



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

June 1, 2006

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held March 14 - 15, 2006 on Event MIR604 Modified Cry3A Protein Bt Corn - Plant-Incorporated Protectant

TO: James J. Jones, Director
Office of Pesticide Programs

FROM: Joseph E. Bailey, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Steven Knott, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Clifford J. Gabriel, Ph.D., Director
Office of Science Coordination and Policy

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia on March 14 - 15, 2006. This report addresses a set of scientific issues being considered by the Environmental Protection Agency pertaining to Event MIR604 Modified Cry3A Protein Bt Corn - Plant-Incorporated Protectant.

Attachment

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SAP Minutes No. 2006-02

**A Set of Scientific Issues Being Considered by the
Environmental Protection Agency Regarding:**

**Event MIR604 Modified Cry3A Protein Bt Corn -
Plant-Incorporated Protectant**

**March 14 - 15, 2006
FIFRA Scientific Advisory Panel Meeting,
held at the National Airport Holiday Inn
Arlington, Virginia**

NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). The meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of the meeting minutes does not represent information approved or disseminated by the Agency. The meeting minutes have not been reviewed for approval by the Agency and, hence, the contents of these meeting minutes do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP is a Federal advisory committee operating in accordance with the Federal Advisory Committee Act and established under the provisions of FIFRA as amended by the Food Quality Protection Act (FQPA) of 1996. The FIFRA SAP provides advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at <http://www.epa.gov/scipoly/sap/> or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Joseph E. Bailey, SAP Designated Federal Official, via e-mail at bailey.joseph@epa.gov

In preparing the meeting minutes, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. This document addresses the information provided and presented by the Agency within the structure of the charge.

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March 14 - 15, 2006

**FIFRA Scientific Advisory Panel Meeting,
held at the National Airport Holiday Inn
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**Kenneth M. Portier, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: June 1, 2006**

**Joseph E. Bailey
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: June 1, 2006**

**Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
March 14 - 15, 2006**

Event MIR604 Modified Cry3A Protein Bt Corn - Plant-Incorporated Protectant

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of Event MIR604 Modified Cry3A Protein Bt Corn - Plant-Incorporated Protectant. Advance notice of the meeting was published in the *Federal Register* on January 25, 2006. The review was conducted in an open Panel meeting held in Arlington, Virginia, March 14 - 15, 2006. Dr. Kenneth M. Portier chaired the meeting. Joseph E. Bailey served as the Designated Federal Official.

The FIFRA SAP met to consider and review human health and environmental issues associated with Event MIR604 Modified Cry3A Protein Bt Corn - Plant-Incorporated Protectant. Syngenta Seeds, Inc. (Registrant) submitted applications for FIFRA section 3 registrations of the plant-incorporated protectant Modified Cry3A protein intended to provide corn protection from western, northern and Mexican corn rootworm larvae.

The agenda for this SAP meeting included an introduction and background of the issues under consideration provided by Mr. Mike Mendelsohn (Biopesticides and Pollution Prevention Division, Office of Pesticide Programs). Tessa Milofsky, MS (Biopesticides and Pollution Prevention Division, OPP) provided an overview of EPA's ecological risk assessment and Ms. Annabel Fellman (Biopesticides and Pollution Prevention Division, OPP) provided an overview of EPA's human health risk assessment. Janet Andersen, Ph.D. (Director, Biopesticides and Pollution Prevention Division, OPP) offered opening remarks at the meeting.

PUBLIC COMMENTERS

Oral statements were presented by:

Dave Nelson on behalf of the National Corn Growers Association
James D. Thrift on behalf of the Agricultural Retailers Association
Demetra Vlachos, Alan Raybould, and Fred Waters, on behalf of Syngenta Seeds, Inc.

Written statements were provided by:

Todd Barlow, Kentucky Corn Growers Association
Wes Beck, Jr., Farm Operator
James E. Betts, Ohio Professional Applicators for Responsible Regulation
William Bond, M.S., C.A.E., Minnesota Crop Production Retailers Association
Dan DeRycke, DeRycke Farms
Steve Ebke, Nebraska Corn Growers Association
Cresswell A. Hizer, Indiana Plant Food and Agricultural Chemicals Association
John Kuhfuss, Illinois Corn Growers Association
Roger Kvols, Kvols Farms
Lloyd R. Lee, Lee Family Farms
Dale R. Ludwig, Missouri Soybean Association
Gary D. Marshall, Missouri Corn Growers Association
Robert W. Prince, M.S., C.C.A., Garst Seed Company
Mindy Larson Poldberg, Iowa Corn Growers Association
B. Sachau, Public Citizen
Ted Vinson, Vinson Farms
Bernie Walsh, Bernie Walsh Farms
Jere White, Kansas Corn Growers Association

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

Panel conclusions regarding the safety of mCry3A were based on several factors. Environmental Protection Agency (Agency) summaries of the process and the data packet provided the overview, with specific details of studies presented by Syngenta Seeds Inc. Data were reviewed from studies on ecological effects (non-target), human health, mCry3A characterization, mammalian toxicity, and allergenicity, as well as a review of requirements for field testing of ecological effects in fields where MIR604 corn is cultivated. Public testimony was also considered.

Overall, the Panel found poor data quality and inadequate documentation in many aspects of the registration packet for mCry3A safety, from amino acid sequence reporting to documentation of natural versus plant incorporated protein homology to toxicity testing protocols. The errors or omissions in some studies were of a nature and degree that many on the Panel believed they should be excluded from consideration in ecological hazard/risk assessment (in particular the earthworm, soil dissipation, and ground beetle studies) since their study designs and data reporting did not meet basic Agency standards. Omissions in several other studies reduced the certainty of estimates to the point where they may not be useful even in Tier 1 hazard assessments. In several instances the hazard quotients or safety factors computed were deemed by the Panel to be too low and/or unreliable. In others, specifically in the rainbow trout, mouse, and quail studies, the safety factors were sufficiently high; but improper or inadequate replication weakened the validity of all three tests and their conclusion of no acute mortality hazard.

Panel members expressed a diversity of opinions concerning the adequacy of the Agency's analysis of the ecological studies submitted by the Registrant. These opinions ranged from nearly unqualified acceptance to qualified rejection. A number of suggestions for improving Tier 1 Maximum Hazard Dose testing for effects of Plant-Incorporated Protectants (PIPs) on non-target organisms (NTOs) were made during the course of the Panel discussion and are listed below. One omission identified was that the endangered Hungerford's crawling water beetle (*Brychius hungerfordi*; Coleoptera: Haliplidae), that exists in some areas of high corn production, was not studied or otherwise considered in this assessment. Panel members strongly supported the Agency's request for supplemental ecological studies in areas where MIR604 corn is being cultivated, and strongly recommended that these studies be designed around sound scientific study principles in order that data generated can be analyzed statistically in order to better meet information requirements of the Agency. The Panel noted from the public comments how use of Bt corn may not necessarily lead to a corresponding reduction in costs associated with application of conventional pesticides. The ecological impact of these combined technologies, which was not discussed in the assessment, is only now being discussed in the scientific literature, and represents an aspect of PIP use that should be studied further.

The Panel was asked to comment on the extent to which mCry3A protein extracted from MIR604 corn (LPMIR604-0103) is similar for risk assessment purposes to that produced from recombinant *E. coli* (MCRY3A-0102). Based on bioassays against first instar western corn rootworm larvae, the Registrant noted that the mCry3A extract from MIR604 corn was more active than the mCry3A produced in *E. coli*. The Panel found that this could be explained by the different mCry3A gene constructs used to synthesize mCry3A in corn versus *E. coli*, and by the subsequent proteolytic processing of mCry3A in corn or non-target invertebrates, especially beetles. Synthesis of mCry3A protein in *E. coli* produced two forms of the protein, one with a mass of 67.7 kDa, (roughly equivalent to LPMIR604-0103 extracted from MIR604 corn) and the other of 69.5 kDa, (a long form containing sixteen additional amino acids at the N-terminus). In corn, however, the two forms of mCry3A detected were a 67.7-kDa protein and a 55-kDa protein, the latter apparently being an activated form of mCry3A that constituted 50% of the mCry3A in corn. It was concluded that the mCry3A proteins are substantially equivalent with respect to their amino acid sequences, lack of glycosylation, other types of potential post-translational modifications and general stability. However, because the surrogate forms of mCry3A produced by *E. coli* were not activated, as was apparently 50% of the mCry3A produced in the mCry3A corn noted above, it was not certain that the different forms of the mCry3A produced in corn versus *E. coli* could be considered substantially equivalent for the purpose of non-target invertebrate studies. The activated forms of mCry3A, i.e., the 55-kDa protein, were considered to be substantially equivalent regardless of whether the source was corn or *E. coli*. Although no direct information was provided about this form from subsequent cleavage of either of the longer forms from bacterial production, one can reasonably make the conservative assumption, but it is only an assumption, that the 55-kDa form is produced from both the long and short forms of *E. coli*-produced mCry3A. However, at least one Panel member believed that the mammal studies were inadequate to support the statement that the activated forms were substantially equivalent.

Low mammalian toxicity was deemed to be supported by the test results presented. Most of the Panel agreed that the exposure assessment showed that the amount of Cry protein that would be consumed in a rather large serving of corn kernels would be several orders of magnitude lower than the highest dose tested in the mammalian toxicity studies – which had no observed effect. The exact calculation of exposure was considered uncertain due to the mixture of short and long forms of the protein in the *E. coli*-derived material used in the assay. It was pointed out that non-oral routes of exposure were not explored and that true allergenicity studies were not performed. The sequence analysis (homology search via NCBI-BLASTP) was poorly documented and difficult for the Panel to interpret and as a result is of limited value in the risk assessment.

A couple of Panel members believed that no conclusion on safety could be derived from the data and that much more information is needed about acute effects and potential effects on children's growth and development. At least three Panel members expressed concern regarding the adequacy of the scientific basis supporting a permanent exemption from the requirement for a tolerance. Such concerns could be alleviated by more rigorous allergenic testing/monitoring of mCry3A among workers or consumers of products generated from this type of corn.

The Panel discussed the following recommendations to focus Agency evaluations of Tier 1 studies related to PIPs.

- The Agency should utilize full technical reports rather than summary reports in the assessment including full documentation of study protocols, implementation findings and inclusion of raw data in technical reports.
- Rely less on descriptive statistics in technical reports and require more formal statistical testing.
- Ensure that Tier 1 hazard assessments meet the minimum regulatory requirements and have robust experimental designs and, in particular, use true replication in study protocols. Study designs should represent good science and be of journal publication quality. The resulting data should support estimation of the margin of exposure (MOE) with acceptable 95% confidence intervals (CIs).
- For PIPs which may exist in multiple forms, require clear characterization of test materials, particularly in terms of structure and toxicity potential.
- Study protocols should be at least as precise and extensive as those required in chemical pesticide assessments. A finding in support of a permanent exemption from the requirement for a tolerance for a PIP in a specific crop should require, in addition to true replication of treatments and in some cases of whole studies, a predominance of no effect findings. In this situation and with major food crops (such as corn), the potential for risk must be demonstrated to be extremely low.
- With respect to hazard assessment in Tier 1 ecological studies, the Agency should reevaluate its current approach of giving study effort and weight to sub-lethal effects only when it has been demonstrated that the $LC_{50} < 10X$ EEC.
- Fate and persistence studies should be performed on multiple soil types, representative of the soils on which the majority of the crop will be grown and soils measured for sufficient time to demonstrate how long the protein persists under field conditions.
- Develop protocols that clearly allow non-allergenicity in PIPs to be demonstrated.

PANEL DELIBERATIONS AND RESPONSE TO CHARGE

The specific issues addressed by the Panel are keyed to the Agency's background documents, references, and the Agency's charge questions.

Charge to the Panel - MIR604 Environmental Assessment

The weight of evidence from the reviewed data indicates that there will not be a hazard to wildlife from the commercialization of Event MIR604 corn. Although the mCry3A protein expressed by Event MIR604 corn is known to affect only coleopteran insect species, EPA assessed the potential risks, to a wide variety of non-target organisms (*i.e.* mammals, birds, fish, invertebrates and plants), that could potentially result from exposure to this *Bt* protein. The emphasis of this non-target risk assessment, however, was on invertebrate species that dominate corn agro-ecosystems. The Agency also evaluated a soil fate study that was intended to provide information on the persistence and rate of degradation of mCry3A protein in the soil environment. After reviewing data submitted in support of the Event MIR604 *Bt* corn registration, EPA concluded that aquatic and terrestrial wildlife, including soil organisms, were not likely to be adversely affected and that this *Bt* corn product is not likely to threaten the long-term survival of any non-target wildlife populations.

