Data Evaluation Report on Atrazine-Induced Hermaphroditism at 0.1 ppb in American Leopard Frogs (*Rana pipiens*): Laboratory and Field Evidence

EPA MRID Number: None

Data Requirement:

EPA DP Barcode None

EPA MRID Not Assigned
EPA Guideline Open Literature

Test material: **Purity:** 98%
Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 29, 2003
Environmental Fate and Effects Division, ERB 4, U. S. Environmental Protection Agency

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:**
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth), U. S. Environmental Protection Agency

Stephanie Irene, Ph.D., Senior Advisor **Date**:
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary Frankenberry, Senior Statistician **Date:**
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 04/29/2003

CITATION: Hayes, T. B., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2002. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives

EXECUTIVE SUMMARY:

The objective of this study was to examine the effects of atrazine on leopard frogs (*Rana pipiens*) under controlled laboratory conditions, and once the effects were identified to examine wild *R. pipiens* from a variety of habitats in areas with reportedly low atrazine use and high atrazine use. Water samples were also collected at each field site to determine atrazine exposure. The combination of both laboratory and field studies was intended to address the ecological significance and relevance of the initial laboratory studies.

In the laboratory, leopard frog larvae were exposed from 48-hrs post-hatch through complete tail resorption (NF-Stage 66) to atrazine at 0.1 and 25 µg/L in 0.0036% ethanol in 10% Holtfreter's solution. Animals were sacrificed; gross morphology and histological analysis of gonads revealed that 36% and 12% of the males treated with atrazine at 0.1 and 25 µg/L, respectively, suffered form gonadal dysgenesis (under-developed testes with poorly structured, closed lobules and low to absent germ cells). Further, 29% of the 0.1 µg/L and 8% of the 25 µg/L animals displayed varying degrees of sex reversal; testicular lobules of sex-reversed males contained oocytes, and males that metamorphosed later contained large numbers of oocytes. In a few cases, testicular oocytes were reported to be vitellogenic, *i.e.*, contained yolk.

In a field reconnaissance survey of leopard frogs in four low atrazine-use and four high atrazine-use sites, testicular oocytes were identified in males from seven of the eight collection sites. All sites with atrazine levels exceeding 0.2 µg/L had males that displayed sex-reversal similar to those abnormalities induced by atrazine in the lab. The highest incidence (92%) and most advanced cases of hermaphroditism were observed in animals collected from the North Platte River in Wyoming where atrazine residues found in water samples were among the lowest recorded. At sites with similar atrazine residues, the incidence of gonadal abnormalities varied considerably suggesting that there was not a clear pattern of response. Even at sites where no residues were reported, the incidence of testicular oogenesis appeared to be as high as 18%. Additionally, atrazine residues reported for each of the sampling sites may not be reflective of actual exposure conditions during larval development. Although young frogs were reportedly sampled, there is no information to support when the animals may have undergone metamorphosis relative to the atrazine residue analysis.

Although the researchers conducted pesticide residue analysis for chemicals that were reportedly used in the watershed, the scope of chemical analyses was on a site-by-site basis. As the authors noted, even atrazine was present where it was not used, and it is likely that other chemical contaminants may have been present. Chemical profiles of the sites and information on the morphoedapic characteristics of the sites would have shed light on the comparability of the study sites.

In their paper, the study authors suggested that the enhanced response at lower doses was consistent with low-dose effects reported for other endocrine-disrupting chemicals; however, the data did not show a clear dose response pattern. In a previous study using *Xenopus* (Hayes *et al.* 2002), there appeared to be a threshold effect for testicular abnormalities at 0.1 µg/L but the response appeared to remain steady across increasing concentrations of atrazine with 16 - 20% of the males exhibiting gonadal abnormalities. In the laboratory study, only two doses of atrazine were tested, and the field study did not indicate a clear trend. From these two studies, the study authors concluded there is an "inverted-U" (parabolic) dose response curve.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description: Chemservice, Chester, PA

Lot No./Batch No. : Not reported

Purity: 98%
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: American leopard frog *(Rana pipiens*)
.
Age at test initiation: 48-hrs post-hatch
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Leopard frogs [eggs] obtained from Sensiba Marsh, Brown County, Wisconsin.

