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Summary of EPA's Review of Amphibian Development Information for Triazines

Introduction

In assessing the potential for adverse effects on non-target organisms (*i.e*., terrestrial/aquatic plants and animals) from exposure to conventional pesticides due to registered or proposed uses, the Environmental Fate and Effects Division (EFED) of the U.S. Environmental Protection Agency (EPA) relies on multiple lines of evidence. These lines of evidence include unpublished studies submitted by the regulated community in compliance with regulatory testing requirements and consistent with Good Laboratory Practice standards, government reports, open literature peer-reviewed studies which meet EPA Office of Pesticide Program standards (USEPA 2011[1]) based on the Data Quality Act, and incident data contained within the Incident Data System. In considering these multiple lines of evidence, EFED uses a weight-of-evidence approach to evaluate data within the context of the whole body of information to reduce bias, improve transparency and ensure that regulatory decisions are evidence-based using the best available science. Consistent with EPA guidance, the weight-of-evidence approach considers all relevant information and accounts for the kinds of evidence available, the quality and quantity of the evidence, the strengths and limitations of each type of evidence. In assembling the available information, EPA considers the relevance (*i.e*., the degree of correspondence between the evidence and the assessment endpoint to which it is applied), reliability (*i.e*., the extent to which the data are compelling) and strength (magnitude) of the effect (USEPA 2016[2]).

As with other taxa and consistent with methods commonly employed in toxicology, a limited number of species are used for evaluating potential effects of various stressors (*i.e*., surrogate species) (USEPA 1995[3]). For example, freshwater fish are commonly used as surrogates for aquatic-phase amphibians and birds are uses as surrogates for terrestrial-phase amphibians (USEPA 2004[4]). Selection of surrogate species is based on multiple factors (*e.g*., availability, ease of handling, ability to thrive under laboratory conditions).

EPA's Reviews of Amphibian Data for Triazines

With respect to triazine herbicides and their potential effects on amphibians, there are multiple studies suggesting potential effects on native and non-native amphibian species where measurement endpoints have provided more sensitive endpoints than are available for freshwater fish and in some cases, EPA has relied on these amphibian-specific endpoints to assess effects to amphibians. Although in the Preliminary Ecological Risk Assessment for simazine (USEPA 2016b[5]) EPA indicated that there were no

[1] USEPA. 2011. Evaluation Guidelines for Ecological Toxicity Data I the Open Literature. Memorandum for D. Brady, Director, Environmental Fate and Effects Division. Dated May 16, 2011. https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/evaluation-guidelines-ecological-toxicity-data-open

[2] USEPA. 2016. Weight of Evidence in Ecological Assessment. Office of the Science Advisor Risk Assessment Forum. In *EPA 100/R-16/001*. https://nepis.epa.gov/Exe/ZyPDF.cgi/P100SFXR.PDF?Dockey=P100SFXR.PDF

[3] USEPA. 1995. Use of Surrogate Species in Assessing Contaminant Risk to Endangered and Threatened Fishes. Final Report September, 1995. Office of Research and Development, Washington DC. EPA/600/R-96/029. January 1995. http://www.cerc.usgs.gov/pubs/center/pdfDocs/90877.pdf (last accessed 01/15/2016).

[4] USEPA. 2004. Overview of the Ecological Risk Assessment Process in the Office of Pesticide Programs, U. S. Environmental Protection Agency, Endangered and Threatened Species Effects Determinations.