The Panel is requested to comment on the Agency's analysis of the currently available data on the potential impacts of Event MIR604 corn on non-target species.

Panel Response

The Panel reviewed the supplemental data provided in support of the Agency's ecological risk assessment. The sections below describe the Panel's conclusions with regard to the data provided and address specific components of the assessment under each subtitle.

The Standard for Maximum Hazard Dose Testing (MOE>10).

Several Panel members expressed the opinion that uncertainty in Margin of Exposure (MOE) estimates for maximum hazard dose testing should be quantified and used in the Agency's ecological risk assessment. Uncertainty in estimates of MOE is by definition a function of the uncertainty in Equivalent Test Concentration (ETC) estimates and Expected Environmental Concentration (EEC) estimates because $MOE = ETC/EEC$. When using surrogate test material, uncertainty in ETC includes consideration of the uncertainty of the correction factor used to adjust for the difference in the effectiveness between the test material and the protein as expressed in the Event MIR604 corn. Uncertainty in EEC can be addressed by making conservative assumptions concerning the diets of non-target organisms (NTO) in the field and incorporating the uncertainty in the estimate of mCry3A protein concentrations in relevant crop tissues. Finally, computing the confidence interval (CI) for the MOE requires estimation of the standard error of the MOE estimate which statistically is constructed as the ratio of the two random variables. A Taylor series expansion approximation of the standard error of this ratio can be computed as demonstrated in Cochran (1977, eq 6.13).

Several Panel members noted methodological and/or analytical inaccuracies in estimating ETCs and EECs. In some cases, it was possible to estimate correction factors to account for these inaccuracies. The Panel's comments on these issues are organized under the following headings: accuracy and standard errors (SE) of ETCs, accuracy and SEs of EECs, and 95% CIs of adjusted MOEs.

Accuracy and SEs of ETCs. A primary issue discussed by the Panel was whether the *E. coli*-derived MCRY3A-0102 test substance was equivalent in toxicity to mCry3A produced by MIR604 corn. Discussions of biochemical function suggested that there is reasonable cause to conclude that the two proteins are not substantially equivalent, with respect to the NTO toxicity tests, for the following reasons. About 50% of the mCry3A in corn is 55 kDa, a mass consistent with the activated form of mCry3A; but because the 55-kDa form of mCry3A was not sequenced, it is not certain that this is actually the activated form. The bacterially derived MCRY3A-0102, however, contains two forms, a short form (SF) and a long form (LF), neither of which is pre-activated. Independent bioassays of, respectively, the SF and LF against the western corn rootworm (WCRW) provided evidence that LF was much more difficult to activate, possibly due to the additional amino acids at the N-terminus. It was pointed out that to make MCRY3A-0102 equivalent to the MIR604 protein, about 50% of MCRY3A-0102 proteins should have been pre-activated with a cathepsin (or activated with some other proteases) in such a way as to yield about 50% of the protein in the 55-kDa activated form before being used in the bioassays. It was also unclear whether the various non-target organisms, especially the insects and other invertebrates, have cathepsins capable of activating the long and short forms of MCRY3A-0102. One Panelist noted that the Agency itself did not seem to agree on this issue. For example, the Agency's human health assessment considered the corn and *E. coli*-derived mCry3A proteins to be equally toxic, whereas the Agency's ecological risk assessment did not.

The western corn rootworm LC₅₀s of MCRY3A-0102 and LPMIR604-0103, a protein extract of mCry3A from leaves of MIR604 corn, were compared based on evaluation of the overlay of the associated 95% CIs. The Panel concluded that this test was inadequate to support the Registrant's conclusion that the tested materials "... demonstrated comparable activities ...". Overlap of 95% CIs is not sufficient to conclude statistically significant differences since the statistical significance of two estimates also depends on effective sample sizes and the form of the comparison test used. For very low sample sizes, and/or when a pooled variance term of the LC₅₀s is used in the hypothesis test, substantial overlap of 95% CIs, even to the degree that one estimate is included in the 95% CI for the other estimate, can occur simultaneously with a finding of significant difference between the population mean LC₅₀s. Also, there is a curious asymmetry in the 95% CI reported for the LC₅₀ of MCRY3A-0102. On a log(dose) scale, the 95% CI for the LC₅₀s should be symmetric. This is approximately the case for log(LC₅₀) of LPMIR604-0103 (-0.7±0.3) but not for log(LC₅₀) of MCRY3A-0102 (-0.4-0.5, -0.4+0.3). If one adjusts the lower limit of the 95% CI for LC₅₀ MCRY3A-0102 to be consistent with the upper limit, the 95% CI changes from 0.14-0.94 to 0.20-0.94 µg/ml, and the clear inclusion of the LC₅₀ for LPMIR604-0103 (0.20 µg/ml) within the 95% CI for MCRY3A-0102 is lost. The number of concentrations tested and the SEs of the LC₅₀s are not reported, so it is not possible to perform the appropriate significance test on the mean LC₅₀s or confirm the validity of the correction of the 95% CI. Conservatively, one must assume that the test material MCRY3A-0102 has a toxicity 0.46 [= (0.20 µg LPMIR604-0103/ml diet)/(0.43 µg MCRY3A-0102/ml

diet)] times that of mCry3A from MIR604 corn. If one makes the generous assumption that five concentrations were used to estimate the LC₅₀s, then a conservative estimate of the SE of this correction factor [with the appropriate adjustment for conversion from log(dose)] is 0.069 [$=0.46 \cdot \sqrt{2} \cdot (\text{AVE}[\frac{(95\% \text{ CI}_{\log})}{2}]) / t_{0.025}(3) = 0.46 \cdot \sqrt{2} \cdot 0.333 / 3.182$]. Thus, except for *Oncorhyncus*, which was dosed with feed formulated from MIR604 grain (see below), the ETC values estimated by the Agency should be multiplied by 0.46 (± 0.07) [mean (\pm SE)].

In the *Eisenia* toxicity tests, there are several problems with the ETC estimates provided. First, solutions for the *Leptinotarsa decemlineata*, Colorado potato beetle (CPB), toxicity tests to verify the presence of mCry3A in the test soil were prepared by adding an agar mixture at 50°C to the MCRY3A-0102-treated soil. This could have reduced the effectiveness of mCry3A by 11% based on Registrant data for temperature stability of MCRY3A-0102. Second, toxicity data in the CPB tests show much temporal variation in LC₅₀s that appears to be contrary to standard acute dose responses. Third, recovery of the nominal applied concentration of mCry3A ranged from 7-18% by ELISA. Analyses that conform to Agency criteria generally require 80-120% recovery (USEPA, 1996; see Tables 13-15). Approximate limits of quantification were reported as 0.06 to 0.28 µg/g for corn leaf and pollen (MRID 4615560-04). It must be noted that these limits are not method of quantification limits, but rather instrument quantification limits multiplied by a dilution factor. This does not account for the presence of interfering compounds or for matrix effects in the samples analyzed, which normally increase quantification limits. One Panel member suggested that a better method to test for concentrations in samples is needed and that quantitative polymerase chain reaction might be a relevant approach to consider. Fourth, the use of crude extracts in analyses that depend on polyclonal antibodies for toxin quantification raised questions regarding interferences. Finally, CPB mortality did not decline over time whereas the concentration of mCry3A did.

Un-aged, MCRY3A-0102-treated soil used in the *Eisenia* toxicity test was fed to CPB at rates of incorporation into the diet of 5% and 10%, and the resulting mortalities were assessed. In the same experiment, the mortality of CPB fed diet with MCRY3A-0102 added directly to the test soil ("neat") was assessed at two concentrations. (Note: the Agency's nominal calculation of 250 µg MCRY3A-0102/g in the test soil is slightly in error: the correct value is closer to the nominal value of 266 µg MCRY3A-0102/g diet.). The probit vs. log[dose] relationship based on the "neat" test data suggests that, in the presence of the test soil, MCRY3A-0102 has 30 (± 4)% the toxicity it has in the absence of the soil (Appendix 1). Thus, in the absence of dose verification with reasonable recoveries, a case can be made that the ETC value estimated by the Agency for *Eisenia* should be multiplied by a factor of 0.30 (± 0.04).

In the *Coccinella* toxicity test, Agral 90, a wetting agent included in the negative control treatment, showed no evidence of toxicity. However, no data are presented to support the presumption that Agral 90 did not denature or otherwise reduce the toxicity of MCRY3A-0102 in the MCRY3A-0102 treatment. Agral 90 and MCRY3A-0102 should have been tested against CPB as an indication that mCry3A toxicity was not reduced by Agral 90.

In the *Apis* toxicity test, the test material was administered as a suspension of 66.4 µg

MCRY3A-0102/ml in 50% aqueous sucrose solution. Viscous solutions or suspensions are known to increase the variability of dose delivery (Deicke and Suverkrup, 2000; Elkheshen et al., 1996). There are many examples of concentration of the test material in a dosing suspension being far lower than the nominal concentration (ca. 0.7X) (Thomas et al., 1987; Thomas et al., 1990; RTI 1992; Brewer et al., 1995; Ramsey et al., 2003). Such an effect can be caused by adsorption to solution containers and to transfer tools such as pipettes. Such errors lower the concentration of the test material in the test substrate and bias test results toward a finding of reduced toxicity.

Accuracy and SEs of EECs. Given the uncertainties of ecological processes, the Agency made appropriately conservative assumptions concerning the diets of NTOs in the field, and the resulting EECs are close to maximum estimates in most cases. However, there was a systematic error in correcting for extraction efficiency of mCry3A from plant tissues as submitted by the Registrant. The efficiency of extraction of mCry3A from plant tissues using the ELISA assay was estimated by assuming that all of the mCry3A remaining in an initial extraction is recovered by a second extraction. If one makes the more realistic assumption that a second extraction recovers mCry3A with the same efficiency as the first, then estimated extraction efficiency is reduced, the estimated concentration of mCry3A in MIR604 corn tissue is increased, the estimated value of EEC is increased, and the estimated value of MOE is decreased (Appendix 2). The Registrant reported extraction efficiencies for leaves, kernels, and silage of 77.1%, 69.7%, and 84.5%, respectively. Variance information for extraction efficiency is provided by the Registrant for leaf tissue only. Assuming that the coefficient of variation (CV) for extraction efficiency of the other tissues is approximately that for leaves, then standard errors can be estimated for all three. Applying the correction for extraction efficiencies (Appendix 2) and making the appropriate adjustments in standard errors for the reciprocal of random variables (Barford 1967), the efficiencies [mean (\pm SE)] of first extraction of leaves, kernels, and silage are estimated to be 70.3 (\pm 1.1)%, 56.5 (\pm 1.5)%, and 81.7 (\pm 0.8)%, respectively. Consequently, the Agency EEC values based on the concentration of mCry3A in MIR604 leaf tissues (*Orius*, *Coccinella*, *Poecilus*, *Apis*, and *Eisenia*) should be increased by 10% [i.e., multiplied by 1.10 (\pm 0.02)], those based on kernels (*Colinus*, *Mus*, and *Onchorhyncus*, as well as the ETC value for the *Onchorhyncus*) by 23% [i.e., multiplied by 1.23 (\pm 0.05)], and those based on silage (*Aleochara*) by 3% [i.e., multiplied by 1.03 (\pm 0.01)].

The Agency made an error in estimating EEC for *Aleochara*. The Agency's conservative assumption is that the diet of *Aleochara* in the field is lepidopteran larvae containing 70% the concentration of mCry3A observed in MIR604 tissue. Assuming that the relevant tissue is leaf tissue, the resulting EEC is 4.7 μ g/g. Thus, the Agency's EEC estimate for *Aleochara* of 1.07 μ g mCry3A/g tissue should be multiplied by 4.4.

95% Confidence Intervals of Adjusted MOEs. Combining the SEs for ETCs and EECs for adjusted estimates of MOEs (Barford 1967) results in MOE < 10 for five of the nine NTOs tested; Lower Bound of MOE 95% CI < 5 for five of the NTOs; and Lower Bound of MOE 95%

CI < 1 for two NTOs (Table 1). It should be reiterated that the data in Table 1 assume that the nominal test concentrations were in fact the concentrations administered.

