spotted ladybird beetle larvae Pollen feeding	PT in pollen	7-d acute toxicity, weight reduction NOEC > 58.52 µg/g diet	461239-12
chronic dietary collembola	MT, Cry34Ab1/Cry35Ab1 mixture(**)	28-d acute toxicity, reproduction NOEC > 12.7 mg/kg diet	457904-06
earthworm	MT, Cry34Ab1/Cry35Ab1 mixture(**)	7- and 14-d acute toxicity NOEC > 76 mg/kg dry soil	453602-01
poultry feeding	PT, grain in diet	42-day feeding study no diet-related effects	461239-11

insecticidal activity spectrum bioassay	MT, Cry34Ab1/Cry35Ab1 mixture	insects from three orders (Lepidoptera, Homoptera and Coleoptera) and four families (Pyralidae, Chrysomelidae, Aphididae and Noctuidae) were tested and only larvae of <i>Diabrotica</i> spp. were affected	457904-06
field survey	PT	no overall differences in abundance of non-target invertebrates	461239-14

\* SDS-PAGE, Western blot, ELISA, N-terminal amino acid sequencing, MALDI-TOF MS peptide mass fingerprinting, glycosylation and bioactivity.

\*\* NOECs for a Cry34Ab1/Cry35Ab1 mixture are expressed as the sum of the Cry34Ab1 and Cry35Ab1 protein concentrations.

**MRID Reports References for Tables A - H**

- 429322-03: Bartnicki, D., P. Lavrik, R. Leimgruber, *et al.* 1993. Equivalence of Microbially-Produced and Plant-Produced *B.t.t.* Protein also Called Colorado Potato Beetle Active Protein from *Bacillus thuringiensis* subsp. *tenebrionis*: Lab Project Number: 92-01-37-07: 93-081E: 12897. Unpublished study prepared by Monsanto Co. 95 p.
- 429322-04: Lavrik, P. 1993. Characterization of Colorado Potato Beetle Active *Bacillus thuringiensis* subsp. *tenebrionis* Protein Produced in *Escherichia coli*: Lab Project Number: 92-01-37-10: 93-081E: 92-448-711. Unpublished study prepared by Monsanto Co. 82 p.
- 429322-05: Bartnicki, D., R. Leimgruber, P. Lavrik, *et al.* 1993. Characterization of the Major Tryptic Fragment from Colorado Potato Beetle Active Protein from *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*): Lab Project Number: 92-01-37-15: 93-081E: 12994. Unpublished study prepared by Monsanto Co. 53 p.
- 429322-06: Rogan, G. and P. Lavrik. 1993. Compositional Comparison of Colorado Potato Beetle (CPB) Active *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) Protein Produced in CPB Resistant Potato plants and Commercial Microbial Products: Lab Project Number: 92--01-37-17: 93-081E: 12988. Unpublished study prepared by Monsanto Co. 46 p.
- 429322-09: Maggi, V. 1993. Evaluation of the Dietary Effect(s) of Purified *B.t.t.* Protein on Honey Bee Larvae: Lab Project Number: CAR 188-92: 92-01-37-03: 92-448-702. Unpublished study prepared by California Agricultural Research, Inc. 52 p.
- 429322-11: Hoxter, K. and G. Smith. 1993. *B.t.t.* Protein: A Dietary Toxicity Study with Parasitic Hymenoptera (*Nasonia vitripennis*): Lab Project Number: 139-349B: WL-92-436: 93-081E. Unpublished study prepared by Wildlife International Ltd. 51 p.
- 429322-12: Hoxter, K. and G. Smith. 1993. *B.t.t.* Protein: A Dietary toxicity Study with Ladybird Beetles (*Hippodamia convergens*): Lab Project Number: 139-348: WL-92-435: 93-081E. Unpublished study prepared by Wildlife International Ltd. 47 p
- 429322-13: Hoxter, K. and G. Smith. 1993. *B.t.t.* Protein: A Dietary Toxicity Study with Green Lacewing Larvae (*Chrysopa carnea*): Lab Project Number: 139-347: WL-92-437. Unpublished study prepared by Wildlife International Ltd. 43 p.
- 429322-14: Cambell, S. J. Beavers, and M. Jaber. 1993. A Dietary Toxicity Study with Russet Burbank Potatoes in the Northern Bobwhite: Lab Project Number: 139-356: WL-93-11: 93-081E. Unpublished study prepared by Wildlife International Ltd. 87 p.
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