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SESSION TITLE: Characterization and Non-Target Organism Data Requirements for Protein Plant-Pesticides

LEAD DIVISION: Biopesticides and Pollution Prevention Division

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In 1994 EPA published a proposed rule for the scope and potential data requirements for plants expressing pesticidal traits (FR 59, No. 225, at 60495). This document included a general description of the types of data that would be required for developing an environmental risk assessment and a summary of the outside scientific reviews of these proposed data requirements. The basic rationale was that plants expressing pesticidal proteins would have to address data similar to that found in the microbial pesticide testing guidelines (OPPTS Harmonized Microbial Test Guidelines - see 1). These requirements were modified to address the non-target toxicity rather than pathogenicity and to take into account that the plant itself rather than another application method was being employed.

Since 1994 EPA has registered eight plant-pesticide active ingredients using data generated by following the proposed requirements. The intent of this SAP is to revisit the types of data that EPA examines for environmental risk assessment especially that related to non-target insect hazards. The general guidance for types of data EPA would require to do a risk assessment as well as the outside scientific review to that date are found in the sections of the 1994 proposal attached (2). EPA intends to revisit the data requirements for human health risk assessment of protein plant-pesticides early in the year 2000. The document you are being asked to examine deals only with the environmental risk assessment and is divided into two parts: one on product characterization and the other on ecological effects testing. Product characterization is an essential element of any risk assessment and is presented first. However, the major thrust of this SAP is the adequacy of the current EPA approach for an environmental risk assessment. The majority of the questions are directed at this topic.

Two documents are essential for the product characterization portion: 1) the USDA/APHIS Canada-United States Bilateral on Agricultural Biotechnology document entitled "Appendix I: Molecular Characterization Data." (Attachment 3), and 2) the OECD document entitled "Consensus Document on the Biology of *Brassica napus* L. (Oilseed Rape)." (Attachment 4). These two documents provide examples of the types of data EPA might consider to adequately describe the proteinaceous plant-pesticide and the nature of any anticipated environmental exposure to the plant-pesticide. With the protein characterization and potential exposure adequately circumscribed, the types of data that need to be generated for an environmental risk assessment can defined.

The ecological effects portion of the presented document is fairly inclusive. The four

attachments are meant to serve only as additional background information. The questions at the end of the document are intended to address the issues "are we doing enough?" and "is there anything missing?"

I. Background

The role of biological (including plant) pesticides in the pesticide regulatory scheme:

EPA's pesticide registration process under Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) includes an assessment of unreasonable risk to non-target species based on data submitted to EPA and on other available information from the scientific literature. EPA routinely consults with USDA and FDA on data reviews of these plant-pesticides.

Background of biological pesticides testing guidelines. The first biological pesticide (*Bacillus popilliae*, a naturally occurring bacterium) was registered in 1948. During the late 1960s and early 1970s, interest in biological pesticides began to increase. More biological products were registered for use in agriculture, forestry, mosquito control, and homeowner situations.

In 1974, in recognition of the growing interest in, and concern about, biological pesticides, EPA began to sponsor a variety of workshops, symposia, and panel discussions aimed at identifying the relevant safety concerns for biopesticides. As early as 1978, at an EPA symposium titled ``Viral Pesticides: Present Knowledge and Potential Effect on Public and Environmental Health," the need for sensitive identification and detection methods for microorganisms as well as quality assurance provisions were clearly identified.

In a Policy Statement on Biorational Pesticides (1978) issued by the Office of Pesticide Programs (OPP), OPP recognized biopesticides (microbial and biochemical pesticides) as distinct from conventional chemical pesticides, and made the commitment to develop appropriate testing guidelines. In 1979, OPP commissioned an American Institute of Biological Sciences' expert panel to develop a ``Human Hazard Evaluation Scheme for Biorational Pesticides." The final report of this expert panel formed the basis for the mammalian toxicology unit of the testing guidelines for microbial pest control agents (MPCA). The environmental effects portion of the guidelines was developed by an in-house workgroup with extensive input by outside experts, followed by final FIFRA Scientific Advisory Panel (SAP) review.

OPP issued these testing guidelines for microbial and biochemical pesticides as Subdivision M of the Pesticide Assessment Guidelines (published through the National Technical Information Service (NTIS) in 1983 (EPA-540/9-82-028)). The microbial pesticide portion of the Subdivision M guidelines was used for both naturally occurring and genetically modified MPCAs. It was decided at that time that any additional data that would be required for the registration of genetically modified microorganisms would be determined on a case-by-case basis.

The Agency gained considerable experience in the risk assessment of conventional and genetically altered microbial pesticides since Subdivision M was published in 1983. Accordingly, the need for revising and updating portions of the guidelines became apparent. After review by the FIFRA Scientific Advisory Panel, a revision intended to better address genetically engineered microorganisms and the needs of both testing laboratories and scientific staff was published in l989 which reflects an extensive updating of testing guidelines for microbial pesticides. In February 1996, the revised guidelines were incorporated into the OPPTS (Office of Prevention, Pesticides and Toxic Substances) Harmonized Microbial Pesticide Test Guidelines and are available on the internet (see Attachment 1 – for the web site address.)

The revised guidelines utilize the tier testing scheme set forth in 1983 to ensure, to the greatest extent possible, that only the minimum data sufficient to make scientifically sound regulatory decisions will be required. The Agency expects most of MPCAs to require testing only in the first tier (i.e. single, maximum hazard dose testing or actual LC/LD50 determination). The Agency believes that the Tier I test requirements represent a reasonable approach to evaluating risk related to the use of biopesticides, and is one in which negative results at high exposure would allow a high degree of confidence in the safety of the test agents. Where Tier I testing shows a hazard at doses approaching the expected environmental concentrations, higher tier testing (to assess population level effects) may be required.

OPP has followed these ecological effects data guidelines for all other biopesticides not strictly falling into the microbial and biochemical pesticide categories on a case-by-case basis, while at the same time working on development of appropriate guidelines. In the case of plant-pesticides, the Agency published a proposed rule for plant-pesticides on November 23, 1994. The SAP comments on the rule are also included (Attachment 2, FR59 at 60516 Section H). OPP uses the guidance on information needs published in the 1994 proposal (59 FR at 60511), together with the existing biopesticide guideline principles, for the ecological risk assessment of protein plant-pesticides. (Note: only protein pesticidal substances have been seen to date).

