UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460
OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

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Date: April 8, 2002

MEMORANDUM:

SUBJECT: EFED Review of 20-day Toxicity Study of Atrazine to Largemouth Bass

To: Kimberly Lowe, PM Team Reviewer
Robert McNally, Product Manager 60
Special Review and Reregistration Division (7508C)

FROM: Thomas Steeger, Ph.D., Senior Biologist Thomas M Steeger
William Rabert, Biologist
Environmental Fate and Effects Division (7507C)

THRU: Kevin Costello, Acting Chief Kevin J Costello
Environmental Risk Branch III/Environmental Fate and Effects Division (7507C)

The Environmental Fate and Effects Division has completed its review of the study entitled "Determination of Potential Effects of 20-day Exposure of Atrazine on Endocrine Function in Adult Largemouth Bass (*Micropterus salmoides*)" (MRID 456223-04). Largemouth bass were exposed to nominal concentrations of technical grade atrazine (purity 97.1%) at 0, 25, 35, 50, 75, and 100 µg/L. Additionally, bass were exposed to commercial grade (purity 42.1%) atrazine at 100 µg/L. After 20 days, plasma concentrations of estradiol, 11-ketotestosterone, testosterone, and vitellogenin (a protein that serves in yolk formation) were measured. The nonguideline study is classified as supplemental and provides useful information on the potential effects of atrazine on endocrine-mediated pathways.

Although the study concluded (see attached Data Evaluation Record) that atrazine treatment did not effect plasma steroid or vitellogenin levels, EFED believes that the study is confounded by the high level of variability in the test results. However, the results show that in spite of high levels of variability atrazine treatment significantly increased plasma estradiol in females and significantly decreased plasma 11-ketotestosterone in males. Additionally, although not statistically significant, vitellogenin levels in atrazine-treated female fish appeared to be elevated relative to controls. The presence of quantitative levels of plasma vitellogenin in male bass is of particular concern since the protein is normally only expressed in females; males can be induced to synthesize vitellogenin if exposed to an estrogenic compound.. Furthermore, the formulated endproduct appeared to have

enhanced effects on plasma steroids and vitellogenin levels relative to technical grade atrazine. These data further substantiate EFED's concerns regarding the endocrine disrupting potential of both technical grade atrazine and its formulated endproduct.

Previous studies examining the endocrine disrupting potential of both technical and commercial grade atrazine have shown that atrazine exposure increased plasma estradiol. Additionally, treatment with commercial atrazine increased plasma vitellogenin levels and decreased plasma testosterone levels at concentrations greater than 50 µg/ml (Gross *et al.* 1997; Grady *et al.* 1998). The current study was undertaken to examine more environmentally relevant doses and exposure routes. To that end, reproductively mature (approximately 2 year old) Florida strain largemouth bass were exposed to technical grade atrazine using a static renewal, no flow system at concentrations ranging from 0 to 100 µg/L and to commercial grade atrazine at 100 µg/L. After 20 days, plasma steroid and vitellogenin levels were measured. Vitellogenin has been recommended as a biomarker for measuring exposure to environmental estrogens since it is a sex-specific protein that is normally synthesized in yolk-producing females following its induction by estrogen; males fish do not typically synthesize vitellogenin unless exposed to an environmental estrogen.

In the current study, female bass treated with 100 µg/L formulated atrazine contained significantly higher plasma estradiol and exhibited plasma vitellogenin roughly 37 times greater (260 µg/ml) than controls (7 µg/ml). Male bass treated with 100 ug/L formulated atrazine contained significantly lower plasma 11-ketotestosterone. While not statistically significant, plasma testosterone (286 pg/ml) was lower than controls (433 pg/ml) and plasma vitellogenin (42 µg/ml) was 7 times greater than control (6 µg/ml).

Male plasma estradiol in atrazine-treated fish was not significantly different than controls; however, levels of estradiol in males fish were surprisingly high. Although there was considerable variability in plasma vitellogenin levels, atrazine-treated fish appeared to have elevated plasma vitellogenin relative to controls. at 50 and 100 ug/L of atrazine. Plasma 11-ketotestosterone was significantly lower in fish exposed to atrazine concentrations greater than 35 ug/L.

Treatment of fish with commercial grade atrazine resulted in a significant increase in plasma estradiol in female fish and a significant decrease in 11-ketotestosterone in male fish. Although not statistically significant, plasma vitellogenin in both female and male fish appeared to be increased in fish treated with technical and commercial grade atrazine.