Table 1. Adjustment of EPA Estimates for Effective Test Concentration (ETC), Expected Environmental Exposure (EEC), and Margin of Exposure (MOE, $MOE \equiv ETC/EEC$) for Toxicity Tests of Non-target Organisms Submitted by the Registrant in Support of an Application for Registration of MIR604 Corn

Non-target Organism	EPA-Estimated MOE	Adjusted, ETC [(SE), $\mu\text{g mCry3A g}^{-1}$ diet]	Adjusted, EEC [(SE), $\mu\text{g mCry3A g}^{-1}$ diet]	Adjusted MOE (SE)	95% CI of Adjusted MOE
<i>Orius</i>	10.6	23 (3)	5.0 (0.2)	4.6 (0.7)	2.3-6.0
<i>Poecilus</i>	11.2	5.5 (0.8)	5.0 (0.2)	1.1 (0.2)	0.55-1.7
<i>Aleochara</i>	15.6	23 (3)	3.3 (0.3)	6.9 (1.0)	3.6-10.1
<i>Coccinella</i>	12.3	4.1 (0.6)	0.78 (0.04)	5.3 (0.8)	2.7-8.0
<i>Apis</i>	36	23 (3)	1.5 (0.1)	15 (2.4)	7.7-23
<i>Eisenia</i>	46	37 (7.3)	6.0 (1.2)	6.2 (1.7)	0.68-12
<i>Oncorhyncus</i>	37	0.17 (0.01)	ETC/37 ^a	37	----
<i>Colinus</i>	1400	300 (45) ^b	0.55 (0.05) ^c	550 (100)	230-860
<i>Mus</i>	5500	1090 (164) ^b	0.52 (0.05) ^c	2100 (380)	900-3300

^aInsufficient data supplied by the Agency to calculate.

^bTest dose, $\mu\text{g mCry3A g}^{-1}$ body weight.

^cDaily dietary dose, $\mu\text{g mCry3A g}^{-1}$ body weight.

According to OPPTS 885.4340 (Microbial Pesticide Test Guidelines for Tier 1 Nontarget Insect Testing), the minimum required concentration for maximum hazard dose testing is 10X EEC. The Panel was split on the importance of the Registrant not adhering to this standard. Several Panel members considered this a serious deficiency but a deficiency that easily could have been avoided. Other Panel members considered the test concentrations/doses adequate to support the Agency's finding of no likely adverse ecological effects.

Estimation of 95% CIs for MOEs, although based on incomplete information, represents an enhancement of hazard assessment that may be useful in the Agency's overall risk assessment process. Adding confidence intervals provides the basis to identify areas of concern on which the Agency may wish to have the Registrant focus additional research. In addition, it would enable future Panels that may be asked to consider related issues to make more definitive decisions regarding the quality of the data supporting the analyses

Experimental Design and Statistics.

Most Panel members thought that the experimental design and statistical analysis/reporting in the Registrant's NTO toxicity testing were frequently inadequate. Several Panel members were of the opinion that standards of experimental design, data analysis, and statistical reporting in studies submitted in support of requests for registration of crops with PIPs should be no less than those required for publication in professional scientific journals. The Panel's concerns are organized into consideration of the following two topics: improper or insufficient replication; and failure to report sample sizes, SEs, and test statistics/P-values or even failure to perform statistical tests of interest.

Replication. In toxicity testing of individuals, one can define a “replicate” as an experimental unit (whether a single subject or a group of subjects) from which a value for the random variable of interest is generated independently of other units. The experimental unit can be a single subject when the random variable of interest is an individual trait (e.g. weight), or it can be a group of subjects when the random variable of interest is a group trait (e.g. % mortality).

Mortality data can be tested for differences among treatment groups using Fisher's Exact Test or Chi-Square—as was done in the vertebrate NTO toxicity tests: *Oncorhynchus*, *Colinus*, and *Mus*. Unfortunately, in these tests, all individuals in a given treatment group were housed in a single tank, pen, or cage, respectively. Consequently, treatment effects are confounded with any differences between the two groups due to uncontrolled factors so that the datum collected from a given subject (“alive” or “dead”, in this case) is not independent of the data collected from the other subjects. Although the requirement of independence of data must be qualified to require independence with respect to sources of experimental error that are considered likely, it was the consensus of the Panel that the vertebrate NTO toxicity tests were deficient in this respect (Table 2). In addition, one Panel member preferred to have replication of groups of experimental units for tests involving percent mortality as an endpoint, and another expressed the opinion that none of the NTO toxicity tests satisfied the requirement of reasonable independence of experimental units because they had not been repeated.

Table 2. Statistical Design/Analysis/Reporting in Non-target Organism and Auxiliary Studies Submitted by the Registrant in Support of Application for Registration of MIR604 Corn.

Non-target Species/ Study	Valid Replication	Sufficient Replication	SEs Reported	Tests/Analyses Performed and Valid
Characterization of MCRY3A-0102	✓	✓	X	X
MCRY3A-0102: Further Analysis	✓	✓	X	X
Quantification of mCry3a in MIR604	✓	X	✓	X
Soil Degradation	X	X	X	X
<i>Orius</i> toxicity	✓	✓	✓	✓
<i>Orius</i> Diet Validation	✓	X	X	X
<i>Poecilus</i> Toxicity	✓	✓	✓	X
<i>Poecilus</i> Diet Validation	✓	X	X	X
<i>Aleochara</i> Toxicity	✓	✓	✓	✓
<i>Aleochara</i> Diet Validation	✓	X	X	X
<i>Coccinella</i> Toxicity	✓	✓	✓	✓
<i>Coccinella</i> Diet Validation	✓	X	X	✓
<i>Apis</i>	✓	X	✓	✓
<i>Eisenia</i> Toxicity	✓	✓	✓	✓
<i>Eisenia</i> Diet Validation	✓	X	X	X
<i>Oncorhyncus</i> Toxicity	X	✓	✓	X
<i>Oncorhyncus</i> Diet Validation	✓	✓	✓	✓
<i>Colinus</i>	X	X	✓	X
<i>Mus</i>	X	X	✓	X

✓=Adequate

X=Inadequate

Insufficient replication reduces the value of data presented as the basis for making ecological risk assessment decisions because the power of statistical comparisons is insufficient to detect treatment effects of potentially ecologically-significant magnitude. The consensus of the Panel was that the number of replicates or sample sizes in the toxicity tests of *Colinus* and *Mus* was too low: 5 individuals per sex X treatment group combination. Other concerns regarding insufficient sample size included the following: all of the ELISA assays of NTO toxicity test diets (except *Oncorhyncus*) were unreplicated, although in two cases (*Poecilus* and *Coccinella*), units of the test diet (fly pupae and aphids, respectively) were composited for the single analysis performed; the feed consumption rate observations for *Colinus* and *Mus* were

unreplicated; and the sample size in the *Apis* toxicity test (N = 4) provided insufficient statistical power given the high level of variance observed in the negative controls (Table 2).

Statistics: Performing and Reporting. Raw data were supplied for all of the NTO toxicity tests, so it was possible for the Panel to calculate SEs and perform statistical tests even when such statistical tests were not reported by the Registrant. However, in the soil degradation study (except for the microbial activity observations) and in all of the CPB assays used in the NTO toxicity tests to verify the presence of mCry3A in the diets, the raw data were not supplied and SEs were not reported. In addition, the Registrant apparently did not perform tests for the sex X treatment effect for *Poecilus* adult weight, the sex X treatment effect for *Colinus* weight gain, and differences in variance of *Oncorhynchus* weight gain over time (Table 2). As discussed above, a valid statistical test to compare LC₅₀s of MCRY3A-0102 and LPMIR604-0103 was not performed. Also, a similar failure occurred in comparing LC₅₀s for MCRY3A-0102-SF and MCRY3A-0102-LF (Table 2). In the opinion of several Panel members, these failures represent a serious deficiency in the value of the evidence presented by the Registrant.

Non-lethal PIP Effects.

If one takes the NTO toxicity assays of this Tier I hazard assessment at face value, none of them reported high acute toxicity. Rather, in most instances mortality was low or did not occur at the ETCs achieved. Although the ETCs frequently did not meet the standard for maximum hazard dose testing, these results do support the conclusion of the absence of a strong, toxic response in the NTOs. However, sub-lethal endpoints (weight gain) measured in the toxicity tests of *Oncorhynchus* and *Colinus* may imply signs of stress that were not translated into mortality within the time frame and conditions of the tests conducted.

The statistical analysis provided by the Registrant for the *Oncorhynchus* toxicity test is incomplete. Although it is stated that homogeneity of variances between treatment and control groups was tested, it was not reported that there was a significant increase in the variances of size and weight for treated fish relative to untreated over the course of the experiment. The variance of weight in the treatment group was 1.88 times that for the control group by day 14 (F = 1.88; df = 39, 39; P < 0.05) and 2.67 times greater by the end of the test on day 28 (F = 1.88; df = 38, 39; P < 0.01) (Fig. 1a). The variance of length in the treatment group was 1.60 times that for the control group by day 14 (F = 1.60; df = 39, 39; 0.05 < P < 0.10) and 2.52 times greater by day 28 (F = 2.67; df = 38, 39; P < 0.01) (Fig. 1b). The increase in variance in the treatment group could be the result of stress due to the PIP acting on genetic variability among the test subjects. A test of this hypothesis can be performed by examining the effect of the PIP on a condition index [$CdI = \text{Length}/(\text{Wt})^{1/3}$] for the data collected on Day 28, the day of the final observation. For the control group, $CdI_C = 39.92 (0.133, 40)$ [mean (SE, N)]; and if an outlier for CI is excluded from the treatment group (Z = 3.432; P = 0.0003), $CdI_T = 39.41 (0.153, 38)$. The variances of CdI_T and CdI_C are not significantly different (F = 1.27; df = 37, 39; P = 0.465), and $H_0: \text{mean}[CdI_T] = \text{mean}[CdI_C]$ is rejected (Z = 2.511; P = 0.0142). Thus, for a given body weight, *Oncorhynchus* fed a diet containing MIR604 corn grain were shorter than those fed the control diet.

Fig. 1a. Weight and SD[Weight] of *Oncorhyncus* in Toxicity Test

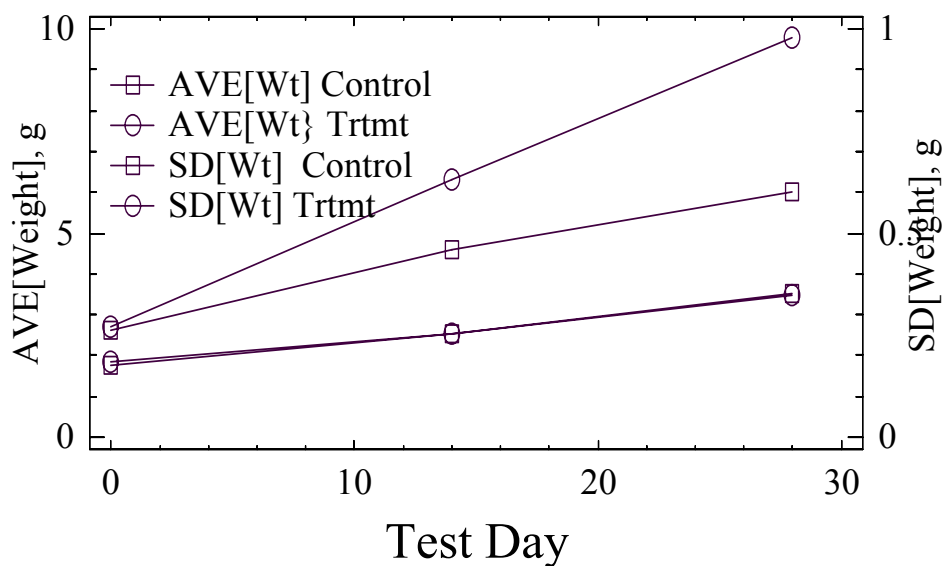
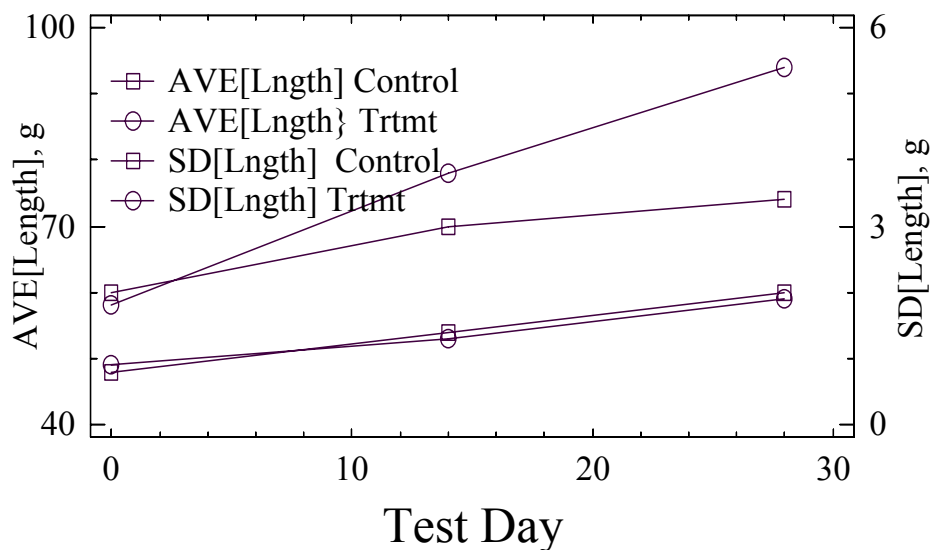