B. STUDY DESIGN:

Objective: To examine the effects of atrazine on leopard frogs (*Rana pipiens*) under controlled laboratory conditions and once the effects were identified to examine wild *Rana pipiens* from a variety of habitats in areas with reportedly low atrazine use and high atrazine use. Water samples were also collected at each field site to determine level of atrazine. The combination of both laboratory and field studies was intended to address the ecological significance and relevance of the initial laboratory studies.

1. Experimental Conditions

a) **Range-finding Study**: Not reported
b) **Definitive Study**

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	Leopard frog eggs were allowed to hatch (conditions not stated) and then apportioned to rearing tanks.
Duration of the test	48-hrs post-hatch through Nieuwkoop-Faber (NF)Stage 66
Test condition static/flow- through Type of dilution system for flow-through method. Renewal rate for static renewal	 static renewal NA complete exposure solution change every 72 hours
Aeration, if any	exposure tanks aerated
<u>Test vessel</u> Material: (glass/stainless steel) Size: Fill volume:	 plastic cages (personal communication, T. Hayes, 2002) 4 L
Source of dilution water quality:	Deionized, distilled water (personal communication, T. Hayes, 2002)

Parameter	Details
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	 not reported not reported not reported not reported not reported not reported not reported not reported not reported not reported 22ºC not reported not reported
Number of replicates/groups: negative control: 0.004% ethanol treated ones:	3 replicates
Number of organisms per replicate /groups:	30 larvae per replicate
Biomass loading rate	30 larvae/4 L
Test concentrations: nominal:	0.1 and 25 µg/L
Solvent (type, percentage, if used)	0.0036% ethanol in 10% Holtfreter's solution Holtfreter's medium:
Lighting	12 hrs light, 12 hrs dark
Feeding	Purina rabbit chow *ad libitum* (Purina Mills, St. Louis, MO)

Parameter	Details
Recovery of chemical Level of Quantitation Level of Detection	 detection limit: 0.1 µg/L
Positive control {if used, indicate the chemical and concentrations}	not reported
Other parameters, if any	

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	mortality, time to metamorphosis, weight and length at metamorphosis, sex based on gross morphology (all animals) and histology (9 females per treatment and on all males).
Observation intervals	not reported
Were raw data included?	No
Other observations, if any	

Field study sites were initially selected based on atrazine sales data and were located between 39°N and 43°N latitude. Counties with less than 0.4 kg/km^2 atrazine use were chosen as potential control sites, while areas with > 9.3 kg/km^2 atrazine use were chosen as potential atrazine-exposed sites. Sampling began in Utah on July 15, 2001 and moved eastward. Sampling stopped at the Iowa-Illinois border because *R. pipiens* populations were reportedly low or threatened in Illinois and Indiana.

A total of 8 sites (4 control and 4 "atrazine-contaminated") were sampled with 100 frogs collected at each site. Small frogs were intentionally collected in order to sample newly metamorphosed animals. Animals were immediately euthanized and fixed in Bouin's for 48 hours and preserved in 70% ethanol.

Back in the lab animals were measured, sex determined and histological analysis conducted on the gonads of 20 males from each site and a subset of females from each site.