Although OPP currently uses the existing guidelines for protocols and data evaluation in determining whether a plant-pesticide can be registered, it believes that the unique and novel aspects of plant-pesticides indicate that there should be testing guidelines and data requirements specific for these products. The Agency plans to begin the process of establishing data requirements and testing guidelines specific for plant-pesticides when it completes the rulemaking process for the series of exemptions proposed in 1994 for these products. In establishing the data requirements, EPA will propose the tests it believes are appropriate, indicating the circumstances under which each test is required, conditionally required, or not required. The test guidelines and data requirements will continue to evolve as the science and policies related to biotechnology mature. The option to request a waiver from any particular study will be retained where the data are not applicable to the specific product, or where a scientific rationale or public literature are found sufficient for risk assessment (as an example see Attachment 5 for a review of the published literature used by OPP to make a Bt/lepidopteran risk assessment). Until data guidelines specific for plant-pesticides are published, EPA will continue to handle the data requirements for new

plant-pesticides on a case-by-case basis.

II. Product Characterization For Risk Assessment of a Protein Plant-pesticide

This section describes the data and information needed to characterize a proteinaceous plant-pesticide for environmental risk assessment. EPA has elected to present to the SAP the data and information for product characterization outlined by the USDA/APHIS in the Canada-United States Bilateral on Agricultural Biotechnology and provided in the attachments entitled "Appendix I: Molecular Characterization Data." (Attachment 3). This same document is also being used in other international fora as a basis for harmonization among countries on risk assessment approaches. The emphasis in the USDA/APHIS data requirements reflect the nature of the specific legal parameters of their regulatory oversight provided by the Plant Pest Act and the Plant Quarantine Act. Their oversight is particularly focused on issues related to traits derived from plant pests. This plant pest focus is evident in the questions regarding the type of transformation system used (e.g., *Agrobacterium* is a known plant pathogen) and specific questions regarding the incorporation and expression of plant virus sequences. In contrast, more specific information is required by EPA to characterize the pesticidal protein and the potential for altered environmental exposure to the pesticidal substance provided by the modified plant. The exposure potential for the pesticidal substance is derived primarily from its expression levels in different plant tissues and a thorough analysis of the recipient plant's biology. An example of the type of plant biology information EPA might rely on in its assessment is found in the attached OECD document entitled "Consensus Document on the Biology of *Brassica napus* L. (Oilseed Rape)." The following is intended as an outline of the types of characterization data required for protein plant-pesticides as a prerequisite for an environmental hazard and risk assessment.

Product Characterization

A knowledge of the pesticidal substance is essential for any identification of the hazards and risks to humans and the environment. This information is termed product characterization and consists of several subject areas for EPA. These subject areas are outlined in the Proposed Plant-Pesticide Rule (Federal Register vol.59, no.225, p 60511-60513, November 23, 1994). The information consists of description of the biochemistry and bioactivity of the pesticidal substance, the molecular biology of trait introduction, the biology of the recipient plant and the source of the introduced gene(s). Protein pesticidal substance expression levels in tissues of the recipient plant and environmental stability are needed to predict exposure for non-target organisms.

A detailed description of the source of the expressed pesticidal substance is required in order to determine the nature of the trait and its previous environmental exposures. This description also identifies any questionable traits (e.g., toxins) that may be present in the source organism in order to confirm that DNA for these questionable traits is not accidentally introduced into the recipient plant. The introduction of genetic material encoding the active pesticidal substance is one of the fundamental features of these novel pest control systems in engineered plants. Therefore, the nucleotide sequence of the open reading frame(s) for the plant-pesticide

and sequences controlling its expression should be included as part of the plant transformation vector description. The introduction of the new trait DNA into the plant genome is usually confirmed by performing Southern blots. To verify stable incorporation of DNA for the new trait into the plant genome, Southern blots are required on progeny for several generations after the initial transformation event.

The basis for much of the product characterization data is to describe the expressed protein pesticidal substance. The DNA simply contains information needed to produce the pesticidal protein and provides a convenient means to track the plant-pesticide trait. To enhance the expression of an introduced trait (e.g., a bacterial gene in a eukaryotic system) changes are made to the DNA construct including different promoters, terminators and codons for the open reading frame of the protein of interest. It is well established that prokaryotic species have DNA enriched in G/C content. Since the genetic code is redundant for certain amino acids (e.g., arginine can be specified by the codons CGA, CGG, AGA and AGG), codon changes can result in an identical amino acid sequence while shifting the G/C content of the DNA and enhancing the expression of the foreign DNA. Changes made in the DNA sequence of the open reading frame resulting in an identical amino acid sequence for the expressed protein should not alter the hazard assessment of the protein. However, changes to the DNA altering the tissue specificity, timing or level of protein expression would require an assessment for a changed exposure profile.

The submitted DNA sequence and amino acid sequence of the open reading frame must indicate any processing that may occur after translation in the plant (e.g., signal sequence removal, protein glycosylation or prenylation). The similarity in biological activity between the protein in the source and its expression in the recipient plant is an essential feature of the characterization because the subsequent toxicology testing can be driven by available information about the trait as it was expressed in the source organism. For example, many published facts about the delta endotoxin crystal protein in *Bacillus thuringiensis* have been relevant for the risk assessment of these proteins expressed as plant-pesticides. Changes in the DNA can result in an altered amino acid sequence for the plant-expressed protein. If this is the case, the plant-pesticide must be shown to be a protein with activity similar to that of the source organism (e.g., bioactivity against the target pest, host range, sensitivity to proteases, immunorecognition) if the available literature on the source is to be cited for evaluation. Another consideration for addressing similarity of expressed proteins would be if the substituted amino acid had similar side chain chemical properties (e.g., valine compared to leucine).

Expression levels for the protein pesticidal substance in various plant parts are critical to determining the expected exposure for both humans and other non-target organisms. Expression data for plants is presented for foliage, seed, pollen and the whole plant at various stages of plant maturity. These expression data are essential for assessing the environmental impact of the pesticidal substance, if it has any non-target toxicity. Expression data for the pesticidal substance have also been critical to the development of a model approach for delaying the appearance of target insects resistant to the expressed Cry proteins. Resistance management related to the Cry proteins has been examined in depth by several FIFRA Scientific Advisory Panels and is an on-

going concern for the Agency (USEPA, 1998). Expression data usually consist of analyses for concentration of the protein active ingredient with an immunological assay which has been standardized for different plant tissues.