[5] USEPA. 2016b. Preliminary Ecological Risk Assessment for Simazine. DP Barcode D424724. Dated April 13, 2016. https://www.regulations.gov/document/EPA-HQ-OPP-2013-0251-0036

direct toxicity data for simazine effects on aquatic-phase amphibians at environmentally relevant concentrations, the Biological Evaluation of simazine, written in support of the federally listed threatened and endangered species assessment, identified a chronic toxicity endpoint (*i.e*., no observed adverse effect concentration; NOAEC) of 1.2 µg ai/L for the African clawed frog (*Xenopus laevis*) to evaluate impacts to aquatic vertebrates (fish and aquatic-phase amphibians) as the endpoint represented the most sensitive toxicity value for aquatic vertebrates (USEPA 2021[6]). The NOAEC was based on an open literature by Sai *et al*. 2016.[7]

Since the early 2000s, there have been competing accounts on how triazine herbicides (*e.g.*, atrazine) could affect amphibian development and in 2003, EPA reviewed a total of 17 studies, 12 of which were sponsored by the technical registrant (*i.e*., Syngenta) and 5 were published in the open literature. The registrant-submitted studies underwent detailed reviews by EPA as raw data and more in-depth descriptions of the underlying methods were available. In contrast, open literature studies (5) were reviewed at face value since the raw data were not available. Each study was individually evaluated with regard their relevance, reliability, and strength (*i.e*., experimental design, protocols and data quality assurance, strength of cause-effect and/or dose-response relationships, mechanistic plausibility, and ecological relevancy of measured endpoints). In the data evaluation records (DERs) for the registrant-sponsored studies, the Agency reviewers outlined the study methodologies, re-analyzed raw data, and documented any uncertainties or differences in conclusions from those of the study authors. Multiple confounding effects were identified across all of the studies and there wasn't a single study where uncertainties were not identified. Consistent with EPA's current process, DERs focused on uncertainties in how the studies were conducted (*i.e*., potential confounding effects) in addition to the statistical results of the studies.

Methodological discrepancies within some of the studies included atrazine contamination of the negative controls; poor water quality, poor growth/development/survival of controls, high variability limiting the power of the study to discriminate treatment effects, poor reproducibility, and poor positive control (*i.e*., 17-*beta* estradiol) response. The field studies represented a variety of study designs/conditions and test species, and there was natural variability between sampling sites. Some of the studies were confounded by atrazine contamination in the controls and the extent to which other pesticides were present was poorly characterized. Also, several of the studies had unusual environmental conditions which included high rain events and introduced predators.

The EPA White Paper[8] summarizing the 17 studies was presented to EPA's Federal Advisory Committee (*i.e*., FIFRA Scientific Advisory Panel; SAP) in 2003. The SAP agreed that there were multiple confounding effects in the available studies, and they agreed that there was sufficient information to formulate a hypothesis regarding potential effects which could be evaluated through additional testing. Therefore, in

[6] USEPA 2021. Final Biological Evaluation Chapter 2—Final Simazine Effects Characterization https://www.epa.gov/endangered-species/final-national-level-listed-species-biological-evaluation-simazine#chap2

[7] Sai L., B. Qu, Y. Li, Q. Jia, C. Bo, Y. Liu, G. Yu, L. Xie, L. Li, J. C. Ng, C. Peng. 2016. Continued Studies on the Effects of Simazine on the Liver Histological Structure and Metamorphosis in the Developing *Xenopus laevis* Bull. Environ. Contam. Toxicol. 97(4): 517-520

[8] USEPA. 2003. White Paper on Potential Developmental Effects of Atrazine on Amphibian. In Support of an Interim Reregistration Eligibility Decision on Atrazine. Submitted to the FIFRA Scientific Advisory Panel of Review and Comment June 17 – 20, 2003. https://archive.epa.gov/scipoly/sap/meetings/web/pdf/finaljune2002telconfreport.pdf

response to input from the SAP, EPA issued a data call-in (DCI) and the technical registrant agreed to conduct two studies consistent with SAP recommendations. EPA inspected both the laboratory-based studies and in 2007, the Agency presented the results of the study to a FIFRA SAP.

In a White Paper[9] written in support of the 2007 FIFRA SAP, EPA evaluated the registrant-conducted studies, which were responsive to the DCI, EPA also evaluated an additional 35 studies which had been published in the open literature. Some of these studies were finalized versions of studies which had been evaluated by EPA as interim reports in 2003. The evaluations were captured in another white paper.