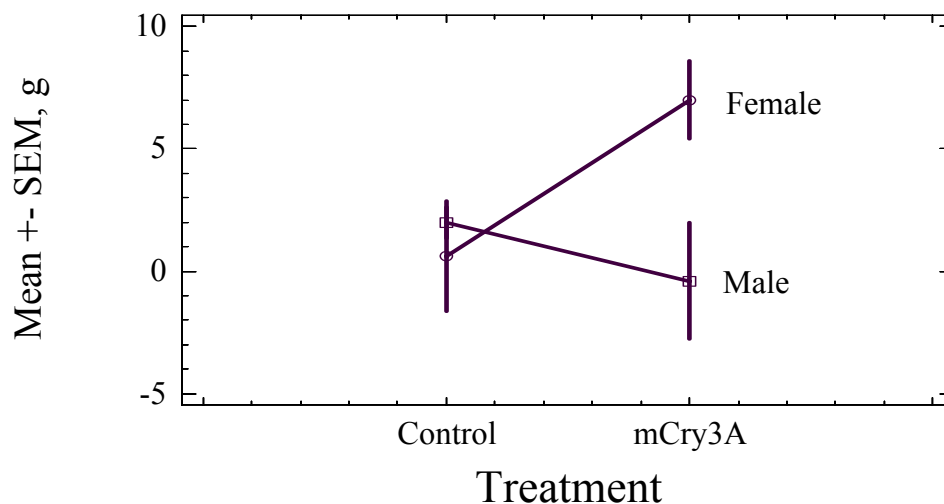
Fig. 1b. Length and SD[Length] of *Oncorhyncus* in Toxicity Test



The statistical analysis provided by the Registrant for the *Colinus* toxicity test is incomplete. It was not reported that there was a significant interaction between Treatment Group (TG) and Sex (S) when weight change (ΔWt) is the dependent variable in an analysis of variance with TG, S, and TG x S as sources of variation ($F = 5.69$; $df = 1, 16$; $P = 0.0298$). For

males in the group fed feed containing MIR604 corn grain, ΔWt was lower than that in the control group while the reverse was true for females (Fig. 2).

Fig. 2. Change in Weight of *Colinus* (by Sex) in NTO Toxicity Test



In both of the toxicity tests in which the subjects were fed MIR604 corn itself, treatment effects were observed. Whether these effects are harmful, beneficial, or neutral for *Oncorhyncus* and *Colinus* is unclear. Also, as noted above, the validity of these tests are in question due to improper replication. However, several Panel members were of the opinion that such responses should trigger subsequent hazard assessments of PIPs at and below 1X EEC.

At present, sub-lethal endpoints observed during Tier I maximum hazard dose testing of NTOs are given no weight and follow-up testing is triggered only for $LC_{50} < 10X$ EEC. However, sub-lethal effects may represent very important ecological information. For example, observations of discoloration, sounding and surfacing in the *Oncorhyncus* test, while not causing increased lab mortality, may significantly increase predation rates in the field. Redundant response across multiple taxa, as was observed here, should be of particular concern. Future study designs should use models that assess the effectiveness of copulation, survival and development of offspring, and subsequent emergence success as adults. The Panel recommends that the Agency reevaluate its current approach with respect to the weight given to sub-lethal effects observed in Tier 1 toxicity testing.

A number of Panel members suggested that the Agency better define its basic approach to data analysis and interpretation for these studies (e.g., is ANOVA or multiple T-tests recommended for comparison of treatment groups?). This assessment also points to a need to develop strategies for evaluating and using evidence in situations where different statistical analyses produce conflicting results.

Endangered Species.

Hungerford's crawling water beetle (*Brychius hungerfordi*; Coleoptera: Haliplidae), an endangered beetle species, occurs within habitats in corn production areas of Michigan's northern Lower Peninsula where they could encounter corn crop residues. Both adults and larvae are herbivorous and live in moderate to fast-flowing streams of the Cheboygan River watershed, but the larvae feed in riverbank soils affording potential exposure to plant residues. It is possible that this endangered species could be exposed to mCry3A from MIR604 corn planted in this region. Several panelists strongly suggested that the Agency consider the potential impact of MIR604 corn on *B. hungerfordi*.

Aquatic Species Testing.

Oncorhynchus Toxicity Test. Findings are generally in agreement with what is known about acute toxicity of *Bacillus thuringiensis* (Bt), *Bacillus thuringiensis israeliensis* (Bti), and *Bacillus sphaericus* (Bs) in fish, fiddler crabs and grass shrimp. Typically, mortality occurs at concentrations that are much greater than the expected environmental concentrations (Lee and Scott, 1989; Dee, 1988; Banks, 1988; Lee, 1987). These studies estimated a MOE of approximately 900 for fish based upon the NOEC and an EEC based on Bt application rates for mosquito control in 12 inches of standing water.

The Agency did not consider the importance of components of feed formulations on carrier molecules in the NTO. Research has shown that antioxidants such as ethoxyquin (very common in most commercial foods) will turn on carrier proteins in fish, such as p-glycoproteins and multi-drug resistance proteins. Up-regulation of these accessory proteins could be very important in affecting uptake of Bt and Cry3A proteins. Up-regulation may result in either greater uptake or lowered uptake depending on the specific protein pathway affected. Greater uptake may result in an overestimation of toxic effects while down regulation may mean that bioeffects have been underestimated.

Waiver of Estuarine and Marine Animal Toxicity Testing. The Agency maintains that estuarine and marine animal studies are not required for this product, because mCry3A is not intended for direct application to estuarine or marine environments and there is very low potential that these ecosystems will be exposed to mCry3A protein in MIR604 corn. Although the risks of exposure in marine/estuarine systems may be low, there could be concern in areas where large acreages of corn are grown in coastal plain habitats that discharge directly into receiving streams. Based upon the discharge of conventional pesticides and plot studies, applied pesticide losses are generally in the < 5% range, but may approach 20% during catastrophic events (> 2 inches of rain immediately after application). The Agency's overall risk assessment for aquatic environments emphasizes the importance of pollen as the primary aquatic exposure route, but non-point source (NPS) runoff from corn fields has not been excluded by direct study. The Agency should commission studies to verify that indeed there is no NPS runoff risk from PIP corn into aquatic ecosystems including marine/estuarine ecosystems. This could be accomplished most efficiently by sampling sediments from retention ponds draining corn fields or streams that border corn fields. An absence of mCry3A proteins in these sites would preclude their occurrence in more distant receiving bodies of water.

Some aquatic testing of marine organisms should be considered if PIP corn is expected to be grown on sufficient acreage where events might conspire to move a significant amount of mCry3A proteins into marine systems where it would otherwise not normally be encountered. Freshwater fish and marine fish differ greatly in their physiology: marine fish may drink several times their body weight per day while freshwater fish do not. Agency Water Quality Criteria documents have recognized this difference as being extremely important. In addition, support for the waiver did not appear to consider that aquatic plants, such as phytoplankton and blue green algae, and bacteria have the greatest potential for gene flow concerns because of their very short growth cycles. Finally, if the Agency does require additional testing in marine systems, it would be important to use a species such as the marine copepod *Amphiascus tenuiremis*, which allows multi-generational assessments on growth, survival, and reproduction as well as population estimations using a Leslie matrix population model linked with Monte Carlo simulation. However, in using copepods for NTO toxicity testing of PIPs, it is important to take into account, by carefully defining control groups, that egg production may be increased as they apparently use the protein as a source of food.

While much of the presented data support the claim for waiver of estuarine and marine animal toxicity testing, the uncertainties and concerns expressed above suggests that the Agency should consider requesting the Registrant provide additional information to decrease these uncertainties before consideration of a waiver request.

Laboratory Soil Degradation Study.

General Comments. As already identified by the Agency, this study is not sufficiently robust to draw conclusions with confidence regarding how long the mCry3A protein is likely to persist and retain insecticidal activity in different soils. Reports suggest that: (i) the Cry1Ab protein binds to clays in soil, thus protecting it from bacterial degradation and increasing its residence time (Tapp and Stotzky, 1998); (ii) the Cry1Ab protein is released from the roots of Bt corn (Saxena and Stotzky, 2000); and (iii) the rate of decomposition of Bt crop residues is lower compared to non-Bt crop residues (Saxena et al., 2004; Flores et al., 2005). These reports have raised public concern and increased scrutiny in this area. Thus, the Registrant should supply a dataset, properly replicated and statistically evaluated, that provides the likely upper and lower limits for the longevity of the protein derived from crop residues in soil. There is a rapidly expanding body of published research that shows Bt residues containing other Cry proteins (e.g., Cry1Ac) degrade readily and quickly in soil (Head et al., 2002; Zwalen et al., 2003). However, there are also a few studies reporting results to the contrary (e.g., Palm et al., 1996). Since public concern is heightened on this specific topic, more information should be supplied to increase confidence in the low risk of the protein persisting in soil post-cropping.

Reports supplied by the Registrant provide results of a single study on a single soil using the purified bacterially-derived MCRY3A-0102 protein. As residues, and potentially root exudates, are a major environmental source of this protein, soil degradation studies should be conducted with the residues themselves, as already proposed by the Agency. The characteristic binding to clays, humic materials and soil organic matter will differ between the purified protein

and its form when released from, or bound to, decomposing residues. Therefore, the likelihood of the protein's persistence in soil should be evaluated using the plant materials in this case.

Although this test was meant to "screen" for potential soil accumulation and longer-term toxicity to susceptible NTOs, there was insufficient evidence supplied to project the decomposition kinetics of residue-derived mCry3A protein with confidence across the range of soil types on which this crop may be grown.

While the Registrant appears to have followed many stipulations of the protocols accepted by the EPA for Tier 1 "screening", these protocols may require some adjustment for future studies as the Cry proteins do differ substantially from chemical pesticides in their likely fate and persistence in soil. Although the Agency did not appear to require statistically robust experimental designs in this Tier 1 "screening" process, attention to detail and full reporting, at a minimum, should be expected.