To determine adverse effects in non-target species a major issue is the type of test substance to be used for toxicity testing in non-target species. The appropriate test substance for all non-target species except mammals is the plant tissue to which the non-target species will be exposed. However, use of plant tissue becomes a problem if subsequent products are developed which have significantly higher levels of expressed plant-pesticide. Therefore, a treatment where the plant tissue test substance is spiked with pure pesticidal protein or is tested at high concentrations as a pure protein is useful. This spiked treatment would cover the non-target organism safety where cultivars are developed with higher expression of the plant-pesticide. The ideal source of this pure form of test substance would be that isolated from plant tissue expressing the plant-pesticide itself. However, obtaining sufficient pure protein from the plant itself is quite involved and has a limited yield. Large quantities of the proteinaceous material are easier to produce in an alternate source (e.g., the source microbe or an *E. coli* or yeast expression system). The similarity or equivalence of this alternate source to that expressed in the plant must be established where an alternate production system is needed for producing adequate test substance for spiking. The equivalence data should show the DNA construct used and demonstrate a protein with the same biochemical and biological activity as that of the plant-pesticide is being produced in the alternate system.

A thorough discussion of the biology of the recipient plant is critical to the assessment of ecological effects from an expressed protein plant-pesticide. This information is available from the OECD for several plant species that have already received new traits. A copy of the OECD document developed for oilseed rape or canola is attached as an appendix to provide an example of a discussion of plant biology. (Attachment 4). Where the OECD document is not available for the recipient plant, the same categories of information must be covered for recipient plant biology. This information would include a general description of the recipient plant, its agronomy including a description of agricultural practices required for the crop in the United States, mention of the weeds, insects and diseases affecting the crop's culture and the possibility that the crop itself presents a problem in rotation as a volunteer. The most recent taxonomy of the recipient plant, including the centers of origin for the species, must be discussed. Any wild relatives or related weeds significant in the United States must be mentioned. The reproductive biology of the crop plant and the possibility for pollination within the crop or cross pollination with related species to form viable hybrids needs to be addressed. The ecology of the crop plant and relatives must be discussed with special reference to the United States. This would include general classification of the plant with respect to its reproductive strategy such as a ruderal, competitor or stress-tolerant species and its presence in different biomes in the United States. This information makes it possible to address the likely escape and exposure scenarios related to the introduced traits in the recipient plant when adverse effects are seen in non-target species testing.

III. Non-Target Organism Data Required for Existing Registrations (Proposed

Requirements)

Summary:

The review process starts with an assessment of the probability of the plant becoming weedy and possible gene outcrossing and its possible ecological effects. This is followed by a non-target wildlife species/population risk assessment Following the principles of the ecological effects and environmental expression testing scheme of the OPPTS Harmonized Pesticide Test Guidelines, the protein plant-pesticide data requirements also have been effectively grouped in tiers. The first Tier requirements consists of maximum dose non-target organisms single-species hazard (toxicity) testing. If adverse effects are observed in the initial round of non-target single-species laboratory testing, the potential exposure to the plant protein is estimated by use of environmental expression and fate data (the concentration and degradation rates of the proteins in soil and plant residues) and the non-target organism population dynamics. These data serve as the basis for assessments of the exposure and potential risk to non-target organisms and the fate of pesticides in the environment.

The Agency desires a high level of confidence that no unreasonable adverse environmental effects will result from actual use of plant-pesticides. Toward this end, the Tier I guidelines reflect a maximum hazard approach to testing. Negative results from tests using this approach provide a high degree of confidence that no unreasonable adverse effects are likely to occur from the cultivation of pesticidal plants. Therefore, data that establishes an LC50 or LD50 that is greater than the maximum hazard dosage level (e.g. LD50 >1,000 mg/kg) is often adequate for the purposes of hazard assessment. In addition, on a case-by-case basis, definitive higher Tier field test data showing that the pesticidal plant does not affect the abundance of non-target species can be submitted as support for a no-hazard risk assessment (see Attachment 6 for an example of this process).

Environmental Risk Assessment Process:

Plants that are expected to be genetically engineered for use as pesticides include row crops, vegetables, fruit, ornamentals, aquatic plants, and forest and rangeland flora. Protein plant-pesticides are biological pesticides, and as such are reviewed according to the regulation scheme outlined above, since no plant-pesticide specific guidelines have been published to date. Currently the OPPTS Harmonized Pesticide Test Guidelines are being applied on a case-by-case basis while taking the following issues into consideration:

The overriding issue which defines the type of non-target data needed for risk assessment from large scale cultivation of genetically engineered pesticidal plants is that the pesticidal property is contained within the plant parts thus resulting in minimal exposure to non-target organisms. This is quite different from spray applications of pesticides. Exposure of non-targets to plant-pesticides would occur primarily when wildlife feed on the pesticidal plants, dispersed pollen or if sexual transfer of the new trait(s) to non-target wild/weedy relatives by cross-pollination

takes place. Therefore the ecological effects data are, in most cases, determined by the biology of the host plant, the inserted gene and geographical use considerations based on the proximity to related cultivars or weedy relatives that can cross-pollinate with the pesticidal plant. This amounts to a case-by-case analysis. Each risk assessment is made from an analysis of the properties of the engineered organism and its target environment, i.e. on the nature of the gene being introduced, the plant receiving the gene, the environment where the plant will be grown and the species susceptible to the effects of the introduced gene. The degree of scrutiny depends on the type of gene product, i.e. the intended mode of action. Protein products are not expected to pose much, if any, non-target hazard because they are biodegradable and more specific in their mode of action (while chemical compounds may be more recalcitrant and toxic).

A. Cross-pollination and Weediness

Potential weediness of transgenic forage, forest or rangeland plants is closely evaluated. It is not expected that the highly hybrid major crops will become weedy as a result of genetic modifications. Cross pollination, however, may transfer the new traits to closely related wild and weedy cousins. This may pose a risk to non-target wildlife in neighboring fields and natural ecosystems. In the US, cross-pollination is a minor problem as few crops originated in the US.

Hybridization of plants producing plant-pesticides with their wild relatives may pose some degree of risk to non-target species. This is especially significant for protection of those species of insects (or other wildlife) that depend on a single plant species for food or deposition of eggs. In addition, the delicate balance of the natural ecosystem may be disrupted if wild plants become toxic/repellent to their natural control organisms (microbial, insect or animal). This may occur where one, or only a few species are responsible for that control. Therefore, information on natural weed control mechanisms is needed, however much of it is yet to be developed.

B. Disease Resistance

In general, a minimal non-target effects review of this category is expected at the present time. A review of theoretical enhanced or reduced disease agent spread needs to be performed. The newly acquired disease resistance genes may also transfer to weedy relatives thus depressing a natural control of some weed population densities. A beneficial aspect is also possible, namely the elimination of plant disease agent reservoirs in weedy plants. A review of the above issues may trigger non-target plant infectivity testing.