Water samples (100 ml) were collected at each site in chemical-free glass jars (Fisher Scientific Co., Houston, TX) and frozen on dry ice immediately upon collection. Atrazine levels were determined by liquid chromatography/mass spectrophotometry (PTRL West, Inc., Hercules, CA); duplicate samples were analyzed using gas chromatography with a nitrogen phosphorous detectro (EPA method 507; Hygenic Laboratory

University of Iowa, Iowa City, Iowa). In addition to parent atrazine, diaminochlorotriazine (DAC), deisopropylatrazine (DIA), deethylatrazine (DEA), two other triazines (simazine and hexazinone) and two other herbicides (diuron and norfluraone) were analyzed at all sites. The following pesticides were analyzed for water collected in Utah, Wyoming and Nebraska:: metolachlor, alachlor, glyphosate, metalaxyl, nicosulfuron, propiconazole, β-cyfluthrin, λ-cyhalothrin, and tebupirimphos.

II. RESULTS and DISCUSSION:

Laboratory Study

Control and atrazine-treated animals were sexually differentiated at metamorphosis, but 36% and 12% of the males treated with atrazine at 0.1 and 25 µg/L, respectively, suffered form gonadal dysgenesis (under-developed testes with poorly structured, closed lobules and low to absent germ cells.) Further, 29% of the 0.1 µg/L and 8% of the 25 µg/L animals displayed varying degrees of sex reversal. Testicular lobules of sex-reversed males contained oocytes, and males that metamorphosed later contained large numbers of oocytes. Males that appear to have undergone complete sex reversal had gonads completely filled with oocytes. In 2 males, oocytes were vitellogenic making the oocytes observable by gross morphology. Control males "never" contained testicular oocytes, although 2 control males contained 2 - 3 degenerating extragonadal oocytes (not in lobule) and a single male showed gonadal dysgenesis.

Field Study

None of the collection sites were atrazine-free (**Table 3**); however, atrazine residues were not reported at site 7. Except for metalochlor at site 5 (York County NE), pesticides were not found at any of the sites. Testicular oocytes were identified in males from 7 out of 8 collection sites. All sites with atrazine levels exceeding 0.2 µg/L had males that displayed sex-reversal similar to those abnormalities induced by atrazine in the lab. The highest incidence (92%) and most advanced cases of hermaphroditism were observed in animals collected from the North Platte River in Wyoming (site 3).

Table 3. Sample site, date, locality, description and atrazine residues for American leopard frog *(Rana pipien*) collections (source: Hayes *et al.* 2002).

Site	Date	State	County	Altitude (m)	Source	Habitat	Atrazine µg/L
1	7/15/01	Utah	Juab	1500	Pond	Grazeland	0.1
2	7/17/01	Utah	Cache	1555	Pond	Golf Course	0.2
3	7/19/01	Wyoming	Carbon	1952	river	Wildlife Area	0.2
4	7/23/01	Nebraska	Cherry	1031	Pond	Prarie	0.3
5	7/22/01	Nebraska	York	480	Ditch	Corn Field	0.8[1]
6	7/26/01	Iowa	Polk	252	Ditch	Corn Field	6.7
7	7/28/01	Iowa	Polk	246	Marsh	Wildlife Area	NA [2]
8	7/28/01	Iowa	Clinton	211	Stream	River Valley	0.5

[1] according to report figure, this value is more likely 8.0 µg/L.
[2] inconsistent results, one lab reported below level of detection

According to the authors, atrazine exposure disrupted gonadal development in exposed larvae as evidenced by poorly developed testicular tubules and reduced germ cells (gonadal dysgenesis) and oocytes developing in testes (testicular oogenesis); in a few cases, oocytes were vitellogenic.

F. REVIEWER'S COMMENTS:

This study is useful in identifying a potential hazard to amphibians and presents information on measurement endpoints, such as gonadal deformities. The study, however, does not show a clear dose response that demonstrates a causal relationship between atrazine exposure and developmental effects. The study authors suggested that the enhanced response at lower doses was consistent with low-dose effects reported for other endocrine-disrupting chemicals, but the data did not show a clear pattern of response. In a previous study using *Xenopus* (Hayes *et al.* 2002), there appeared to be a threshold effect for testicular abnormalities at 0.1 µg/L. The response, though, appeared to remain steady across increasing concentrations of atrazine with 16 - 20% of the males exhibiting gonadal abnormalities. Additional information is needed to support the authors conclusion that of an "inverted-U" (parabolic) dose response curve.