Although high variability confounds this study's ability to resolve the effects of atrazine on plasma steroids and vitellogenesis, the study has demonstrated that technical grade atrazine affects plasma 11-ketotestosterone in males and that the formulated product affects plasma estradiol in females.

Literature Cited

Grady, J., C. Wieser, J. Wiebe, and T. S. Gross. 1998. An evaluation of atrazine as a potential endocrine disruptor in largemouth bass. 19th Annual meeting of SETAC, Charlotte, NC, Nov 15 - 19.

Gosss, T. S. , S. Shrestha, C. Wieser, J. Wiebe, N. Denslow, C. Chow, W. E.. Johnson, and R. Stout. 1997. Evaluation of potential endocrine-disrupting effects of water-soluble herbicides in largemouth bass. Abstract, 18th Annual meeting SETAC, November 16 - 20.

DATA EVALUATION RECORD
§ 70-1(A) -- TOXICITY TEST WITH A WARMWATER FISH

1. **CHEMICAL**: Atrazine technical PC Code No.: 080803

2. **TEST MATERIAL**: Atrazine Purity: 97.1%

3. **CITATION**

Authors: Carla M. Wieser and Tim Gross
Title: Determination of Potential Effects of 20 Day Exposure of Atrazine on Endocrine Function in Adult Largemouth Bass
Study Completion Date: February 15, 2002
Laboratory: University of Florida Wildlife Reproductive Toxicology Lab
7920 NW 71st Streeet
Gainsville, FL
Sponsor: Syngenta Crop Protection, Inc.
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419
Laboratory Report ID: Wildlife Number NOVA98.02e
MRID No.: 456223-04
DP Barcode: D281928

4. **REVIEWED BY**: Thomas M. Steeger, Ph.D., Senior Biologist, Environmental Risk Branch 4, Environmental Fate and Effects Division, U. S. Environmental Protection Agency

Signature: Thomas M Steeger **Date:** 4/8/02

5. **APPROVED BY**: William Rabert, Biologist, Environmental Risk Branch 3, Environmental Fate and Effects Division, U. S. Environmental Protection Agency

Signature: William Rabert **Date:** April 12, 2002

6. **STUDY PARAMETERS**

Scientific Name of Test Organism: *Micropterus salmoides*
Age or Size of Test Organism: Approximately 2 years old
Definitive Test Duration: 20 days
Study Method: Static Renewal
Type of Concentrations: Mean measured

7. **CONCLUSIONS**: This nonguideline study is classified as supplemental. Atrazine-treated female largemouth bass exhibited a dose-dependent increase in serum estradiol Female plasma vitellogenin concentrations, while not significantly different were minimally four times greater in atrazine-treated female bass

(range: 29 - 260 ug/ml) than in controls (7 ug/ml).

Male plasma estradiol in atrazine-treated fish was not significantly different than controls; however, levels of estradiol in males fish were surprisingly high. Although there was considerable variability in plasma vitellogenin levels, atrazine-treated fish appeared to have elevated vitellogenin relative to controls at 50 and 100 ug/L of atrazine. Male plasma 11-ketotestosterone was significantly lower in fish exposed to atrazine concentrations greater than 35 ug/L.

Female bass treated with 100 µg/L formulated atrazine contained significantly higher plasma estradiol and exhibited plasma vitellogenin roughly 37 times greater (260 µg/ml) than controls (7 µg/ml). Male bass treated with 100 ug/L formulated atrazine contained significantly lower plasma 11-ketotestosterone. While not statistically significant, male plasma testosterone (286 pg/ml) was lower than controls (433 pg/ml) and male plasma vitellogenin (42 µg/ml) was 7 times greater than control (6 µg/ml).

Exposure to technical grade atrazine resulted in decreased 11-ketotestosterone levels in male bass. Treatment of fish with commercial grade atrazine resulted in significant increase in plasma estradiol in female fish and a significant decrease in 11-ketotestosterone in male fish. Although not statistically significant, plasma vitellogenin in both female and male fish appeared to be increased in fish treated with technical and commercial grade atrazine.

Although high variability confounds this study's ability to resolve the effects of atrazine on plasma steroids and vitellogenesis, the study demonstrates that technical grade atrazine decreases plasma 11-ketotestosterone in males and that the formulated product significantly increased plasma estradiol in females.

