## STUDY TITLE

Waiver Request: Fish Toxicity Test With Transgenic Maize (Corn) Containing *Bacillus thuringiensis* var. *aizaqoi* (Bt) Cry 1F Delta-Endotoxin

## DATA REQUIREMENTS

N/A

## AUTHORS

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## STUDY COMPLETED ON

01-DECEMBER-1999

## PERFORMING LABORATORY

Global Toxicology Laboratory
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

## LABORATORY STUDY ID

GH-C 5016

# STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry 1F Delta Endotoxin

Title: Waiver Request: Fish Toxicity Test With Transgenic Maize (Corn) Containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F Delta-Endotoxin

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A)(B), or (C).*

Company: Dow AgroSciences LLC

Company Agent: Diane Shanahan

Title: Regulatory Manager

Signature: Diane Shanahan

Date: 11/30/99

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

# STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Waiver Request: Fish Toxicity Test With Transgenic Maize (Corn) Containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F Delta-Endotoxin

Study Completion Date: November 1999

This study does not meet the definition of a GLP study as it appears in.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organization for Economic Co-Operation and Development
ISBN 92-64-12367-9, Paris 1982

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Waiver Request: Fish Toxicity Test With Transgenic Maize (Corn) Containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F Delta-Endotoxin

## SUMMARY

A waiver is requested for a fish toxicity test with transgenic maize (corn) containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F delta-endotoxin. The waiver is requested because of the lack of exposure of fish to Cry 1F delta-endotoxin in commercially manufactured fish diet. This conclusion is based on the low content of Cry 1F endotoxin in corn kernels, the limitations on the amount of corn meal incorporated into commercial fish diets and, the lack of measurable concentrations (by both ELISA and bioassay) of Cry 1F delta-endotoxin in commercially prepared fish diet prepared with corn expressing Cry 1F delta-endotoxin.

## Introduction

A waiver is requested for a fish toxicity test with transgenic maize (corn) containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F delta-endotoxin. The waiver is requested because of the lack of exposure of fish to Cry 1F delta-endotoxin in commercially manufactured fish diet.

Previous registrations indicate that the Environmental Fate and Effects Branch (EFEB) is concerned about the possible acute toxicity of commercial fish diets prepared with grain from transgenic corn expressing Bt endotoxin(s) in various tissues. Therefore the EFEB has, in the past, required registrants to conduct acute toxicity tests with fish fed a diet prepared with corn kernels expressing the specific Bt endotoxin. The EFEB has waived this requirement when it has been demonstrated that the Bt protein is denatured or inactivated during a typical commercial fish diet manufacturing process. Therefore Dow AgroSciences/Mycogen contracted Purina Mills, a major manufacturer of fish diets, to produce a diet manufactured with kernels from corn plants expressing the Bt Cry 1F delta-endotoxin. Analysis of the finished product indicates that a fish diet prepared with kernels expressing the Bt Cry 1F delta-endotoxin will not be a source of exposure of fish to this endotoxin. Further, a study in which daphnids were acutely exposed to pollen derived from Bt maize plants (a potential source of exposure of aquatic organisms to Bt endotoxins) as well as purified Bt Cry 1F endotoxin, has been conducted. The results indicate neither the pollen nor the purified protein is toxic to daphnids (Drottar and Krueger, 1999)

Transformed maize line contain a gene encoding Cry 1F delta-endotoxin, an insecticidal protein derived from *Bacillus thuringiensis* var. *aizawai.* The Cry 1F endotoxin is expressed differentially in maize tissues. Cry 1F endotoxin is detectable in kernels by enzyme-linked immunosorbent assay (ELISA) at a concentration of approximately 3-4 ng/mg (Young and Herman, 1999)

The composition of a fish diet is dependent on the species being cultured, but all fish diets must contain sufficient amounts of protein and essential vitamins, fatty acids, and minerals (National

Research Council, 1983, 1993; Robinson, 1989). In addition to these essential ingredients, fish diets contain varying amounts of starch, which is frequently provided by corn. The maximum amount of corn found in fish diets is 34% to 49% in diets for warm water fishes, such as catfish. Corn content of diets for cold water species, such as trout, generally contain 10 to 15 % corn because corn is not as efficiently metabolized by these species (National Research Council, 1983, 1993).

Fish diets are typically processed into pellets or they are extruded. The bulk of catfish diets are extruded and are preferred by many fish farmers because they float (National Research Council, 1983). In this process mixed mill is ground through a screen followed by steam conditioning (180 to 212° F) for about 30 seconds. The meal is moved to an extruder barrel where it is processed under pressure and heat up to 350°F. Water is added to both the conditioned product and the barrel of the extruder. The product is extruded through a die and cut into ¼" pellets and is dried to less than 11% moisture in a batch dryer (generally 15 to 30 minutes). Once cooled at ambient temperature it is bagged for shipment.