Access to the raw data supporting this study would also help the Agency verify the study's conclusions regarding time to metamorphosis, growth, and gonadal development. Although the study authors report that in some cases animals exhibited a mix of testicular and ovarian tissue and that the oocytes were vitellogenic,

i.e., contained yolk, they provide no evidence that any differential staining was done to verify that the oocytes were indeed vitellogenic. To better support their argument, a stain capable of differentiating phospholipids or glycolipids should have been used. The authors go on to state that induction of vitellogenesis in males would be consistent with upregulation of aromatase resulting in the increased production of endogenous estrogen. Upregulation of aromatase, according to the authors would also account for the failure to induce spermatogenesis (demasculinization) and induction of oocyte growth (feminization). The authors note that testicular oogenesis was not observed in any of the control animals nor in any of the *R. pipiens* routinely studied in their laboratories.

In this study, total atrazine residues (including atrazine degradates) are presented in graphical form and required extrapolation. According to this graph, parent atrazine residues (0.2 µg/L) at site 3 (highest incidence and most advanced cases of hermaphroditism) were similar to atrazine residues (0.2 µg/L) at site 2 (second lowest incidence of hermaphroditism. At site 4 (second highest incidence of testicular oogenesis), atrazine residues (0.3 µg/L) were relatively similar to site 3; however the atrazine degradates DEA, DIA and DAC resulted in total residues of approximately 1.2 µg/L. The highest residues of atrazine and its degradates occurred at site 5 [the study reports residues at 0.8 µg/L, but , the figure suggests that the reported value is actually 8 µg/L]. This site had the third highest rate of gonadal abnormalities, and the abnormalities were primarily gonadal dysgenesis rather than hermaphroditism. The second highest site (site 6) for atrazine residues (6.7 µg/L) corresponded to the third lowest incidence of gonadal abnormalities. Although the study showed gonadal effects in frogs, there was no clear pattern of response. Atrazine residues were not reported at Site 7 , and at least one of the two labs measuring atrazine reported that residues were below the level of detection; however, testicular oogenesis was reported in roughly 18% of the males examined.

In its evaluation, the Agency had questions concerning the use of ethanol as a co-solvent Atrazine concentrations used in the exposures are less than the 30 mg/L solubility limit of atrazine, and a co-solvent should not have been necessary . Previous studies conducted by the authors (personal communication: Tyrone Hayes 2002) have used dihydrotestosterone and 17-β estradiol as positive controls and required ethanol as a co-solvent. The current study, though, did not use these steroids, and a co-solvent should not have been needed.

EPA also needs more specific information concerning the methods used to determine the presence of vitillogenic oocytes. To better support their argument, a stain capable of differentiating phospholipids or glycolipids should be be used. In this study, the authors state that induction of vitellogenesis in males would be consistent with upregulation of aromatase resulting in the increased production of endogenous estrogen. Upregulation of aromatase, according to the authors would also account for the failure to induce spermatogenesis (demasculinization) and induction of oocyte growth (feminization). At the same time, the authors note that testicular oogenesis has not been observed in any of the control animals nor in any of the *R. pipiens* routinely studied in their laboratories.

Small frogs were intentionally sampled to assure that animals had recently metamorphosed implying that measured atrazine residues may have been reflective of exposure conditions during larval development. Additional information concerning the weights and age of the animals is needed to support this contention. Actual exposure conditions may have been considerably different than those reported in the study.