8. ADEQUACY OF THE STUDY

A. Classification: Supplemental

B. Rationale: Nonguideline Study

C. Repairability: NA

9. GUIDELINE DEVIATIONS

Not applicable.

10. SUBMISSION PURPOSE: Determine plasma gonadal sex steroid (estradiol, 11-ketotestosterone and testosterone) and vitellogenin concentrations in adult largemouth bass after a 20-day aquatic exposure to atrazine.

11. MATERIALS AND METHODS

A. Test Organisms

Guideline Criteria	Reported Information
Species	*Mircropterus salmoides* (Florida subspecies)
Mean Weight 0.5-5 g	males: 160 ± 1.5 g females: 153 ± 1.3 g
Mean Standard Length Longest not > 2x shortest	Mean: males: 241 ±0.7 mm females: 236 ±0.6 mm Range: males 195 - 280 mm females: 202 - 283 mm
Supplier	Florida Caribbean Science Center
All fish from same source?	Yes
All fish from the same year class?	Not reported

B. Source/Acclimation

Guideline Criteria	Reported Information
Acclimation Period	3-day acclimation to exposure tanks
Wild caught organisms were quarantined for 7 days?	N/A
Were there signs of disease or injury?	Not reported
If treated for disease, was there no sign of the disease remaining during the 48 hours prior to testing?	NA
Feeding	Fish maintained on commercial diet Floating Fish Nuggets (Ziegler Brothers Inc.)

Guideline Criteria	Reported Information
Pretest Mortality No more than 3% mortality 48 hours prior to testing	% mortality prior to testing.

C. Test System

Guideline Criteria	Reported Information
Source of dilution water Soft reconstituted water or water from a natural source, **not** dechlorinated tap water	well water
Does water support test animals without observable signs of stress?	Yes
Water Temperature 17°C or 22°C	18 - 25.1 °C
pH Prefer 7.2 to 7.6	7.0 - 8.6
Dissolved Oxygen Static: ≥ 60% during 1st 48 hrs and ≥ 40% during 2nd 48 hrs, flow-through: ≥ 60%	5.2 - 9.1 mg/L
Total Hardness Prefer 40 to 48 mg/L as $CaCO_3$	not reported
Test Aquaria 1. Material: Glass or stainless steel 2. Size: Volume of 19 L (5 gal) or 30 x 60 x 30 cm 3. Fill volume: 15-30 L of solution	Polyethylene tanks (122 cm diameter, 91-cm depth, 900-liter capacit

Guideline Criteria	Reported Information
Type of Dilution System Must provide reproducible supply of toxicant	static renewal, no flow, conditions with a 50% water change/replacement daily.. Water replaced at a flow rate of 3.8 liters/minute, Treatments were administered when the water volume was at 75% of final volume by the addition of the appropriate 1-liter stock solution
Flow Rate Consistent flow rate of 5-10 vol/24 hours, meter systems calibrated before study and checked twice daily during test period	0.5 volume/24 hours
Biomass Loading Rate Static: ≤ 0.8 g/L at ≤ 17°C, ≤ 0.5 g/L at > 17°C; flow-through: ≤ 1 g/L/day	not reported
Photoperiod 16 hours light, 8 hours dark	not reported
Solvents Not to exceed 0.5 ml/L for static tests or 0.1 ml/L for flow-through tests	Solvent: water

D. Test Design

Guideline Criteria	Reported Information
Range Finding Test If LC_{50} >100 mg/L with 30 fish, then no definitive test is required.	Range finding test based on study with formulated product
Nominal Concentrations of Definitive Test Control & 5 treatment levels; dosage should be 60% of the next highest concentration; concentrations should be in a geometric series	0, 25, 35, 50, 75, and 100 µg ai/L techncial grade atrazine and 100 µg/L formulated product.