## RESULTS

Mycogen contracted Purina Mills to prepare an experimental fish food to be processed by extrusion, the most common method for the preparation of diets for warm water species, using the maximum corn content found in fish diets (35 to 40%). Diets using either corn expressing the Bt Cry 1F endotoxin or non-transgenic corn were formulated (Table 1). Processed diets were shipped to Dow AgroSciences for analysis by ELISA and for bioassays with first instar tobacco budworm (TBW). A copy of the Purina's process report is provided in Appendix 1.

Table 1. Ingredients in Commercially Prepared Fish Diet

| Major Ingredients | |
|---|---|
| Soybean Meal | 50% |
| Corn Meal | 38.72 % |
| Fish Meal | 8% |
| Animal Fat | 1.5% |

Four separate bioassays were conducted. Each bioassay consisted of the following treatments:

fish diet prepared with corn expressing the Bt Cry 1F endotoxin, control corn fish diet, a positive control (spinosad), a carrier control (2:1 mixture of acetone:water, the carrier for spinosad) and an agar control. TBW larvae were exposed in small plastic cups containing an agar-based TBW diet to which the test substance was applied and allow to dry. Mortality and growth data were assessed after 7 days of exposure (Young and Herman, 1999). The results of the bioassays were somewhat variable, however statistical analysis did not demonstrate a difference between treatments indicating that there was no statistically significant biological activity associated with the diet containing corn meal expressing Cry 1F endotoxin. A summary of the results is provided in Table 2.

Table 2. Effects of fish feed containing grain from Cry1F expressing *Bt* maize on first instar tobacco budworm, *Heliothis virescens.*

| Sample | Percent mortality in each bioassay | | | | Total weight of 16 larvae in each bioassay (mg) | | | | Treatment mean weight |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | mg |
| Bt maize feed (TSN101835) 7.7% | 0 | 0 | 6.3 | 12.5 | 1089.3 | 1029.1 | 1050.0 | 334.5 | 875.7[a] |
| Control feed (TSN101834) 7.7% | 12.5 | 0 | 0 | 0 | 1647.0 | 532.1 | 1597.6 | 352.5 | 1032.3[a] |
| 2:1 acetone/water | 0 | 0 | 0 | 0 | 1583.1 | 919.9 | 1517.9 | 994.0 | 1253.7[a] |
| Agar Control | 0 | 0 | 0 | 0 | 1616.3 | 587.5 | 1746.2 | 909.6 | 1214.9[a] |

[a] Means were not significantly different (alpha=0.05) based on analysis of variance.

The Cry 1F endotoxin protein content of kernels before processing into feed was 2.2 – 3.5 ng/mg. Analysis of the fish diet samples by ELISA demonstrated that Cry 1F endotoxin was not detectable in the feed samples with a detection limit of 0.04 ng/mg. These data are summarized in Table 3. (Young and Herman, 1999)

Table 3. Cry1F protein level in maize grain and formulated fish feed as measured by ELISA

| Test Material | ng Cry1F/mg maize |
|---|---|
| | |
| TSN101792 – Cry1F (TR) maize | 2.2 - 3.2 |
| | |
| TSN101791 – Control maize | nd[a] |
| | |
| TSN101835 – Fish food containing Cry1F maize | nd |
| | |
| TSN101834 –Fish food containing control maize | nd |
| | |

[a] not detectable – below the limit of detection of the ELISA (0.04 ng/mg), 5mg sample extracted.

## CONCLUSION

Based on the above discussion Dow AgroSciences/Mycogen requests that the fish testing requirement be waived because fish fed commercial diets containing corn meal from kernels expressing Cry 1F endotoxin will not be exposed to biologically active or detectable amounts of Cry 1F endotoxin. This conclusion is based on the low content of Cry 1F endotoxin in corn kernels, the limitations on the amount of corn meal incorporated into commercial fish diets and the lack of measurable concentrations (by both ELISA and bioassay) of Cry 1F endotoxin in commercially prepared fish diet.

# REFERENCES

Drottar, K. R. and Krueger, H. O. (1999). Bt Cry 1F Delta-Endotoxin: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*) Using Bacterially Expressed Bt Cry 1F Delta-Endotoxin, and Pollen from Maize Expressing Bt Cry 1F Delta-Endotoxin. Wildlife International, Ltd. Easton, Maryland. Unpublished report 354A-111 dated 3 September 1999.

National Research Council. (1983). Nutrient Requirements of Warmwater Fishes and Shellfishes, Revised Edition. Subcommittee on Warmwater Fish Nutrition. National Research Council; National Academy Press, Washington, D.C.: 102 pp.