C. Insect Resistant/Repellant Plants

Insect resistance by a plant may result from two primary modifications: (a) new trait gene insertion and expression and (b) modification of expression of existing traits. These may result in production of toxic chemicals or proteins, which may be confined to the plant parts or be released into the environment. They may be biodegradable or persist in the environment. A hazard to non-target populations feeding on the new plants may exist in the event that the traits are passed on to

wild/weedy relatives by cross-pollination. Plant-bound protein toxins, however, are expected to be biodegradable, but data to this effect must be submitted. The anticipated exposure of non-target wildlife should dictate the amount of testing to be performed. Previously undefined toxins should be subject to characterization by minimal Tier I aquatic and terrestrial non-target toxicity testing.

D. Basic Issues In Ecological Risk Analysis

The non-target organism endpoints of concern for plant-pesticides are initially based on containment of the gene product to the modified plant (except for pollen dissemination). The containment limits the exposure of the gene product to non-target organisms which are expected to interact with the plant. If exposure occurs, then the possible toxicity to non-target organisms is evaluated through non-target wildlife testing. If toxicity to the non-target wildlife in question is observed, then the amount of exposure is determined to ascertain if adverse effects to any non-target could occur under field conditions. The concentration of the proteins in plant tissues and soil residues and their degradation rates are also measured to more accurately determine exposure.

1. *Hazard and risk analysis considers the following questions*:

- Can the plant with the pesticide become a weed/pest through dispersal and persistence?
- What plant parts contain the gene product? Do pollen and seeds?
- Is the gene product released from the plant or from flowering parts?
- Is the plant a copious producer of mind-borne pollen?
- Is the plant-pesticide in a plant that is naturally self- or cross-pollinated, or both?
- Is the plant-pesticide containing pollen transmitted by wind, insects and/or other vector?
- Are sexually compatible, non-target plants nearby? If so, then can the plant actually transmit the newly acquired trait to non-targets plants?
- If transmission to non-target plants occurred, what would be the ecological consequences?
- If the new trait is pest resistance would there be significant ecological consequences if it were transferred to related plants?
- Would natural control of the wild plant/weed populations by insects or disease be curtailed, and if so, what would be the ecological consequences?
- Will the gene product (pesticide) persist and move in the soil environment?
- What effect does the plant protein have on beneficial soil invertebrates?
- Will wild birds/mammals feed on the plants or moribund insects?
- Will non-target animals/insects feeding on, or exposed to the pesticidal plants be adversely affected?
- What effect would the pollen have on pollinating insects?
- When the active principle is a metabolic product, (e.g. chitinase, protease inhibitor, a lectin, hypersensitive response proteins, etc.) will it pose a hazard to insects or beneficial fungi?
- Can animals, birds or bats distribute seed to locations containing weedy relatives?
- When in aquatic plants, will these be consumed by fish or aquatic invertebrates?

- When in aquatic plants, will these be released from plants into water?
- Is the gene product expected to reach the estuarine/marine environment in significant concentration?

2. *Effects on non-target organisms and fate in the environment*:

The basic ecological effects testing requirements on the representatives of non-target terrestrial and aquatic species listed in the OPPTS Harmonized Pesticide Test Guidelines must be addressed by submission of data or waiver requests with credible justification:

(Note: the OPPTS Harmonized Pesticide Test Guidelines protocol parameters are equally applicable to microbial toxins and to protein toxins contained in plant tissues. OPP relies more on microbial pesticide protocol parameters rather than conventional chemical pesticide protocols primarily because the microbial protocols are geared more for use of organic test materials and are generally of a longer duration. In addition, the conventional chemical testing guidelines do not have non-target insect protocols, except for pollinators. Currently, case-by-case dosing and test substance adjustments are made with OPP's concurrence prior to testing.)

The required tests and recommended representative species are:

Tier I: Avian oral toxicity test (on upland game bird and waterfowl species)
(Avian injection test)*
(Avian inhalation test)*
Wild mammal oral toxicity test (rodent species)
Freshwater fish oral toxicity test (on cold water and warm water species)
Freshwater invertebrate testing (on *Daphnia* or aquatic insect species)
Estuarine and marine animal testing (grass shrimp and fathead minnow species)
Non-target plant studies (terrestrial, aquatic and out crossing issues)
Non-target insect testing (predators and parasites, most commonly green lacewing larvae, ladybird beetle and parasitic wasp)
Honey bee testing (larval and adult toxicity)**
Terrestrial environmental expression testing (environmental fate/degradation rates of the proteins in soil)
Plant tissue expression data (and degradation rates of the proteins)
(Earthworm and springtail/Collembola toxicity testing)***

If the results of Tier I testing show adverse non-target species effects at field use rates, then testing of additional species and/or testing at a higher Tier level may be required:

Tier II Freshwater and marine or estuarine environmental expression testing (aquatic environmental fate).

Tier III: Chronic, reproduction, life cycle and population effects (host range) testing.

Tier IV: Simulated (microcosm) or actual field testing
(Field scouting for non-target insect abundance is currently recommended as a Tier I test)

* Conditionally required

**Representative of pollinator species. In the honeybee study, effects studies on brood as well as adults may be required.

*** (Not required by OPP prior to plant-pesticides registration. Data on *Collembola* and earthworm species are required at this time where crop residue incorporation into the soil is a possibility.)

If the results from environmental fate studies show that a plant protein that is toxic to non-target species persists in the environment at significant levels, Tier III studies are designed to show effects of chronic exposure to these levels on fish and wildlife. (Tier III studies are also used to determine non-target effects of plant proteins designed to inhibit insect molting, reproduction, etc.) Tier IV studies (simulated or actual field studies) are used to determine if there is a problem under field use conditions. Tier IV tests are designed on a case-by-case basis to evaluate any specific problem that cannot be resolved by lower tier testing.

OPP recognizes the potential value of Tier IV field tests as a further check on the safety of pesticidal plant proteins. These tests could be conducted concurrently with full-scale efficacy testing, and OPP strongly encourages such testing. This would provide the opportunity to evaluate pesticidal effects (both direct and indirect) on a much broader spectrum of non-target species, under more natural exposure conditions than is possible in Tier I testing.