Although the researchers conducted pesticide residue analysis for chemicals that were reportedly used in the watershed, the scope of chemical analyses was on a site- by- site basis. As the authors noted, even atrazine

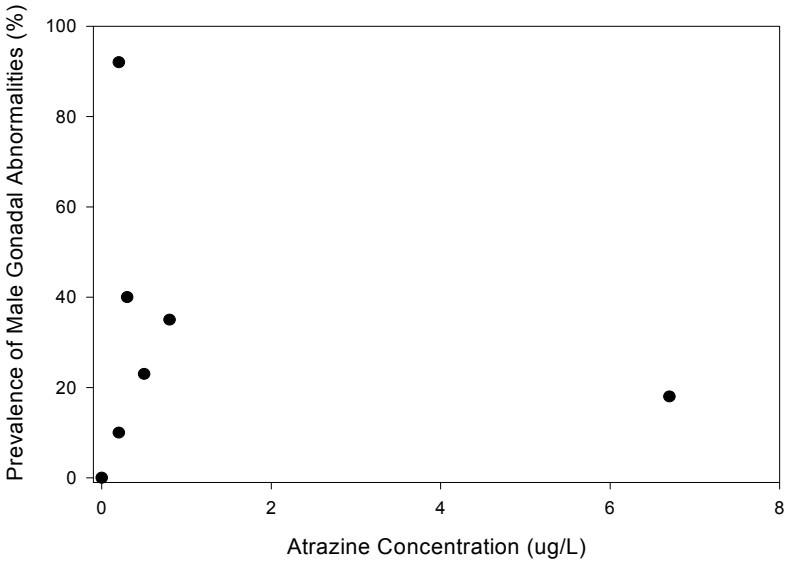

Figure 1. The prevalence of gonadal abnormalities in seven sites plotted against measured atrazine concentrations. One site is omitted because of a lack of atrazine analysis. Prevalence data are estimated from graphical representations of the data. This analysis also assumes that atrazine concentrations at site 5 are 0.8 ug/L, although figure 11A of the publication indicates that it was actually 8.0 ug/L.

was present where it was not used ,and it is likely that other chemical contaminants may have been present. Additional information on the chemical profile and morphoedapic characteristics of the sites is needed in order to compare the study sites.

The relationship of the potential atrazine exposure at the critical developmental stages is unknown because the surface water sampling was conducted during the post-metamorphic period. When the prevalence of male gonadal abnormalities is plotted as a function of atrazine concentration, there is no clear relationship (**Figure 1**). Additional data would help clarify this problem.

The Agency also needs clarifying data concerning the number of males and females collected at each site. Presumably at least 20 males were analyzed histologically, but it is unclear how these organisms were selected. If they were selected based on gross observations, then there is a strong possibility that the sample set was biased. Because a subset of organisms was selected and prevalence calculations were based on this selection, the subset should have been selected randomly.

In the laboratory study, 30 organisms per replicate with 3 replicates were exposed per treatment level. The prevalence of gonadal abnormalities was evidently based on combining the data of the replicates. It would be useful to understand the variance among the replicates and whether responses in individual tanks are driving the analysis. The Agency also needs data that indicates the distribution of males and females in the study and the sample sizes to gauge the robustness of the analyses. A further note concerning the laboratory study is the use of 30 organisms with an estimated maximal weight of 2.5 g in 4 liters of test solution. These conditions would result in a loading rate of 18.8 g/L, which is 37 times the recommended loading rate for static renewal toxicity tests.

The highest prevalence of gonadal abnormalities was observed at the site in Wyoming. Is it possible that there is a genetic component to this phenomenon? The authors state that in 7,000 organisms collected from four different states, no gonadal abnormalities [of the type described in the study] have ever been noted. Given the ubiquitous nature of atrazine contamination suggested in the paper, it is hard to imagine that these were all from atrazine-free environments, particularly in Wisconsin. If it is, in fact, true, then these data should have been published, as they would have provided great weight to the analysis.

As the study authors noted, the ecological relevancy of these findings is unclear because the researchers did not have problems in locating frogs. Further information is needed to determine if gonadal abnormalities impair the reproductive success, growth, and survival of amphibians.

H. REFERENCES:

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Science, 99 (8): 5476 - 5480.

Nieuwkoop, P. D. and J. Faber. 1994. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.