Specific comments about the conduct of the laboratory soil degradation study are provided as follows:

1. A single soil sample to a depth of 6" was collected from a single farm site and transported to the laboratory.
 - a. Is this just a 'random' soil? Why was it selected? Information on how the soil was selected and collected is expected. From what total area was the soil collected? Was the sample a composite of multiple samples across the field site?
 - b. Information on the soil history is warranted. What was grown on this land prior to sampling? Was the soil subjected to any treatment that might interfere with the degradation study (enhance or reduce binding or metabolism of the protein)?
2. The soil selected had several properties that made it a good choice as a test soil. Published reports of the protein binding to clays and soil organic matter (SOM) indicate that the protein is likely to have increased persistence in soils with higher clay content, cation exchange capacity (CEC) and SOM, thus higher clay soils should be targeted. The soil tested had a clay content of 30%, CEC of 19.7 meq/100 g and 3.8% SOM, which are within ranges where binding of the Bt protein to the soil complex is likely.
 - a. Five other soil types which may contain 30% or greater clay contents (clay, silty clay, sandy clay, clay loam and sandy clay loam) could be targeted for testing to give better range of persistence across varying environments.
 - b. Target specifically those soil types that dominate the regions where this crop will be grown. If the dominant soil types have lower clay contents and SOM, then these should be selected.
 - c. Aim for a range of soil types to yield understanding of how the protein derived from plant tissue behaves in different environments.
3. The soil was acclimatized for 10 days, which decreases sampling effects on soil respiration and stabilizes the soil moisture content. All units tested were sub-samples from this single soil sample. This is typical for many controlled studies.
4. The Registrant prepared a single aqueous solution of the protein in de-ionized water. Sub-samples of this solution were then used to treat all experimental units.

- a. Is the solubility of the MCRY3A-0102 protein in de-ionized water sufficient to ensure that each 5 ml sub-sample taken contains the concentration targeted ($230 \mu\text{g g}^{-1}$ soil) for each experimental unit (50 g soil)?
5. Twenty-four experimental units were treated with the protein, and destructive sampling of 2 units at 0, 1, 3, 7, 12, 30, 45, 60 and 90 days was intended.
 - a. Data were presented only for units sampled up until 30 days. What happened to the 45, 60 and 90 day samples?
 - b. Samples from the 0, 1, 3, 7, 12, 30 day time-points were frozen until used to prepare a diet for follow-on toxicity tests on CPB.
6. Only 1 experimental unit was left untreated (de-ionized water only added) as a “control”, and this unit was repeatedly sub-sampled at each time point. Thus, the control treatment was essentially not replicated.
 - a. It is unclear how many sub-samples were taken from this single experimental unit at each time point.
 - b. It is unclear how many of these control samples were eventually tested.
 - c. How was the mean value of 18% mortality in the untreated control derived? Since there is only a single experimental unit, all sub-samples are repeated measures, thus a true variance for this treatment cannot be derived.
7. An additional 6 untreated experimental units were set up to demonstrate that there was an active microbial community present in the test soil. Substrate induced soil respiration (SIR), using standard protocols, was measured on 3 untreated units at 0 time. The remaining 3 untreated units were incubated along with the main body of the experiment and then assayed for SIR at 42 days.
 - a. Why was the second SIR measurement made 12 days after the last reported data from the main experiment?
 - b. Why were only untreated soils tested?
 - c. To demonstrate that an active microbial community potentially capable of degrading the protein is present in the test soils, those samples in which the protein was incorporated should also be tested. It is easy to do this test and it answers the question more directly.
 - d. If only 3, 1-g sub-samples from each experimental unit are used for toxicity testing on CPB, the remaining soil can be used to measure SIR.
 - e. Use of two or more true experimental units, each sampled over time would allow formal statistical analysis to estimate uncertainty and test for effects/trends.
8. At each time point, 2 experimental units were sampled. Thus, if use of a single aqueous solution as the starting treatment is considered sufficiently independent, there are only two replicates for each sampling time for the treated soils.
 - a. Three, 1-g soil samples were taken from each experimental unit. These were used to prepare CPB media that contained 10% soil (1 g in 9 ml of medium).
 - b. Each of these preparations was used to pour two Petri dishes. Thus, each dish contained approximately 0.5 g soil and 4.5 ml of CPB medium.
 - c. For each sampling time, 12 Petri dishes were prepared. However, these represent duplicate plates of a single sample and 3 sub-samples from each of 2 experimental units. No matter how it is viewed, there are never more than 2 replicates per time point, not the 12 suggested in the report.

9. Purified MCRY3A-0102 protein was incorporated on its own into the CPB medium as a positive control. This treatment was replicated 4 times. No tests were done on the medium itself (without soil or purified protein added). The mortality of CPB on the soil control treatment was high (18%), but we do not know if this was related to adding soil to the medium or some problem with the medium itself. It is important to know the level of survivorship under the general experimental conditions in order to properly evaluate the mortality rate on the soil control (and in the treated soils and in media to which the protein was added directly).
10. Both a bar chart and a table are presented with mortality values derived as a straight mean of 12 plates per time point for the treated soils, a total of 4 plates for the purified protein across the whole experiment, and an unknown number of control soil samples. The Agency and the Panel should have access to the full dataset.
 - a. What was the between plate and within plate variability for the treated soils?
 - b. What was the variance observed between the two actual replicates?
 - c. How repeatable are the numbers we see in both Table 1 and Figure 1?
 - d. The Registrant should supply all data, and in particular, any data used in a statistical analysis.
11. The data used to model the degradation kinetics and derive a DT_{50} value are questionable. The “observed” values on the graph were derived by subtracting the “mean” value of the Day 30 mortality from the “mean” mortality of CPB on treated soils at each time point. This leads to a calculation of zero mortality at Day 30, but it is not at all certain that there is no toxicity to CPB after 30 days based on this test system.
 - a. A number of different decline models could have been fit to these data with the same or similar precision.
 - b. Given that the DT_{50} value was improperly derived, the reported value is not a good indicator of what the kinetics of degradation are likely to be.
12. More details on the CPB medium formulation are needed. Four different antibiotics were added to the medium at 0.1 ml g^{-1} diet. No concentrations of these antibiotics are reported. Thus, we have no capacity to replicate this experiment.

Interaction between mCry3A and Conventional Insecticides.

Two additional recommendations to the Agency were made by Panel members concerning potential functional and economic interactions between mCry3A and conventional insecticides.

Functional Interaction. The use of other chemicals in Bt corn needs to be considered. Studies of *Bti* and fenoxycarb have shown greater than additive toxicity in grass shrimp and mosquitoes (Dee, 1988). Multiple factor interactions, including additive toxicity with other chemicals, must be rigorously evaluated by looking at conventional pesticides already registered for use on the crop and conducting basic mixture toxicity tests. This may happen with other chemicals used in conjunction with MIR604 corn in the coastal zone.

Economic Interaction. Information provided in public testimony before the Panel indicated that the costs of conventional pesticide use has not dramatically changed with the

introduction of Bt corn. While this testimony did not provide quantitative and statistical analyses of pesticide cost/usage data, this information is important for assessing the relative risks and benefits of PIPs in general. The testimony described increased Bt corn yields as a benefit, but costs associated with conventional chemical pesticide usage had not dropped appreciably in Bt corn production. While this situation could be a result of sequential corn planting without rotation to soybeans, it will affect the cost/benefit analysis and should be evaluated. In general, the Agency should consider quantitatively assessing the effect PIPs may have on the economics of conventional pesticide use when considering future PIP registrations, if a justification for discounting safety concerns with a PIP is asserted based upon decreased use of conventional pesticides.

Additional Data Requirements

The Agency's ecological risk assessment states that "The Agency has sufficient information to believe that there is no risk from the proposed uses of mCry3A corn to non-target wildlife, aquatic, and soil organisms. However, in response to the August 2002 SAP recommendations, the Agency is requesting supplementary studies that will evaluate the persistence of mCry3A in the soil and the long range effects of cultivation of mCry3A on the invertebrate community structure in corn fields. This will facilitate identification of potential adverse effects which may result from long-term use of this product." In particular, the following two types of supplemental studies were identified:

Ecosystem effects: Long-term field studies should be conducted based on recommendations of the August, 2002 SAP.

Soil fate studies: Long-term soil degradation field studies should be conducted. Studies should follow guidelines outlined by the August 2002 SAP.

Ecosystem Effects Studies

The August 2002 FIFRA SAP recommended field-scale census studies of 3-4 years duration to provide information on the general ecological attributes of PIP crops. In addition, they provided at least 13 suggestions for improving the effectiveness of such studies. The consensus among current Panel members strongly supported the Agency's request for such supplemental studies because they could provide an evaluation of the Agency's primarily laboratory-based toxicity/bioassays and predictive modeling approach to environmental hazard assessment.

Non-target impacts of PIPs in corn agro-ecosystems are likely to affect ecosystem function if soil food web structures, ecosystem working order, or numerical species relationships in leaf- and root-zone guilds are shifted. The temporal and spatial scales of these changes are just as likely to accrue advantages as disadvantages to farmers and society but will go undetected without sustained record-keeping. Even though ecosystem function changes are likely to be subtle, they do lend themselves to detection in routine Integrated Pest Management (IPM) and Insect Resistance Management (IRM) monitoring systems tuned to observe and record natural

enemies as well as typical pest species monitored by industry representatives, IPM consultants, extension agents and/or scouts. These effects compared and contrasted with similar measures in nearby conventional IRM fields provide an ideal replicated contrast. Such a long-term ecological transition study may yield valuable insight especially where shifting national priorities lead to more “corn on corn” acreage associated with biofuels policies as public testimony conveyed.

Soil Fate Studies

The August 2002 SAP recommended that the PIP persistence be monitored in the field in at least three different soil types until they become undetectable, but for a minimum of one growing season after harvest. The consensus among current Panel members strongly supported the Agency’s request for such supplemental studies. However, the current Panel sees a need for the Agency to design the requisite, statistically defensible tests that will be used to establish a condition of "undetectable" in these field studies. Public awareness and concern regarding the issue of PIP persistence in soil is high and needs to be addressed. However, the time and resources of the Registrant, the Agency, and independent reviewers will be completely wasted unless said field trials are properly designed, replicated, and sampled and unless the samples are analyzed in a robust experimental system with proper design and replication. Attention to statistical and analytical designs in the field and laboratory is urgently needed.

First, soil types tested in proposed field trials should not be restricted to those with high clay contents, especially if MIR604 corn is unlikely to be planted on these soil types. Soils chosen should represent at least the three major soil types on which this crop will be grown.

Second, to assess the persistence of the mCry3A protein (derived from plant tissue or possibly root exudates) through successive cropping years and the duration of its bioactivity in soil requires that a method be developed to track proteins released from residues of varying age. The Agency has requested that the Registrant sample fields where MIR604 corn has been grown for at least 3 years. If the protein is not bio-available (no detection) after a specified time post-cropping, then age of inputs is irrelevant. However, if active protein is detected in tested soils, how will the age of the proteins detected be determined to establish ‘longevity’? How will this potentially confounding factor be handled? The Agency should be sure the design is sufficiently robust to generate the data requested, such that it will satisfy the data requirements of the Agency and enable more thorough reviews by any future panels that may consider related issues.

Third, the temporal bioavailability of Cry protein in soil is a factor that will strongly influence toxicity to NTOs. Thus, results of the CPB assay are more relevant in field studies than are ELISA assays. The ELISA results are clearly confounded by low extraction efficiencies and high interference from other soil components and thus not sufficiently robust for use in field tests. Other methods to track protein persistence in soil should be developed.

Charge to the Panel - MIR604 Human Health and Characterization - Protein Equivalence

Previously submitted studies for Event MIR604 demonstrated the equivalence of the plant- and bacterial- produced test substances by showing similar molecular weights, purity of 90.3%, a lack of post-translational glycosylation of mCry3A from either source, and comparable toxicities toward western corn rootworm (WCRW). However, two forms of mCry3A (designated as mCry3A-sf and mCry3A-lf) were found in the bacterial-produced test material (MCRY3A-0102). The molecular weights of the short and long form of mCry3A were 67.5 kDa and 69.1 kDa, respectively, determined via SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) and MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight) mass spectrometry. The lesser of the two components, with the lower molecular weight, corresponded to the intended mCry3A protein with 598 amino acids. The other component contained the same 598 amino acids as the first component but also contained an additional 16 amino acids at the N-terminal end of the protein. Both mCry3A forms were insecticidally active against WCRW. On this basis, and taking into account the high degree of structural homology (97.4% amino acid identity), the two forms of mCry3A in test material MCRY3A-0102 were considered to be equivalent.

Please comment on the Agency's conclusion that the mCry3A proteins from corn event MIR604 and from recombinant E. coli are substantially the same for the purpose of the Agency's risk assessment; and that the equivalence is confirmed for the two forms of the bacterial-produced mCry3A test material (MCRY3A-0102).