[Note: Indirect support for this view was provided by the subpanel of the Federal Insecticide Fungicide Rodenticide Act Science Advisory Panel's (SAP) held March 1, 1995 (final report dated March 16, 1995) which encouraged the use of *Bt*-potatoes, because of the preservation of beneficial insects. Dr. Galen Dively, Dr. Casey Hoy and Dr. Gary Reed, among others, explained in their comments that use of *Bt*-crops is a sound IPM strategy especially in areas where CPB infestation is high. In addition, there is a high survival of beneficial insect populations in association with the use of the transgenic potato variety expressing the Bt *tenebrionis* δ-endotoxin. Both Hoy and Reed note that survival of beneficial insect populations will likely lead to a reduction in the use of chemical insecticides to control aphids and leafhoppers. A number of entomologists commented on how this potato variety expressing the Btt δ-endotoxin can be used with a number of IPM strategies including crop rotation.]

Data waiver requests:

The complete set of data requirements will not always be appropriate for every product.

Some products have biological properties or use patterns which would make particular data requirements inappropriate because it would not be possible to generate the data or because the data would not be useful in risk evaluation. OPP may waive data requirements it finds are inappropriate on a case-by-case basis in response to written requests. Generally the written waiver requests have to address and satisfy the environmental safety endpoint associated with each non-target data requirement with information other than the required testing data.

40 CFR part 158 contains provisions for granting waivers for data requirements in response to specific written requests by applicants (40 CFR 158.45). OPP encourages applicants to discuss their preliminary testing plans with OPP scientists. Tailoring of the testing battery on a case-by-case basis relies on both an accurate description of the expressed plant protein, description of the mode of action of the toxin, and knowledge of the range of species affected. Some compounds, such as Bt delta endotoxin (present in registered microbial pesticides), have been more closely examined than others and have a larger data base from which to draw conclusions. Where the proteins might not be as well studied or described, it may be difficult to predict their properties. In this case, it may be more difficult to justify waiving test requirements.

An additional factor in determining the extent of testing necessary for risk assessment is the degree of pest species specificity shown by the protein in question. This is of primary importance in assessing ecological risk. Most protein plant-pesticides produce adverse effects against a specific target species. Careful scientific consideration on a case-by-case basis must be given to the selection of non-target species to be tested (e.g., beneficial insects, environmentally or commercially significant plants, and wildlife) in order to include species that are most likely to be susceptible.

IV. Candidate Test Organisms

The purpose of non-target organism testing is to develop data necessary to assess potential hazard to terrestrial wildlife, aquatic animals, plants, and beneficial insects. The OPPTS Harmonized Pesticide Test Guidelines recommend testing the following species:

Avian test species. The guidelines provide that young bobwhite quail or mallard ducks be tested in Tier I tests. Birds between 14 and 28 days of age at the beginning of the test period should be used in the avian oral toxicity.

Aquatic animals. The Agency recognizes that considerable judgment will be required to properly employ the location of the modified plant as a criterion. While some aquatic plants may also be made pesticidal, OPP also recognizes that less obvious or borderline uses will result in aquatic exposure. Some examples that fall into the latter category are applications in forests, drainage ditches, riverbanks, and partially aquatic crops such as rice. Widespread use in major crops such as cotton, soybeans, and corn could also warrant aquatic testing if these crops are grown near bodies of water. To the extent possible, the Agency will rely on its experience with the conventional chemical pesticides in distinguishing between terrestrial and aquatic use patterns

in borderline situations.

Freshwater fish species. The guidelines provide that the species tested be selected from the list of species recommended with the exception of goldfish (warmwater species--*bluegill sunfish, channel catfish, and fathead minnow*; coldwater species--*rainbow trout, brook trout, coho salmon*). These species are desirable test organisms for several important reasons: They frequently are used to evaluate chemical and microbial pesticides; EPA has considerable background data on these species; standard methods for the care and handling of these species are available; and the species are widely distributed, are generally available, and have a variety of food habits and habitat requirements. Consideration may be given to testing species representative of the geographic region or ecosystem where the pesticidal plant will be cultivated. Fish species likely to scavenge intoxicated insects or the modified plant tissue (such as in farmed fish food) should be tested when appropriate. Unless there are other overriding considerations, the rainbow trout is the recommended as the freshwater fish test species. It is a desirable test animal because it is partially insectivorous.

Aquatic invertebrate species. The most likely plant tissue to be tested is pollen. Due to the broad phylogenetic spectrum from which the investigator may choose, it is difficult to select the most appropriate aquatic invertebrate. *Daphnia*, a Cladoceran, has the advantage of having considerable background data for comparative purposes. In addition, Daphnia exhibits a bioconcentration effect. This results from the filter feeding habits of Daphnia and is a desirable feature in terms of assuring that the test animal ingests the toxin containing tissue. Both Daphnia and certain aquatic insects have the advantage of a short life cycle and are useful for assessment of reproductive effects.

Non-target insect testing.

The purpose of the Tier I non-target insect testing is to assess toxicity to the honey bee and to three species of predaceous and parasitic insects. Selection of the predator/ parasite species to be tested should take into account such factors as the likelihood of exposure to the plant protein, phylogenetic proximity of the test species to target pest species, and similar relationships. A rationale for selection should be developed by the registrant. The chosen species must be reasonably representative and available and must survive under laboratory conditions.

(1) Terrestrial insects. Assessment of potential non-target insect hazard is complicated by a number of factors. Many plant-pesticides are expected to be specifically chosen for their ability to control pest insects. Other insects are the non-target group most at risk, being relatively closely related to the pest species in most cases. While there are few non-target insects that have been shown to be economically important to humans, there are many non-target insects which have an important role in ecological processes and may benefit humans indirectly.

Plant-pesticides will exert their effect on non-targets insect through consumption of either plant tissues or moribund pest insects. The high test dose Tier I tests should suffice for most

hazard evaluations. When Tier I toxicity is noted and field exposure is anticipated, an accurate LD50 will be determined. When the LD50 is close to anticipated field exposure doses, chronic and reproductive effects testing may be in order on a case-by-case basis.

The host range is an important factor in hazard evaluation for a protein plant-pesticide. A problem here is that extrapolation, even across species lines, is often not dependable. For this reason, the Agency provides for testing with representatives from a number of ``beneficial insect'' taxa. *Testing should be performed on the honey bee and three other species of insects, representing at least two of the following groups--parasitic dipterans, predaceous hemipterans, predaceous coleopterans, predaceous mites, predaceous neuropterans, parasitic hymenopterans.* Information from these tests will be used in conjunction with host range data (developed during efficacy testing and submitted as part of product characterization data) to develop a clearer idea of the overall susceptible insect host range.