National Research Council. (1993). Nutrient Requirements of Fish. Committee on Animal Nutrition. National Research Council; National Academy Press, Washington, D.C.: 114 pp.

Robinson, E.H. 1989. Channel Catfish Nutrition. Reviews In Aquatic Sciences. 1:3, 365-391.

Young, D. L. and Herman, R. A. (1999). Characterization of Expressed Cry 1F Protein in Maize Tissues (Pollen, Grain, Grain Containing Feed, and Purified Maize-Expressed Cry 1F Protein) and Microbial Expressed Cry 1F delta Endotoxin by Biological and Biochemical Procedures. Dow AgroSciences, LLC unpublished report GH-C 5006, dated 18 November 1999.

# Appendix I
## Protocol and Final Report for Preparation of Experimental Catfish Diet

EXPERIMENTAL PROTOCOL



Chow Process Research Experiment No.: 3562
CPR Project No.: 20
Study Director: Kent J. Lanter
Location: Research Manufacturing Unit, Gray Summit, MO
Number of Treatments: 2
Number of Formulations: 2
Start: May, 1999
Confidentiality: Standard Research Program

TITLE: Bt Corn

PROCEDURE

1. Manufacturing
   a. Extrusion: Sufficient amounts of variable A will be processed so that optimal or prescribed processing conditions are achieved and the following conditions are maintained:
      1. The attainment of stabilized temperature at the conditioner and extruder discharges.
      2. A constant flow rate at the meal feeder and water injection point.
      3. Stabilized steam addition and loading of the extruder main drive.
      4. Variable B will have very little excess material available. At the end of the running of ration A, the feeder will be allowed to run empty, then ration B will be immediately added to the feeder. After 1.5 minutes, Ration B will be coming out of the extruder die. After 3.0 minutes, sample collection will begin.
2. Observations
   - Extruder speed (rpm)
   - Feeder speed (%), setting and production rate (wet)
   - Conditioned meal temperature at conditioner discharge
   - Ammeter reading on extruder motor
   - Head jacket control, (water or steam)
   - Water addition rate to conditioned meal
   - Water addition rate to meal in extruder barrel
   - Wet product density
   - Dryer temperature
3. Laboratory/sample data: Sample of finished product will be analyzed for moisture and bushel weight.
4. Equipment specifications
   a. Extruder: Wenger X-20 extruder; model no. 68001-000, serial no. 8704-8943, equipped with a 30 h.p. motor variable speed drive, feeder and conditioner.
   b. See attachment for barrel set up. Eight head aggressive screw will be used.

c. Steam harness and water addition system (see attachments).
d. The three drawer batch dryer will be used.
e. Die #016 - 3/16"
f. Three blade knife

5. Corn will be ground in the Fitz Mill through a 4/64" screen.

ESTIMATED MEAL REQUIREMENTS
Ration A 150 pounds
Ration B 33 pounds

OBJECTIVE
To produce small samples of extruded catfish feed using Bt Corn from Dow AgroSciences.

NEED
We have been asked by Dow AgroSciences to produce product for their testing.

VARIABLE TABLE

| | A | B |
|---|---|---|
| Normal Corn | + | |
| Bt Corn | | + |

INGREDIENTS
Bt Corn will be supplied by Dow AgroSciences. Other ingredients are available at the RMU.

ANALYSIS
Moisture, Bushel weight.

DISTRIBUTION
Standard plus M. Griffin, G. Ballam. Monte Mayes and Diane Shanahan of Dow AgroSciences

STUDY DIRECTOR Kent Lamb (KJL) DATE 4/26/99

APPROVED K. G. Pohl (KEP) DATE 4/26/99

APPROVED Diane Shanahan (Dow) DATE 5/15/99

# Custom Formula: Dow Agro Sciences
# BT Corn Project

| Ingredient | BT + Diet | BT - Diet |
|---|---|---|
| BT + Corn | 38.8472 | -- |
| "Normal" Corn | -- | 38.8742 |
| Soybean Meal | 49.8796 | 49.8796 |
| Fish Meal - Menhaden | 8.0000 | 8.0000 |
| Dicalcium Phosphate | 1.0000 | 1.0000 |
| Animal Fat Preserved w/BHA | 1.5000 | 1.5000 |
| L-ascozbyl-Z-polyphosphate | 0.0714 | 0.0714 |
| Vitamin Premix | 0.0770 | 0.0770 |
| Ethoxyquin | 0.0125 | 0.0125 |

# Custom Catfish Formula - Dow AgroSciences
## BT Corn Project

### Calculated Nutrient Composition

**NUTRIENTS**

| | |
|---|---|
| **Protein**, % | **32.5** |
| Arginine, % | 2.18 |
| Cystine, % | 0.45 |
| Glycine, % | 1.65 |
| Histidine, % | 0.81 |
| Isoleucine, % | 1.71 |
| Leucine, % | 2.64 |
| Lysine, % | 2.07 |
| Methionine, % | 0.57 |
| Phenylalanine, % | 1.56 |
| Tyrosine, % | 0.99 |
| Threonine, % | 1.27 |
| Tryptophan, % | 0.44 |
| Valine, % | 1.72 |
| **Fat**, % | **4.3** |
| **Fiber** (Crude), % | **2.7** |
| **Nitrogen-Free Extract** (by difference), % | **44.8** |

Quality Controlled by PMI Nutrition International, a subsidiary of America's oldest and largest animal nutrition company.