Panel Response

Cry3A is a protein endotoxin produced naturally by the *tenebrionis* strain of *Bacillus thuringiensis* subsp. *morrisoni* (H 8a8b) that upon ingestion and proteolytic activation exhibits a high level of insecticidal activity to certain species of coleopteran insects, but otherwise is apparently non-toxic to other types of insects, as well as vertebrates. The basic biology of this protein is reasonably well known. The wild type protein is produced as a 67 kDa protoxin protein, and has been engineered into other subspecies of *B. thuringiensis*, *E. coli*, and a few crop plant species, mainly potato and corn. The wild type protein has served as a model Cry toxin for basic studies of Bt, and was the first Bt endotoxin for which the 3-dimensional structure was solved. The availability of this structure along with site-directed mutational analysis of Cry3A and other Cry proteins based on this structure provided important insights into the selectivity and mode of action of these insecticidal proteins. Specifically, these studies showed that the selectivity of binding to receptors on the midgut microvilli is controlled by domains II and III of this three-domain molecule. After ingestion by a sensitive insect, the molecule must be activated by proteolytic cleavage carried out by midgut proteases. This activation by proteolytic

cleavage occurs at both the N- and C-termini. Once the protoxin molecule is activated, the protein binds to specific receptors on midgut microvilli, typically glycosylated enzymes or glycolipids, after which it inserts into the membrane, oligomerizes, and forms cation-selective pores that lead to midgut cell lysis and insect death. The Cry3A midgut receptor in coleopteran insects has not been identified, but for lepidopteran insects, depending on the species, receptors include amino peptidases, alkaline phosphatases, and cadherins.

The Event MIR604 corn being considered for registration and the subject of this SAP, contains a modified Cry3A in which a cathepsin G cleavage site was created beginning at amino acid 155 by replacing the valine-serine-serine sequence with alanine-alanine-proline followed by an additional amino acid, phenylalanine. The new cleavage site occurs between alpha-helices 3 and 4 of domain I, just upstream from the trypsin and chymotrypsin sites present in wild type Cry3A. The Registrant named this genetically modified endotoxin “mCry3A” to differentiate it from the wild type Cry3A. The cathepsin cleavage site was introduced into the molecule because it enhances the toxicity of Cry3A against the western corn rootworm, *Diabrotica virgifera virgifera*, which apparently uses a cathepsin protease as one of its key midgut proteases. *In vitro* digestion experiments comparing cleavage of mCry3A versus Cry3A with chymotrypsin show that both are cleaved to a 55-kDa protein. However, this cleavage occurs much more quickly, within 30 minutes, for mCry3A, compared to four hours for wild type Cry3A. The receptor binding regions in domains II and III are the same in Cry3A and mCry3A, and thus the binding properties of these two molecules - the primary regions of the molecule responsible for insect target spectrum - should be substantially similar, if not the same. Thus, the addition of a cathepsin cleavage site should not alter the spectrum of activity of mCry3A compared to that of Cry3A. It is, however, possible that this cleavage site could decrease the environmental stability of mCry3A compared to Cry3A. In fact, the finding by the Registrant that the dominant forms of mCry3A in event MIR604 corn are peptides of 67 and 55 kDa in approximately equal amounts shows that the protein is cleaved/activated in corn tissue, and therefore could be considered “less stable” in the plant compared to Cry3A. This processing in plant tissue to the activated form of mCry3A probably accounts for a substantial portion of the higher activity of mCry3A corn extract (LPMIR604-0103) compared to mCry3A (MCRY3A-0102) produced in *E. coli*, as determined by the Registrant in bioassays against first instar western corn rootworms.

In addition to adding a cathepsin cleavage site, to optimize synthesis in corn, the Registrant constructed a synthetic *mCry3A* gene based on plant codon usage. The wild type *Cry3A* gene encodes an endotoxin protein of 73-kDa. Rather than synthesize the entire gene, as the full-length protein encoded by the wild type gene is not required for toxicity, the Registrant synthesized a truncated gene that encodes a protein beginning at methionine-48 of wild type Cry3A. For expression in *E. coli*, the gene was cloned into a pUC expression vector that used a *cryIA* promoter system that included 16 amino acids upstream from the 67.7 mCry3A open reading frame. Expression of this construct in *E. coli* yielded two forms of the mCry3A, one of the predicted mass, 67.7 kDa, referred to as the short form (mCry3A-SF), and a second product of 69.5 kDa, referred to as the long form (mCry3A-LF). These occurred in a ratio of 2 parts SF to 3 parts LF in the mCry3A inclusions isolated from *E. coli*. Importantly, the long form of mCry3A appeared significantly less toxic ($LC_{50} = 361 \mu\text{g/ml}$ diet) than the short form ($LC_{50} = 82 \mu\text{g/ml}$ diet) to first instar western corn rootworms. However, the Registrant noted this difference was within the 95% confidence level of the assay and did not consider these forms different in activity. The substantially lower toxicity of the long form in the *E. coli*-derived mCry3A,

especially compared to the corn-derived mCry3A, of which approximately 50% was already in an activated form, is another factor that likely contributed to the approximately 2-fold difference between the toxicity values of the corn-derived mCry3A (LPMIR604-0103, $LC_{50} = 0.20 \mu\text{g/ml}$ diet) versus the *E. coli*-derived protein (MCRY3A-0102, $LC_{50} = 0.43 \mu\text{g/ml}$ diet) against first instars of the western corn rootworm.

Owing to the low levels of mCry3A synthesized in corn tissue, it is impractical to purify sufficient quantities of this protein for Tier I safety studies carried out against non-target invertebrates and vertebrates. Thus, the *E. coli*-derived mCry3A is used as a surrogate protein for the corn mCry3A in these studies. The quantities of *E. coli*-derived mCry3A to be used in these studies is set by using multiples of 10X or higher than that which the non-target test species are likely to encounter in nature. Therefore, the equivalence of the *E. coli*-derived and corn-derived mCry3A along with the amount of mCry3A that occurs in corn are important determinants of the quantities of *E. coli*-derived mCry3A to be used in non-target tests.

The presence of two distinct forms of the microbially-produced test material which differ in molecular weight is due to the presence of an additional 16 amino acids at the N-terminus of the short form of the protein. The long form is due to the presence of an ATG start codon in the promoter sequence which is in addition to the desired start codon.

It is regrettable that the presence of this unwanted start codon was not detected prior to the full-scale production of the test substance and initiation of biological testing. It would have been a trivial matter to have scanned the sequence proposed to be incorporated into *E. coli* to detect any part of the sequence that could cause potential perturbations. This would have averted the need to consider the potential negative effects of the non-native (i.e. long version) protein on health. It is strongly recommended that such screening for untoward effects be carried out in the future prior to the full production of the test substance. In the same vein, it is also strongly recommended that highly discriminatory characterization tools, such as MALDI-TOF, be applied to test substances such as the mCry3A protein prior to the use of the substance in risk assessment trials.

As noted above, the test material included two forms of the protein – one that was equivalent to the intended plant expression product and one with additional N-terminal amino acids. Because the longer form is not produced in the plant, and will not be consumed, the relevant issue is not the human safety of this form, but whether the presence of the longer form affects our ability to consider the bacterially-produced mixed material as equivalent to the shorter protein in the plant. Since the longer form was the more predominant form derived from *E. coli* (the long/short ratio was 3:2), there is concern that testing may have been done with a potentially less active form of the mCry3A protein.

As a general consideration, it would be unlikely that addition of the extra N-terminal sequence would affect the biological activity of a non-glycosylated protein such as mCry3A in a major way, since many previous studies on a variety of proteins have indicated that short N- or C-terminal extensions, including materials such as immunological flags, markers such as green fluorescent protein and purification aids such as His-tags, added at either the N- or C-terminus typically have negligible effects on activity. However, one is reminded that even single amino acid substitutions in a protein can have major deleterious effects on occasion (for example the change in sickle cell hemoglobin and other single

site mutations in a variety of genetic diseases).

In the present instance one might anticipate that the presence of the additional 16 amino acids at the N-terminus could affect the folding of the protein. This in turn might affect the biological activity if the protein folded in an incorrect manner. In the present instance this might have involved differences in the propensity to crystallize. This issue was addressed, to a degree, by the submissions from the Registrant. However, it was found that the long form of the protein was more than four-fold less active than the short form in at least one study with WCRW. This difference in activity causes difficulties in analysis of other test results for both the environmental and health effects studies. However, it was stated that the relative activities in the WCRW test were within the 95% confidence levels. Given the experimental difficulties of working with WCRW it was suggested to the Panel that from these data the long form and short form of the protein are substantially equivalent.

However, from the data presented it is not possible to ascertain whether the two forms are essentially identical in bioactivity (refer to Panel response to ecological risk assessment). No adverse effects were seen in a mouse study. Therefore, when evaluating mammalian data this issue seems to be of greatest importance in calculating the level of exposure in the mouse studies. Should human exposure calculations be based on the total protein level, or only the level of the short form? The most conservative approach would be to calculate exposures based only on the amount of the short form present.

Moreover, with respect to the equivalence of the two proteins based on the toxicity of the corn-derived versus *E. coli*-derived mCry3A, the approximately 2-fold higher activity of the corn-derived mCry3A toxicity is likely due to the factors noted above. Specifically, these factors are that (1) approximately 50% of the mCry3A in corn is activated, i.e., cleaved to the activated 55-kDa form after synthesis, and (2) the *E. coli*-derived mCry3A contains two types of unactivated mCry3A, a short form (67.7 kDa) and a long form (69.5 kDa), the latter of which is of considerably lower toxicity than the short form. Whereas about 50% of the corn-derived mCry3A is activated, all of the *E. coli*-derived mCry3A must be activated. The bioassay data noted above suggest that this occurs much more slowly in the long form of mCry3A, and possibly to a significantly reduced extent. In the end, all of the mCry3A, whether corn-derived or *E. coli*-derived, should be processed to the activated 55-kDa form. However, it is reasonable to assume, based on the bioassay data, and aside from the problems with the statistical analyses discussed elsewhere in this report, that the differences in the LC₅₀s of the corn-derived and *E. coli*-derived mCry3A are due largely to the differences in the activated state of these proteins upon consumption, and the rate at which the two forms of the *E. coli*-derived protein are processed after ingestion by first instar western corn rootworm. From the standpoint of toxicity, therefore, it can be concluded that the *E. coli*-derived and corn-derived mCry3A are substantially equivalent with respect to their likely effects on the western corn rootworm. However, this equivalence may not transfer to non-target species that do not have cathepsin to readily activate Cry proteins.

The question of the relative bioactivity of the long and short forms of mCry3A could have been further addressed by the use of insects such as the Colorado potato beetle, which are sensitive to mCry3A but which show markedly less variability in bioassay studies. It is also recommended that a receptor binding assay be developed where the long and short forms of *E. coli* expressed mCry3A could be more quantitatively compared to each other as well as to corn derived mCry3A. This would not totally rule out the possibility that once the receptor is bound the long form might still be less active, but it could provide additional criteria that the long and short forms have equivalent biological activity.

Additionally, the Panel thought that the relationship between the bacterial test material and the protein produced *in planta* needs to be considered. The presence of a significant amount of a putative break-down product, apparently the activated form of mCry3A, in the plant material represents a difference between the test material and the *in planta* material. No data were presented suggesting that this break down product was characterized in any way. As far as the Panel can tell, the only information available is that this form reacts with the same antibodies as the intact form in a Western blot. The Panel believes that this form should be characterized to insure that it is, in fact, a simple breakdown product and that it has not been modified in any other way. Without more information on this protein, it is difficult to conclude that the test material was fully equivalent to the plant material.

In addition, whether the surrogate mCry3A produced in *E. coli* and used in all target and non-target studies is substantially equivalent to the mCry3A produced in MIR604 corn is another matter. In fact, there is reasonable cause not to consider these two forms of mCry3A substantially equivalent. Specifically, about 50% of the mCry3A in corn is already activated, whereas the *E. coli*-derived mCry3A contains two forms (SF, short form; LF, long form), with neither form being pre-activated. Moreover, the independent bioassays of, respectively, the SF and LF against the WCRW conducted by the Registrant provide evidence that the latter form is much more difficult to activate perhaps due to the additional amino acids at the N-terminus. Therefore, to make the mCry3A produced in *E. coli* equivalent, about 50% of the *E. coli*-derived mCry3A should have been pre-activated with a cathepsin before being used in bioassays (or activated with other proteases to yield about 50% of the protein in the 55-kDa activated form). Another reason for doing this would be that it is not known whether the various non-target organisms, especially the insects and other invertebrates, have cathepsins capable of activating the long and short forms of mCry3A.