Guideline Criteria	Reported Information
Number of Test Organisms Minimum 10/level, may be divided among containers	3 replicates per treatment, 50 bass per tank (minimally 20 males and 20 females per tank)
Test organisms randomly or impartially assigned to test vessels?	Yes
Biological observations made every 24 hours?	Not reported
Water Parameter Measurements 1. Temperature Measured constantly or, if water baths are used, every 6 hrs, may not vary > 1°C 2. DO and pH Measured at beginning of test and every 48 h in the high, medium, and low doses and in the control	pH, dissolved oxygen, conductivity and temperature were monitored twice daily, once prior to water replacement and approximately 2 hours post water replacement
Chemical Analysis Needed if solutions were aerated, if chemical was volatile, insoluble, or known to absorb, if precipitate formed, if containers were not steel or glass, or if flow-through system was used	Water samples collected on each day of water/atrazine renewal

12. REPORTED RESULTS

A. General Results

Guideline Criteria	Reported Information
Quality assurance and GLP compliance statements were included in the report?	Yes
Recovery of Chemical	92 - 126%
Control Mortality Not more than 10% control organisms may die or show abnormal behavior.	< 1 % (1 fish / 150 fish)

Guideline Criteria	Reported Information
Raw data included?	Yes
Signs of toxicity (if any) were described?	Yes (in terms of mortality)

Plasma Biomarkers for **females**

Concentration (ppb)		Number of Fish	Plasma Concentrations			
Nominal	Mean Measured		Estradiol pg/ml	11-ketotestosterone pg/ml	testosterone pg/ml	vitellogenin µg/ml
Control			738	333	411	7
25	27.55		759	316	367	103
35	37.62		836	442	339	74
50	48.04		903	317	302	65
75	80.21		829	350	512	105
100 (tech)	91.75		1029	327	437	29
100 (form)	125.58		1245*	403	230	260

(Tech) = technical grade (purity 97.1%)
(form) = formulated endproduct (purity 42.1%)
*Significantly different than controls (α = 0.05)

Plasma Biomarkers for **males**

Concentration (ppb)		Number of Fish	Plasma Concentrations			
Nominal	Mean Measured		Estradiol pg/ml	11-ketotestosterone pg/ml	testosterone pg/ml	vitellogenin µg/ml
Control			573	1038	433	6
25	27.55		582	822	387	8
35	37.62		552	762	357	7
50	48.04		563	489*	342	18
75	80.21		518	544*	515	5
100 (tech)	91.75		596	526*	472	18
100 (form)	125.58		644	505*	286	42

(Tech) = technical grade (purity 97.1%)
(form) = formulated endproduct (purity 42.1%)
*Significantly different than controls ($\alpha = 0.05$)

Study concludes that for female bass plasma testosterone and 11-ketotestosterone were not altered regardless of atrazine dose. Results for female bass did indicate a dose-dependent increase in plasma estradiol with a significant increase occurring in the highest treatment: 100 µg/L commercial grade atrazine, but not for the 100 µg/L technical grade. Although plasma vitellogenin appeared to be increased for female fish exposed to 100 µg/L commercial grade atrazine, no significant effect was detected at any concentration of technical or commercial grade atrazine. In contrast, results for male bass did not indicate any effects of atrazine for plasma estradiol, testosterone nor vitellogenin regardless of dose or atrazine grade. Plasma 11-ketotestosterone in bass was, however, decreased in a dose-dependent manner at concentrations of technical atrazine greater than or equal to 50 ug/L.

Regression analyses were utilized to determine potential dose-dependent relationships between biomarker responses and water or plasma atrazine concentration. These analyses did not detect dose dependent responses for testosterone or vitellogenin, regardless of sex or atrazine does. Dose-dependent responses were noted for plasma estradiol in female bass and for plasma 11-ketestosterone in male fish. The dose-dependent response for effects of atrazine in bass are, however, not strong or clear. The 11-ketotestosterone response in male fish is, in general, more reflective of a threshold response with a similar response at atrazine concentrations greater than 48 µg/L in the water and 199 µg/L or greater for plasma. The dose-dependent response for estradiol in female bass is even less clear and is characterized as a response at atrazine concentrations of 126 µg/L or greater in water and 416 µg/L or greater in plasma.

14. REVIEWER'S COMMENTS:

As correctly noted by the authors, vitellogenin is the primary egg yolk precursor protein produced in the liver in response to estrogen. Vitellogenin is also considered a sex-specific protein since its presence requires exposure to estrogen. Thus, male fish do not synthesize vitellogenin unless they have been exposed to an estrogen-like compound. It is for this reason that vitellogenesis has been proposed as a biomarker for estrogenic compounds. Although the report notes that results indicate a high variance in vitellogenin and detectable concentrations in male fish, it concludes that plasma vitellogenin was not significantly altered by atrazine treatment regardless of dose or sex. This reviewer

believes that the high variability confounds the study's ability to detect treatment effects; however, the detectable levels of vitellogenin in male fish (**Figure 1**) clearly indicates that the animals have been exposed to an estrogen-like compound that resulted in the hepatic synthesis of vitellogenin. If laboratory male fish from this testing facility "normally" express vitellogenin in what they depict as normal seasonal fluctuations in plasma estradiol levels, then the fish from this laboratory are not suitable for testing vitellogenesis as a biomarker. Although not statistically significant, plasma vitellogenin in both female (**Figure 2**) and male fish appeared to be increased in fish treated with technical and commercial grade atrazine.