Honey bee toxicity testing is used to assess hazard to pollinator species. In the honeybee study, effects studies on brood as well as adults may be required. Endangered and threatened species hazard is also addressed by testing related species, and possible risk is mitigated by preventing field exposure. In some cases, OPP considers potential impacts on non-target insects which are neither predators nor parasites.

(2) Aquatic insects. Tier I testing will include toxicity testing with Daphnia, or a species of aquatic insect, or both, depending on use pattern. Detection of toxicity in Tier I testing with anticipated exposure from the proposed use pattern will automatically lead to expanded testing which, if the impacted site is fresh water, will most likely involve testing with aquatic insects.

The Agency recognizes that Tier I testing may be more extensive in some cases than anticipated for plant proteins. However, there should be very few protein plant-pesticides which require effects testing beyond the Tier I level because the pesticidal proteins are confined to pollen and plant tissues which are readily degraded by soil microorganisms limiting non-target exposure. Selection of the test species for any specific plant construct is done jointly by the Agency and the registrant during a pre-registration conference. Rationale for selection is discussed. The final test species list may be smaller or greater than the guideline data requirements.

The selection of test species is limited by their availability, the inability to rear them in captivity in sufficient quantities for testing or their inability to survive captivity without unacceptable mortality levels.

V. Test Substances and Dosing

To determine adverse effects in non-target species a major issue is the type of test substance to be used for toxicity testing. Pesticidal plant proteins could be applied in any one of a combination of forms. It is preferable that the test organism be exposed to the most hazardous form. The test organism should be exposed to a form of the protein in which the toxin would be

produced in the greatest amount and most readily available. Ideally the test substance for toxicity testing in all non-target species is the modified plant tissue to which the non-target species will be exposed. Unfortunately, there is no easy way to administer the most natural form of the substance (i.e. whole plant tissue). The overriding consideration in the case of plant-pesticides is to administer a sufficiently high dose of the test material to obtain a realistic measure of intrinsic toxicity of the test substance to non-target species. The problem of quantity arises because the amount of introduced protein in transgenic plants is usually a small proportion of the total plant tissue. In many cases unrealistically high amounts of plant tissue are required to administer meaningful amounts of the active ingredient. In other instances the test animals refuse to consume plant tissue. This limited exposure when using plant tissue also causes problems with determination of the "maximum hazard dose" (or the 10X to 100X "safety factor"). In addition, the use of modified plant tissue with low toxin levels becomes a problem if subsequent products are developed which have significantly higher levels of expressed plant-pesticide.

Therefore, for some non-target species OPP has accepted dosing with pure pesticidal protein, or where the plant tissue test substance is spiked with pure pesticidal protein. The ideal source of this pure form of test substance would be isolated from the modified plant itself. However, obtaining pure protein from the plant itself without inactivation is quite involved and has a limited yield. It is often easier to produce sufficient quantities of the proteinaceous material in an alternate source (e.g., the source organism or an *E. coli* or yeast expression system). In the cases where an alternate production system is needed for producing adequate test substance for spiking, it is imperative that the similarity or equivalence of this alternate source is the same as that expressed in the plant. The equivalence data development involves showing a similar DNA construct being introduced or expressed and a protein of the same biochemical and biological activity being produced.

The use of pure test substance may be inadequate since it does not test for inadvertent, possibly harmful changes in the plant tissue itself (pleiotropic effects). To overcome these undefined factors OPP has often asked for test data developed on the pure substance, as well as on plant tissue and pollen, or on a mixture of plant tissue and purified toxin. The data often show that pollen, for example, does not contain sufficient protein to show toxicity, where the pure substance does.

An additional issue to be resolved is the state of the plant tissue: i.e. wet weight or lyophillized plant material reconstituted prior to testing.

Maximum hazard dosage levels. The maximum hazard dose for Tier I testing is based on some safety factor times the maximum amount of active ingredient (toxin) expected to be available to terrestrial and aquatic plants and animals in the environment. Whenever feasible, the dosage required is 10-100 x the expected environmental concentration (EEC). In most cases, testing at one maximum hazard dosage level is expected to be sufficient to perform a hazard assessment. If there are no adverse effects at the maximum hazard dose, low doses testing or precise LD50 determination is not required.

. *Maximum hazard routes of administration*. Various routes of administration (dosing) are provided for in the OPPTS Harmonized Test Guidelines and are chosen to reflect ``natural'' exposure routes. OPP believes that for plant proteins the oral route can best define the hazard to non-target organisms in the wild.

Test substance treatment. The lot of the substance tested should be the same throughout the duration of the study, and the test sample should be stored under conditions that maintain purity and stability. If the stability of the test substance cannot be maintained for the duration of the study or if, for other reasons, it is not possible to use the same lot throughout the test, subsequent lots of the test substance shall be selected to be as nearly identical to the original lot as practical. Chemical or biological assays must be performed to ensure composition identity and consistency.

Each lot of the test substance shall be analyzed, to the limits of technical feasibility, and the name and quantities of ingredients, contaminants, and impurities listed. The determination shall include the quantity of unknown material, if any, so that 100 percent of the test sample is accounted for. The test substance shall be within the limits of purity, if any, certified in accordance with OPPTS Harmonized Testing Guidelines.

If the test or control substance is to be incorporated into feed or other vehicle, the period during which the test or control substance is stable or viable in such a mixture must be determined prior to the start of the study. No mixture of test or control substance with the feed or vehicle shall be maintained or used during a period exceeding the known stability or viability of the test or control substance in the mixture. Alternatively, determinations of the stability or viability of the test or control substance in random samples of the diet or vehicle mixture shall be made at least monthly during the study to ensure that proper mixing, formulation, and storage procedures are being followed and that the appropriate concentration of the test or control substance is contained in the mixture.

If the test or control substance is incorporated into feed or other vehicle, its homogeneity and concentration in the diet shall be determined prior to the start of the study and, each time a new mixture is prepared. Random samples of the mixture shall be analyzed at least monthly to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

In addition to or in lieu of data otherwise required by this guideline, the Agency may require, after consultation with the applicant, data derived from testing to be conducted with:

An analytically pure grade of an active ingredient.
The inactivated form of the active ingredient
Other protein products expressed as a result of the transformation.
A contaminant or impurity.

A metabolite from the plants or degradation product of an active ingredient.
Any additional substance that may enhances the toxicity of the product for which registration is sought.
Any combination of the substances mentioned above.

Administration or application of test substance and vehicles. The manner of administration or application of the test and control substance for biological or environmental testing shall be selected to maintain accuracy of the dosage or treatment. A vehicle used to dissolve or dilute the test substance or positive control substance shall be chosen to possess the following characteristics if possible:

It does not alter the absorption, distribution, metabolism, or retention of the test substance.
It does not alter the chemical or biological properties of the test substance or enhance, reduce, or alter the toxic characteristics of the test substance.