The Panel had some questions regarding information in MRID 461556-05. In this document, it is noted in the original analysis that a single N-terminal sequence was obtained for the *E. coli* expressed mCry3A protein which corresponds to the expected N-terminal sequence. However in an addendum it notes two proteins referred to as the long and short forms of *E. coli* expressed mCry3A that have different N-terminal sequences.

Although the fact that the sequences of the two forms of the protein are 97% identical is a strong argument for equivalence, it is important to realize that this similarity has a different biological meaning if the differences are distributed throughout the protein or concentrated in one region. Interestingly, the data submitted by the Registrant describing the mass spectrometry studies show that it was necessary to

digest the two forms with different enzymes to release N-terminal fragments, suggesting that there may be structural differences between these forms.

The fact that the proteins (i.e. the long and short forms) have 97.4% amino acid identity was considered of less consequence than the fact that both forms do have substantial biological activity, albeit with an approximately four-fold difference between them.

Based on the data available it is likely, yet not fully proven, that the two forms of the mCry3A are of relatively comparable biological activity for the purposes of the human health assessments. That is, the mCry3A proteins are substantially equivalent with respect to their amino acid sequences, lack of glycosylation, other types of potential post-translational modifications and general stability. There is, however, a question as to the equivalence of how readily they are activated. Thus, for the purposes of NTO vertebrate (including human health assessment) and non-insect invertebrate toxicity assessment, data from Tier I non-target effects studies based on mCry3A produced in *E. coli*, which in no case showed existence of a hazard, may be assumed to be equivalent to those that would have been obtained had mCry3A from transformed corn been used to conduct these studies. The situation would be much improved if more complete data were available. For example, in order to more firmly draw this conclusion, the data supplied for the plant and *E. coli* expression systems used to produce mCry3A and the associated bioassays using the WCRW would require a more detailed analysis than that provided in the various materials supplied to EPA by the Registrant.

As regards the mammalian and human effects of the mCry3A protein, several Panel members noted that with respect to the equivalence of both forms of the protein, there are no data in the mammalian testing that utilized each of the forms separately (the long form and the short form). The testing was done with a mixture that included a ratio of 3:2 of long form versus short form. It has been stated that the form expressed in the plant is the short form of the protein.

One Panel member noted that no data were provided in the mouse study on equivalence and since there is a 16 amino acid difference between the two proteins, the following three concerns were raised: 1) It is well known that two different proteins are not guaranteed to have the same activity in human and other mammals. 2) While no allergenicity testing was done in animals, it was noted that a difference of one amino acid may make the difference between an allergenic protein and a non-allergenic protein. This is particularly relevant if the different proteins fold differently, which may bring different sequences of amino acids together in proximity and form new allergenic epitopes. 3) A difference of one amino acid may make a difference in the degree of toxicity. Even proteins with completely similar structure (no amino acid differences) but of different spatial configurations can have vast differences in their toxic effects on humans and animals. So, without testing, one cannot assume equivalence.

This Panel member believed that, given the research presented (feeding of 10 mice with the test substance that contained a mixture of the short and long forms of the protein and that there were no other studies presented that utilized the short form alone or compared the activity of both forms in a live mammal), no data were presented that would allow them to state that the Agency's conclusion is justified, namely, that the mCry3A proteins from corn event MIR604 and from recombinant *E. coli* are

substantially the same for the purpose of the Agency's risk assessment; and that the equivalence is confirmed for the two forms of the bacterial-produced mCry3A test material (MCRY3A-0102) as regards toxicity and allergenicity to mammals and humans within the scope of the Agency's risk assessment. That Panelist suggested that the Agency proceed with testing the short form in a rigorous and well controlled study design.

Charge to Panel - MIR604 Human Health and Characterization - Mammalian Toxicity

Previously submitted studies demonstrated the lack of toxicity of the mCry3A protein following acute oral high-dose exposure to mice, rapid degradation of mCry3A upon exposure to simulated mammalian gastric fluid, and the lack of significant amino acid sequence homology of the mCry3A protein to proteins known to be mammalian toxins or human allergens. Moreover, little to no human dietary exposure to mCry3A protein is expected to occur *via* transformed corn. Therefore, dietary exposure to mCry3A is not anticipated to pose any dietary risk to the U.S. population.

Please comment on the Agency's conclusions regarding the lack of mammalian toxicity and allergenicity of mCry3A.

Panel Response

The Panel agreed that the assessment of potential human toxicity rested on five analyses: acute studies in mice, evidence of rapid degradation in-vitro, sequence analysis, analysis of glycosylation, and an exposure assessment.

Several Panel members believed that the results of the acute mouse studies reasonably support the conclusion of the risk assessment. Given that the number of mice tested is considered adequate by EPA as a point estimate of LD₅₀, the data show a lack of adverse effects at the concentrations tested. The only issue that these Panel members identified was the exact calculation of exposure, based on the question of the long-form of the protein discussed in the previous question. These Panel members also noted that it is important to obtain data showing that the shorter form of the protein found *in planta* is, in fact, a simple break down product with the expected sequence and that it has not been modified in any other way.

One Panel member believed that this study did not support the drawing of any conclusions about human safety and elaborated that only ten mice were tested, that there were no longer-term studies or chronic exposure studies, and that there was no consideration of the possibility that the presence of the test protein may have contributed in some way to the death of the one mouse that died as the result of mechanical damage during gavage. This Panel member also believed that other, non-oral, routes of exposure such as inhalation should have been considered and suggested that the fact that several pollen-borne proteins are known allergens suggests that, even given the low level expression in pollen, this issue should have been considered. This Panel member also expressed doubts about the ability to extrapolate from animal studies to human toxicity, particularly allergenicity.

Most of the Panel members believed that the digestion data are indicative, but could have been presented in a stronger manner. Sensitivity to degradation is a relative measure – one protein is more or less sensitive than another under certain conditions. The data provided do not compare sensitivity of the modified protein to the native protein, or to any other protein. Further, these studies were done at a relatively high enzyme to protein ratio. It would have been more informative if the test could have been carried out with lower enzyme concentrations, so that potential breakdown products could have been detected. Given the poor quality of the images in the documents, it is difficult to unequivocally determine that there were no stable breakdown products produced. For example, one can compare undigested protein extracted from plants shown in Fig. 1, Lane 3 in MRID 461556-03 (characterizations study), with a loading of 3.9 µg protein, to Lanes 3 and 4 of Figure 2 of MRID 461556-07 (digestion study) which would have 1 µg of the protein. The second gel does not show any of the many lower molecular weight bands that are seen in the former (most of which should be visible even taking the different loadings into account). This makes it difficult to feel great assurance that any breakdown products would have been observed in the digestion study. This could be an issue related to image reproduction rather than with the actual data, but it is of interest. However, given that digestion stability does not correlate with actual allergenicity, these shortcomings are not significant.

One Panel member suggested that the Agency consider the fact that some humans may have lower than normal acidity in the stomach. Those people may be exposed to intact, or partially degraded, protein.

The Panel believed that the sequence analysis as described was difficult to fully evaluate because the document was more a summary abstract than a technical description. The construction and contents of the allergen sequence database were not fully described. For example, it is not clear what is meant by "redundant sequences were removed from the database." Does redundant mean 100% sequence identity, or some lower percentage homology? How were iso-allergens treated? How many food and non-food allergens are included in the database? The short sequence (8 mer) testing was carried out using a propriety program, but no information is given on how it worked or how it was validated. The long sequence testing (80 mer) was carried out using only a single scoring system with FASTA. It is known that changes in the scoring matrix used can make a difference in the ability of the program to find regions of homology. Also, homology is not an absolute situation. Although the Registrant used the cut-off value suggested by the FAO/WHO expert consultation, it should be noted that this value has not in fact been adopted by FAO/WHO. It would have been much nicer to see the actual results of the testing.

The Panel also believed it is worth mentioning that the sequence analysis related to similarity to known toxins provided even less information. For example, it was not specified which version of the National Center for Biotechnology Information database was tested, nor were descriptions provided of the parameters used in the Basic Local Alignment Search Tool - Protein (a protein sequence analysis tool). The information as actually presented makes it

difficult to appropriately analyze this study and therefore to scientifically support a permanent exemption from the requirement for a tolerance.

One Panel member indicated that, in general, sequence comparison is of limited value. This Panel member suggested that difference of one amino acid may make the difference between an allergenic protein and a non-allergenic protein. This would be particularly relevant if the different proteins fold differently, which may bring different sequences of amino acids together in proximity and form new allergenic epitopes. This Panel member also said that a one amino acid difference may make a difference in the degree of toxicity.

The Panel had no comments on the glycosylation study.

Most of the Panel believed that the exposure assessment appeared to be straightforward. It showed that the amount of Cry protein that would be consumed in a rather large serving of corn kernels would be several orders of magnitude lower than the highest dose tested in the mammalian toxicity studies – which had no observed effect. Even taking into account the questions about quantification that were raised previously, this assessment indicates that the level of human exposure presents a reasonable certainty of no harm.

Having said all this, most Panel members believed that the data did not raise “red flags” regarding human toxicity. On the other hand, a couple of Panel members agreed that no conclusion on safety could be derived from these data and that much more information is needed about acute effects and potential effects on children's growth and development. At least three Panel members indicated that far too little data was made available to support scientifically a permanent exemption from the requirement of a tolerance, especially in a widely used food crop such as corn.

There was a broad consensus among the Panel members that there were significant data quality issues. In many cases, it was not possible to fully evaluate the results because the methods were inadequately described in the documents given to the Panel. In other cases, the methods were used in an inconsistent manner. For example, two documents (MRID 462656-05 and MRID 462656-06) reported Western Blot studies that appear to have been performed within a short period of time that used different detection systems, with clearly different sensitivities. This raises questions about whether all relevant bands were detected. The Panel also suggested that better quality reproductions of data images be used in the future and that all of the data obtained (such as for sequence analyses) be included in the material presented to the Panel.

REFERENCES

- Banks, J. 1988. The acute toxicity of diflubenzuron, methoprene, temephos, fenoxycarb, *Bacillus thuringiensis* var. *israelensis* (BTI), and a fenoxycarb/Bti mixture on the nontarget, saltmarsh fiddler crab, *Uca pugnator*, with an emphasis on the sublethal effects of fenoxycarb and the fenoxycarb/Bti mixture on energy metabolism. Master Thesis, University of South Carolina, School of Public Health: 72 pp.
- Barford, N. 1967. Experimental Measurements: Precision, Error and Truth. Addison-Wesley, London, England.
- Brewer RA, Carlock LL, Brewer LW, Hooper MJ, Cobb GP, R.J. Kendall. 1995. Toxicity Survivability and Activity Patterns of Northern Bobwhite Quail Dosed with the Insecticide COUNTER®. Environ. Toxicol. Chem., 15:570-573.
- Cochran, W.G. 1977. Sampling Techniques, 3rd Edition, John Wiley and Sons. Equation 6.13.
- Dee, J. 1988. The acute toxicity of four commonly used mosquito control larvicides, a microbial larvicide (BTI) and pesticide mixture on the grass shrimp, *Palaemonetes pugio*. Master Thesis, University of South Carolina, School of Public Health: 63 pp.
- Deicke, A.; Suverkrup, R. 2000. Dose uniformity and redispersibility of pharmaceutical suspensions. 2. Assessment of three commercial erythromycin ethyl succinate oral liquids. European Journal of Pharmaceutics and Biopharmaceutics 49(1), 73-78.
- Elkheshen, Seham A.; Badawi, Sabry S.; Badawi, Alia A. 1996. Optimization of a reconstitutable suspension of rifampicin using 24 factorial design. Drug Development and Industrial Pharmacy. 22(7), 623-630.
- Flores, S., Saxena, D., Stotzky, G., 2005. Transgenic Bt plants decompose less in soil than non-Bt plants. Soil Biology and Biochemistry 37, 1073-1082.
- Head G., J.B. Surber, J.A. Watson, J.W. Martin, and J.J. Duan. 2002. No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use. *Environmental Entomology* 31: 30-36.
- Lee, B. 1987. The acute toxicity of four chemical mosquito larvicides (temephos, fenoxycarb, diflubenzuron, and methoprene) and a microbial mosquito larvicide (*Bacillus thuringiensis* var. *israelensis*) on a nontarget, estuarine fish species - the mummichog (*Fundulus heteroclitus*). Master Thesis, University of South Carolina, School of Public Health: 53 pp.