EFED is unclear why plasma estradiol was almost as high in male control fish (average 573 pg/ml) as female control fish (average 738 pg/ml) (**Figure 3**). However, **Figure 3** clearly illustrates the dose response in estradiol concentrations relative to atrazine exposure in females.

Exposure to technical grade atrazine resulted in decreased 11-ketotestosterone levels in male bass. Treatment of fish with commercial grade atrazine resulted in dose-dependent increase in plasma estradiol

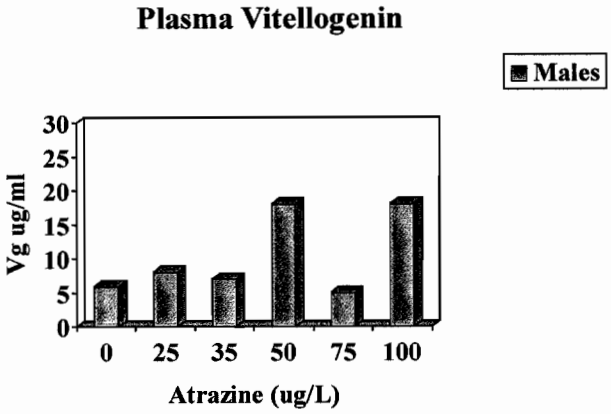

Figure 1 Average plasma vitellogenin in male fish exposed to technical grade atrazine for 20 days.

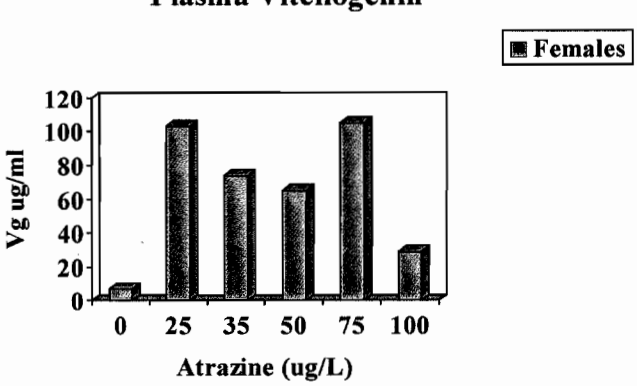

Figure 2 Average plasma vitellogenin in female fish exposed to technicnal grade atrazine for 20 days.

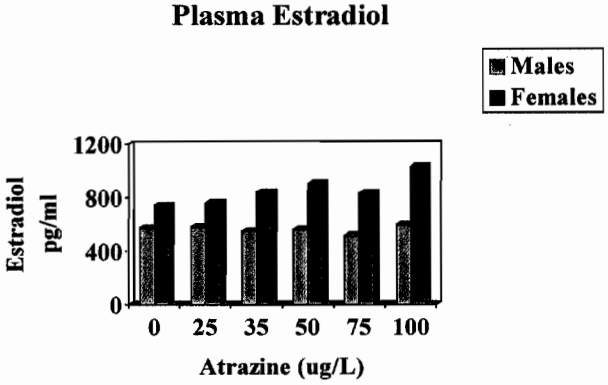

Figure 3 Average plasma estradiol in male and female fish exposed to technical grade atrazine for 20 days.

in female fish (**Figure 3**) and a significant decrease in 11-ketotestosterone in male fish.

Although high variability confounds this study's ability to resolve the effects of atrazine on plasma steroids and vitellogenesis, the study demonstrates that technical grade atrazine decreases plasma 11-ketotestosterone in males and that the formulated product increased plasma estradiol in females and decreased plasma 11-ketotestosterone in males. Although the study collected gonadal tissue for future aromatase assay, the tissues were discarded. It is unforturnate though that aromatase was not assayed as it may have provided an insight on the mechanism of atrazine's action on plasma steroid levels.