At the levels used in the study, it does not produce physiological effects and is nontoxic.

Insect Test substance. The actual form of the material to be regarded as the test substance is discussed in OPPTS 885.0001 (see Attachment 1). Whenever feasible, dosage shall be 10-100 x the recommended field dosage. The best route of administration for adult insects is oral acquisition via a sugar solution. Predaceous insects could also be fed in this manner, but feeding them live, intoxicated prey, or an insect egg slurry laced with the pure toxin may be required.

Note: In the case of insect or aquatic invertebrate studies, the actual amount of test substance consumed cannot be accurately determined.

Additional test requirements. Since it is not possible to foresee the types of products submitted for registration, in addition to the general data requirements listed above, data derived from additional tests may be required by the Agency in order to make judgments regarding safety to non-target organisms on a case-by-case basis. Such data may also be required where special problems with Tier I testing are encountered. Test methods will usually be derived from protocols already described or cited in OPPTS Harmonized Pesticide Test Guidelines or other guidelines, such as the OECD Guidelines, or developed on a case-by-case basis. Such data requests may relate to a proposed geographical use pattern where outcrossing issues arise, a unique mode of action (e.g. gene products affecting physiological or metabolic process common to many life forms, etc.), or a unique chemical property. The data requested will be specific to the problem.

VI. Conduct of the Studies

The data are to be generated according to protocols adhering to these guidelines and which are developed with prior consultation with OPP to ensure their appropriateness to the plant-pesticidein question. All studies have the following elements in common.

(1) Treatment animals and controls for environmental studies.

Controls in biological or environmental studies are required by the OPPTS Harmonized Guidelines to ensure that observed effects are associated with the test substance exposure. The appropriate treated test animal and control groups shall be identical in every respect except for exposure to the test substance. In studies involving animals or plants all controls shall, to the extent possible, be from the same source, be of the same age, receive the same care, and receive the same nutrients as the animals or plants receiving the test substance. To prevent bias, a method to assign organisms to treatment and control groups randomly is required and must be referenced in the report.

Untreated (negative) controls. Untreated (negative) control groups are required. Untreated controls receive neither the test substance nor any ancillary material (vehicle). In the case of plant-pesticides, a control group treated with the unmodified host plant will be performed. In certain circumstances, deleterious effects may be produced in test animals through a mechanism other than toxicity of the introduced pesticidal protein (pleiotropic effects). Untreated controls to ascertain effects of transgenic plant tissues lacking the introduced protein are not available at this stage of guideline development.

Vehicle control groups. If a vehicle other than water or saline is used to administer the test substance, a concurrent vehicle control group may be required. Vehicle control groups receive treatment with the vehicle alone, and the vehicle is usually administered at the highest level that the vehicle is administered in any test group in the study. If required, a vehicle should be selected on the basis of information establishing that it is nontoxic at the levels used in the study, has no independent physiological effects, and does not alter the chemistry or toxicity of the test substance. If, however, there are insufficient data on the effects of the vehicle, testing of the vehicle is required..

Positive controls. Positive controls generally are not required. These serve as internal quality controls, and demonstrate known test organism sensitivity and/or compare the response to known toxic agents. They are also used to ascertain if a strain or species reacts similarly to another strain or species when exposed to the same known or standard toxicant. Individual species protocols and the Agency should be consulted to determine in which tests a positive control is required or recommended.

Additional controls. Additional controls may be required as dictated by test design.

(2) Reporting of data.

Each test report submitted under these guidelines must satisfy the following reporting requirements, unless specific instructions direct otherwise. Data should be submitted to the Agency in hard copy format. In addition, whenever possible, copies should be submitted in machine readable form by computer disk or via direct electronic lines.

General requirements. Each test shall identify the name and address of the laboratory or site where the test was performed and the party or parties primarily responsible for any written or other matter contained in the report, and the portions of the report for which each party is responsible. Each test report shall be signed by each of the senior scientific personnel, including the laboratory director, responsible for performing and supervising the testing and preparing, reviewing, and approving the test report. Each test report shall be certified by the applicant or an authorized agent of the applicant as a complete and unaltered copy of the report provided by the testing laboratory, whether independent or owned, operated, or controlled by the applicant.

Format and content.

The test report shall include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. The test report shall contain at least four parts: A summary and evaluation of the test results, a description of the test procedures, a listing of the data and information required by each applicable section of this guideline, and a section in which data and findings are discussed. Metric units of measurement must be used although English units may be included where appropriate. The systems may not be mixed (e.g. milligrains per quart).

Summary of test results. This section of the test report is to contain a summary of the data and significant findings.

Description of the test procedure. This section of the test report is to contain a full description of the test procedure. If applicants believe any of the reporting requirements are not applicable, they must submit an explanatory statement to this effect. A full description of the test procedure should include but not be limited to:

(a) Deviation from standards. The report must indicate all ways in which the test procedure fails to meet applicable standards for acceptable testing contained in this guideline, and must state the reasons for such deviations.

(b) Test methods. Specification of test methods, including a full description of the experimental design and procedures, and the length of the study (including the dates on which the study began and ended) is to be stated.

(c) Substance tested. Identification of the test substance is to be provided, including: name and, to the extent possible other appropriate designated type, and, to the extent possible, a qualitative and quantitative determination of composition (including names and quantities of known contaminants and impurities, within technically feasible limits). The determination shall also include quantities of unknown materials, if any, to account for 100 percent of the sample.

(d) Manufacturer and lot number of the test substance or record of chain of custody.

Animal and plant data. Test animal and plant data should include:

Species and strain used and reasons for selection of species (if the species is other than the species recommended or required by the Agency); source of supply of test organisms; disease history of the test animals; description of any pretest conditioning; method used in randomly assigning animals or plants to test or control groups; numbers of animals of each sex in each test or control group; age and condition of animals or plants at beginning of study.

Environmental conditions. A description of the environmental conditions under which the testing was conducted is to be reported. Further details may be provided by specific testing methods.

Treatment or doses. For studies where test substance applications, treatments, or dosings are made, a complete description of such is to be reported. Further details may be provided by specific testing sections.

Treatment for diseases not caused by the test substance. Test animals or plants with a history of disease are not to be used for plant-pesticide testing.

Observations. Method, frequency, and duration of observations made during the study are to be reported. Other related specific information to be reported may be provided by specific testing methods.