- Lee, B. & G. Scott. 1989. The acute toxicity of 4 chemical mosquito control larvicides and a microbial larvicide (BTI) on the estuarine fish, *Fundulus heteroclitus*. *Bulletin of Env. Contamination and Toxicology* 43:827-832.
- Palm, C.J., Schaller, D.J., Donegan, K.K., Seidler, R.J., 1996. Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki* d-endotoxin. *Canadian Journal of Microbiology* 42, 1258-1262.
- Ramsey Janine M; Cruz-Celis Adriana; Salgado Liliana; Espinosa Luis; Ordonez Rosalinda; Lopez Rene; Schofield C J. 2003. Efficacy of pyrethroid insecticides against domestic and peridomestic populations of *Triatoma pallidipennis* and *Triatoma barberi* vectors of Chagas' disease in Mexico. *Journal of Medical Entomology* 40(6), 912-20.
- RTI (1992). Developmental toxicity evaluation of N,N'-methylenebisacrylamide administered by gavage to Swiss (CD-1) mice on gestational days 6 through 17., Research Triangle Institute. RTP, NC 198 pp.
- Saxena, D., Stotzky, G., 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn *in vitro* and *in situ*. *FEMS Microbiology Ecology* 33, 3539.
- Saxena, D., C.N. Stewart, I. Altosaar, Q. Shu, and G. Stotzky. 2004. Larvicidal Cry proteins from *Bacillus thuringiensis* are released in root exudates of transgenic corn, potatoes and rice but not of *Bacillus thuringiensis* canola, cotton and tobacco. *Plant Physiology and Biochemistry* 42: 383-387.
- Tapp, H., Stotzky, G., 1998. Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. *Soil Biology and Biochemistry* 30, 471476.
- Thomas, Peter T.; Ratajczak, Helen V.; Eisenberg, Walter C.; Furedi-Machacek, Marianna; Ketels, Kathleen V.; Barbera, Peter W. 1987. Evaluation of host resistance and immunity in mice exposed to the carbamate pesticide aldicarb. *Fundamental and Applied Toxicology* 9(1), 82-9.
- Thomas P; Ratajczak H; Demetral D; Hagen K; Baron R. 1990. Aldicarb immunotoxicity: functional analysis of cell-mediated immunity and quantitation of lymphocyte subpopulations. *Fundamental and applied toxicology*. 15(2), 221-30.
- Thompson, S.K. 1992. Sampling. Chapter 7. John Wiley and Sons, Inc. New York.
- USEPA. 1996. Microbial pesticide test guidelines for tier I nontarget insect testing. Office of Prevention, Pesticides and Toxic Substances. Harmonized Testing Guideline 885.4340. EPA 712-C-96-280.
- Zwahlen, C., A. R. Hilbeck, P. Gugerli, and W. Nentwig. 2003. Degradation of Cry1Ab protein

within transgenic *Bacillus thuringiensis* corn tissue in the field. *Molecular Ecology* 12:
765-775

Appendix 1

Effect of the *Eisenia* Test Diet on Toxicity of MCRY3A-0102

$$j := 0..1 \quad \text{DoseNeat}_j := \begin{array}{|c|} \hline 12.5 \\ \hline 50 \\ \hline \end{array} \quad \text{MortNeat}_j := \begin{array}{|c|} \hline 0.87 \\ \hline 0.97 \\ \hline \end{array}$$

Concentration of MCRY3A-0102 (“DoseNeat”, µg/g) and resulting mortality (“MortNeat”, fraction) following “neat” (without soil) addition to CPB test diet.

$$\text{LogDoseNeat}_j := \log(\text{DoseNeat}_j) \quad \text{PrbtMortNeat}_j := \text{qnorm}(\text{MortNeat}_j, 0, 1)$$

$$\text{LogDoseNeat} = \begin{pmatrix} 1.097 \\ 1.699 \end{pmatrix} \quad \text{PrbtMortNeat} = \begin{pmatrix} 1.126 \\ 1.881 \end{pmatrix}$$

Log base-10 of DoseNeat (“LogDoseNeat”) and probit mortality of MortNeat (“PrbtMortNeat”), where probit mortality is the inverse of the cumulative distribution for the underlying standard normal tolerance distribution (+5).

$$\left(\left[\frac{1}{\sigma} \right] \right)_j := \frac{(\text{PrbtMortNeat}_1 - \text{PrbtMortNeat}_0)}{(\text{LogDoseNeat}_1 - \text{LogDoseNeat}_0)} \quad \left[\frac{1}{\sigma} \right] = \begin{pmatrix} 1.253 \\ 1.253 \end{pmatrix}$$

Slope of the probit mortality-log(dose) plot, where the variable name used acknowledges that the slope is equal to the reciprocal of the standard deviation of the underlying standard normal tolerance distribution.

$$\left(\left[\frac{\mu}{\sigma} \right] \right)_j := \text{PrbtMortNeat}_j - \left(\left[\frac{1}{\sigma} \right] \right)_j \cdot \text{LogDoseNeat}_j \quad \left(\left[\frac{\mu}{\sigma} \right] \right) = \begin{pmatrix} -0.248 \\ -0.248 \end{pmatrix}$$

Y-intercept of the probit mortality-log(dose) plot, where the variable name used acknowledges that the y-intercept is equal to the ratio of the mean to the standard deviation of the underlying standard normal tolerance distribution.

$$i := 0..100 \quad \text{LogDose}_i := \frac{i}{50} \quad \text{PrbtMort}_i := \left(\left[\frac{1}{\sigma} \right] \right)_1 \cdot \text{LogDose}_i + \left(\left[\frac{\mu}{\sigma} \right] \right)_1$$

Definition of the probit mortality-log(dose) line between the two “neat” observations.

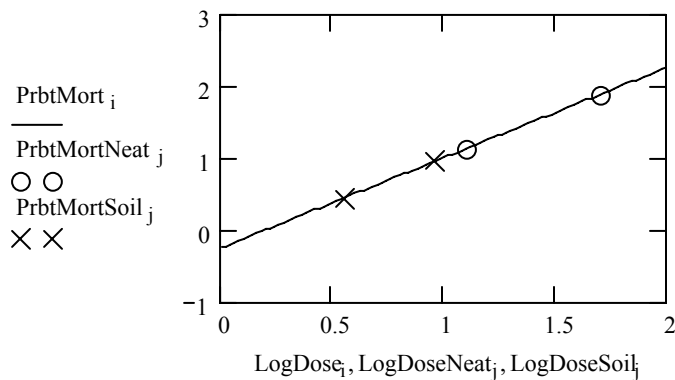
$$\text{MortSoil}_j := \text{PrbtMortSoil}_j := \text{qnorm}(\text{MortSoil}_j, 0, 1)$$

$$\begin{array}{|c|} \hline 0.67 \\ \hline 0.83 \\ \hline \end{array}$$

Mortality observed following addition of MCRY3-0102 in test soil to the CPB test diet (“MortSoil”, fraction) and probit mortality of MortSoil (“PrbtMortSoil”).

$$\text{LogDoseSoil}_j := \frac{\left[\text{PrbtMortSoil}_j - \left(\left[\frac{\mu}{\sigma} \right]_1 \right) \right]}{\left(\left[\frac{1}{\sigma} \right]_1 \right)}$$

Calculation of the Log(DoseSoil) (“LogDoseSoil”) values predicted by the probit mortality-log(dose) relationship based on the “neat” data and the observed PrbtMortSoil values.



Plot of the probit mortality-log(dose) relationship based on the “neat” data and the observed PrbtMortSoil values.

$$\text{DoseSoil}_j := 10^{\text{LogDoseSoil}_j} \quad \text{DoseSoil} = \begin{pmatrix} 3.54 \\ 9.109 \end{pmatrix}$$

Antilog(LogDoseSoil) to give the effective concentrations of MCRY3-0102 added with soil to CPB test diet (“DoseSoil”, µg/g).

$$\text{NomDoseSoil}_j :=$$

$$\begin{matrix} 13.3 \\ 26.6 \end{matrix} \quad \text{FracNomDoseSoil}_j := \frac{\text{DoseSoil}_j}{\text{NomDoseSoil}_j} \quad \text{FracNomDoseSoil} = \begin{pmatrix} 0.266 \\ 0.342 \end{pmatrix}$$

Nominal concentrations of MCRY3-0102 added with soil to CPB test diet (“NomDoseSoil”, µg/g) and DoseSoil as a fraction of NomDoseSoil (“FracNomDoseSoil”).

$$\text{CF} := \text{mean}(\text{FracNomDoseSoil}) \quad \text{CF} = 0.304$$

Average concentration of MCRY3-0102 relative to nominal concentration (“CF”).

$$\text{SECF} := \frac{\text{Stdev}(\text{FracNomDoseSoil})}{(2)^{.5}} \quad \text{SECF} = 0.038$$

Standard error of CF.

Appendix 2

Estimation of Extraction Efficiency from a Series of Two Extractions

The Registrant estimated extraction efficiency (“EE”) of mCry3A from tissues of MIR604 corn using the following formulation:

$$EE = X/(X+Y) \quad (X \equiv \text{concentration mCry3A recovered in first extraction})$$

$$\quad \quad \quad (Y \equiv \text{concentration mCry3A recovered in second extraction})$$

If alternatively one assumes that the fraction of residual mCry3A recovered in successive extractions is constant, then EE overestimates the true extraction efficiency (“AEE”).

For total extractable mCry3A present in the corn tissue (“T”), one has the following:

$$AEE = X/T \quad (\text{definition of AEE})$$

$$Y = (X/T) \cdot (T-X) \quad (\text{applying constant extraction efficiency assumption})$$

$$Y = X \cdot [1 - (X/T)] \quad (\text{rearranging})$$

$$X/T = (X-Y)/X = 2 - EE^{-1} \quad (\text{solving for X/T and substituting Registrant's def. for EE})$$

$$AEE = 2 - EE^{-1}$$

$$i := 0..100 \quad EE_i := 0.5 + \frac{i}{100} \quad AEE_i := 2 - \frac{1}{EE_i}$$

Calculation of AEE for various tissues of MIR604 corn:

Leaf tissue:

$$EE := 0.771$$

$$AEE := 2 - \frac{1}{EE} \quad AEE = 0.703$$

Kernel tissue:

$$EE := 0.697$$

$$AEE := 2 - \frac{1}{EE} \quad AEE = 0.565$$

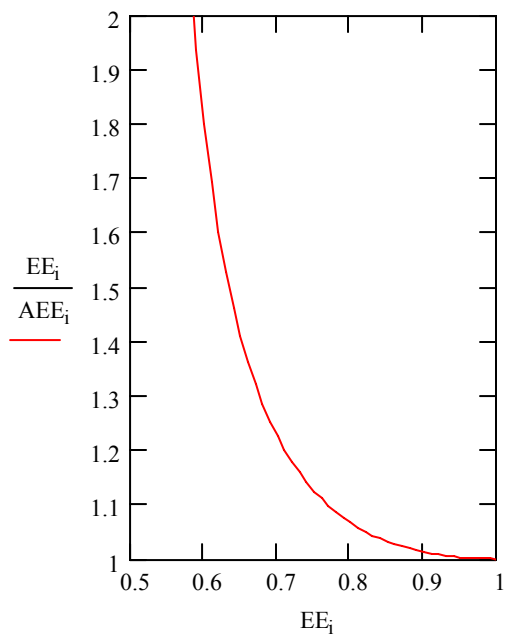
Silage tissue:

$$EE := 0.845$$

$$AEE := 2 - \frac{1}{EE} \quad AEE = 0.817$$

Factor by which the Registrant’s estimates for EEC should be multiplied to give EEC based on AEE (“AEEC”):

$$AEEC/EEC := (X/AEE)/(X/EE) := EE/AEE$$



Graph of factor to convert from EEC to AEEC.

Leaf tissue: $EE/AEE = 0.771/0.703 = 1.10$

Kernel tissue: $EE/AEE = 0.697/0.565 = 1.23$

Silage tissue: $EE/AEE = 0.845/0.817 = 1.03$