**MINERALS**

| | |
|---|---|
| **Ash**, % | **5.7** |
| Calcium, % | 0.85 |
| Phosphorus, % | 0.83 |
| Phosphorus (non-phytate), % | 0.52 |
| Potassium, % | 1.25 |
| Magnesium, % | 0.20 |
| Sulfur, % | 0.29 |
| Sodium, % | 0.06 |
| Chlorine, % | 0.13 |
| Iron, ppm | 330 |
| Zinc, ppm | 140 |
| Manganese, ppm | 100 |
| Copper, ppm | 19 |
| Cobalt, ppm | 0.39 |
| Iodine, ppm | 1.6 |
| Selenium, ppm | 0.39 |

**VITAMINS**

| | |
|---|---|
| Vitamin K (as menadione), ppm | 4.0 |
| Thiamin Hydrochloride, ppm | 10 |
| Riboflavin, ppm | 25 |
| Niacin, ppm | 57 |
| Pantothenic Acid, ppm | 35 |
| Choline Chloride, ppm | 1,800 |
| Folic Acid, ppm | 6.0 |
| Pyridoxine, ppm | 18 |
| Biotin, ppm | 2.0 |
| Vitamin $B_{12}$, mcg/kg | 44 |
| Vitamin A, IU/gm | 4.4 |
| Vitamin $D_3$ (added), IU/gm | 2.0 |
| Vitamin E, IU/kg | 60 |
| Ascorbic Acid, ppm | 250 |

FINAL REPORT



Chow Process Research Experiment No.: 3562
CPR Project No.: 20
Study Director: Kent Lanter
Location: Research Manufacturing Unit, Gray Summit, MO
Number of Treatments: 2
Number of Formulations: 2
Date Run: 6/3/99
Confidentiality: Standard Research Program

TITLE: Bt Corn

OBJECTIVE
To produce small samples of extruded catfish feed using Bt Corn from Dow AgroSciences.

METHODS
Product was extruded on the Wenger X-20 extruder using the 8 head aggressive setup, one 3/16" hole and 6 blade knife. Corn was ground through a #6 Fitz Mill screen. At the end of the running of ration A, the feeder was allowed to run empty, then ration B was immediately added to the feeder. After 1.5 minutes Ration B was coming out of the extruder die. After 3.0 minutes sample collection began.

RESULTS AND CONCLUSIONS
Product extruded normally. The Bt corn was more dense (39 vs 32-33 pounds/bushel) possibly due to higher oil content of corn but this was not analyzed.

VARIABLE TABLE DATA SUMMARY

| | A | B |
|---|---|---|
| Normal Corn | + | |
| Bt Corn | | + |

Extrusion Conditions

| | A | B |
|---|---|---|
| Conditioned Meal °F | 192-205 | 206 |
| Steam Flow Control Valve, % | 78 | 78 |
| Water Conditioner lbs/min | 0.26 | 0.27 |
| Water Barrel lbs/min | 0.31 | 0.31 |
| Extruder RPM | 567 | 566 |
| Extruder Amps | 22 | 21 |
| Feeder Setting, % | 20 | 20 |
| Production Rate (wet) lbs/min | 5.6 | 5.2 |
| Density Wet lbs/bu | 32-33 | 39 |
| Die # | #016, 3/16" round | |
| Number of Holes | 1 | 1 |
| Number of Knife Blades | 6 | 6 |
| Barrel Setup | 8 head aggressive | |
| Jacket Control | Water on all heads | |

FORMULATION

| Major Ingredients | % |
|---|---|
| Soybean Meal | 50.0 |
| Corn | 38.72 |
| Fish Meal | 8.0 |
| Animal Fat | 1.5 |

DISTRIBUTION
Standard plus M. Griffin, G. Ballam, Monte Mayes and Diane Shanahan of Dow AgroSciences.

ACTION
Samples are being sent to Dow AgroSciences. No further testing is planned at this time.

STUDY DIRECTOR Kent Lamb (KJL) DATE 6/7/99

APPROVED K. E. Pike (KEP) DATE 6/8/99