Availability of raw data, specimens, and samples of the test substances. The location of all raw data, specimens and samples of the test substances which are retained in accordance with 40 CFR, part 160 and OPPTS 885.1200, and the name and address of the individual responsible for the archives must be reported.

References. References must be provided for the statistical and other methods employed for analyzing the data, and for any published literature used in developing the test protocol, performing the testing, making and interpreting the observations, and compiling and evaluating the results.

Reporting the results and evaluation of specific tests. The test results and any evaluations of test results should be reported in accordance with the requirements of the individual specific testing protocols. Such results and evaluations include all data, information, and analysis necessary to support the registration application and its corresponding product label claims, directions, and precautions. *The report must be sufficiently detailed that a reviewing scientist has sufficient information to reach an independent conclusion from the data*.

Discussion section. The discussion section of the test report must contain a full scientific discussion of any and all positive or unexpected negative results and findings. All aberrant data must be noted and explanations based on sound scientific principles must be presented. Any

conclusions arrived at by the study authors should be included.

Statistical procedures. Appropriate statistical methods are to be used to summarized experimental data, to express trends, and to evaluate the significance of differences in data obtained from different test groups. The methods used shall reflect the current state-of-the-art. All data averages or means must be accompanied by standard deviations, to indicate the amount of variability in the data. In addition, the standard errors of the means should also be calculated, to compare means from different test groups; however, notations of statistically significant differences accompanied by the confidence level or probability should also be used in place of standard error determinations. Other methods of expressing data dispersion may also be used when appropriate.

VII. Questions

PRODUCT CHARACTERIZATION:

1. Are the presented characterization data requirements adequate to describe the introduced trait for risk assessment purposes?

2. It is well known that expression of an ingredient in a plant as a percentage of fresh weight is subject to gross errors due to the variable water status of plant tissue. To provide a more consistent basis to compare expression levels between tissues or products, is it more appropriate to express protein plant-pesticide levels as a percentage of total protein or as a percentage of dry weight tissue?

NON-TARGET ORGANISM DATA REQUIREMENTS:

3. Is a non-target insect risk assessment based on three selected representative single species and the honey bee adequate? (Considering the improbability of being able to test all of the insects exposed to pesticides? e.g. there are >750 species of butterflies in the USA)

   If not:

   a. What additional non-target insect species should be tested (taking into consideration the availability of laboratory colonies of the insects)? What criteria should be used to make the selection? (Such as for non-target Lepidoptera)

   b. Considering that it is not single species laboratory toxicity which is the basis for for the risk assessment, and since it is not practically possible to determine the toxicity to all of the exposed non-target insect species, can definitive higher Tier field scouting data showing the effect of the pesticidal plant on the abundance of non-target species be submitted as support for a request for waiver of some or all of Tier I testing requirements? (or should both single species and field scouting data

be required for a risk assessment?)

4. What would be an acceptable number of animals for maximum hazard dose (limit dose) testing vs. the number per replicate for LD50/LC50 determinations? (The current recommendation is 10 per replicate for LD50/LC50 determinations and 30 for maximum hazard dose testing for avian and fish studies. For insect studies the numbers range up to 100 per test group for maximum hazard dose testing).

5. Should OPP extend non-target insect effects testing requirements to include secondary exposure scenarios, like pollen covered milkweed and lupine or intoxicated aphids?

6. Is the maximum hazard dose at 10-100 x the EEC sufficient for non-target insect hazard determination?

7. Are the currently used test duration times adequate? Should they be changed? Currently the longest practical time is required and therefore, for insects, is dependent on how long the species can survive under laboratory conditions (avian - 8 days; fish - 20 days; daphnia - 2 to 21 days; honey bee - 8 to 15 days; earthworm - 14 days; Collembola - 28 days; lady bird beetle 21 days; parasitic wasp- 15 days; green lacewing larvae - 7 to 9 days). Alternatively, is a conventional acute, short duration study acceptable?

8. Current plant-pesticide environmental expression and fate studies (the concentration and degradation rates of the proteins in soil and plant residues) are required to be submitted as Tier I data with the registration application, while the guidelines for other biopesticides list them as Tier II data. Should we continue to require soil degradation data in Tier I for plant-pesticides?

9. The overriding consideration in the case of plant-pesticides is to administer a sufficiently high dose of the test material to obtain a realistic measure of intrinsic toxicity of the test substance to non-target species. Plant tissue toxin levels are often too low to detect toxicity in insects when pure toxin testing shows a hazard. What are the Panel's recommendations to the following test dosing for insect testing?

   (a) Dose with transgenic plant tissue whenever possible

   (b) Dose with purified (bacterial) protein

   (c) Dose with transgenic plant tissue/pure toxin combination to detect possible plant tissue secondary effects

   (d) Dose with both the (b) and (c) regimen above

10. Should OPP continue requiring earthworm and Collembola testing for protein plant-pesticides?*

* It was originally thought that since long-term exposure of soil organisms to plant-pesticides is possible when crop residues are incorporated or left upon the soil surface, EPA would require studies evaluating effects upon the representative soil organisms Collembola and earthworms. (This testing was not required by the Agency for registration of conventional pesticides or spray *Bacillus thuringiensis* products.) However there is no evidence that Bt toxins are exuded into the soil by Bt crops, or that Bt proteins are in a form that cannot be readily degraded by soil biota. One of EPA's reasons for requiring the non-target soil invertebrate tests was the concern that adverse effects on these species would cause a build up of plant detritus in cotton fields. However, in reconsideration, EPA discovered that the long term soil use of highly toxic chemical insecticides, such as aldicarb, terbufos, phorate and carbofuran, which have long term effects on soil invertebrate species, has not resulted in the build-up of plant detritus in soils based upon available information on current routine agronomic practices. Some of these materials had half-lives of 10 or more years. Thus protein plant-pesticide crops, which are expected to have less impact on these species than the highly toxic chemical pesticides, should not result in any increased build up of plant detritus. Supporting this conclusion are data which indicate that Bt toxin production in plant-pesticides ceases at plant senescence in the majority of registered Bt corn crops, allowing some time for protein degradation prior to harvest. Additionally, the environmental fate data indicate that for currently registered Bt corn crops only <1 to 90 grams of Bt protein per acre would enter the soil as a result of post harvest incorporation of Bt plants. Since proteins are known to degrade rapidly in the soil (and in-house and published data show a soil half-life of approximately 5 days), the potential for significant soil buildup and hazard to non-target soil organisms is not anticipated from the growing of crops containing protein plant-pesticides.