Again, measurement endpoints evaluated in the studies included time to metamorphosis, growth, gonadal abnormalities, laryngeal dilatory muscle area, sex ratios, plasma steroid concentrations and tissue aromatase activity. Other than the two studies conducted in response to the DCI, study designs of the laboratory-based studies were not consistent with the SAP-recommended protocol and loading rates were a consistent issue where the ASTM-recommend rate of 1 tadpole/L or 1 gram/day was exceeded. For some of the studies, there was infrequent or incomplete renewal of exposure solutions. Exposure chambers were constructed of a variety of materials (*e.g*., plastic cages) some of which could have impacted study results. Also, environmental conditions were not well controlled/documented. A total of 9 field studies were reviewed; all of these had been interim reports reviewed as part of the 2003 SAP. Again though, triazine concentrations were poorly characterized as was chemical/mineral loading (*e.g.*, excessive chromium levels) and larval exposure conditions were not well characterized. The studies were generally not designed to address/limit potential sources of variability and they had a limited ability to correlate chemical exposure to effects on measurement endpoints.

Recent Reviews of Sai et al. Amphibian Studies Involving Triazines

EFED recently re-reviewed the Sai *et al*. 2016[10] paper cited in the simazine BE along with several other papers on both simazine (Sai *et al*. 2015[11]) and atrazine (Sai *et al*. 2016b[12]) by this researcher. As noted in the revised open literature study reviews (OLSR), the Sai *et al*. papers contained similar uncertainties as those discussed in the 2003 and 2007 EPA white papers limiting the ability of the study to evaluate effects. Although EPA evaluated the three publications in separate open literature study reviews, the two papers on simazine appear to rely on the same single study. The Sai *et al.* studies utilized static-renewal conditions and there is uncertainty as to the extent to which exposure solutions were completely

[9] USEPA. 2007. White Paper on the Potential for Atrazine to Affect Amphibian Gonadal Development. In Support of an Interim Reregistration Eligibility Decision on Atrazine. Submitted to the FIFRA Scientific Advisory Panel for Review and Comment. October 9 – 12, 2007.
https://archive.epa.gov/scipoly/sap/meetings/web/pdf/2007_amphibian_white_paper.pdf

[10] Sai L., B. Qu, Y. Li, Q. Jia, C. Bo, Y. Liu, G. Yu, L. Xie, L. Li, J. C. Ng, C. Peng. 2016. Continued Studies on the Effects of Simazine on the Liver Histological Structure and Metamorphosis in the Developing *Xenopus laevis* Bull. Environ. Contam. Toxicol. 97(4): 517-520

[11] Sai L., Y. Liu, B. Qu G. Yu; Q. Guo, C. Bo, L. Xie, Q. Jia, Y. Li, X. Li, J.C. Ng, C. Peng. 2015. The Effects of Simazine, a Chlorotriazine Herbicide, on the Expression of Genes in Developing Male *Xenopus laevis* Bull. Environ. Contam. Toxicol. 95(2): 157-163.

[12] Sai L.,Z. Dong, L.Li, Q. Guo, Q. Jai, L. Xie, C. Bo, Y. Liu, B. Qu, X Li, H. Shao, J.C. Ng and C. Peng. 2016b. Gene expression profiles in testis of developing male *Xenopus laevis* damaged by chronic exposure to atrazine. Chemosphere 159: 148 - 152.

renewed. Although dimethyl sulfoxide (DMSO) is a relatively common solvent for *in vitro* studies, it can affect the absorption, distribution, metabolism and excretion (ADME) of a compound and typically EFED recommends against the use of DMSO for *in vivo* studies. The Sai *et al*. studies with both simazine and atrazine did not include a negative control, the loading rate exceeded the ASTM rate of 1 larva/L, the environmental conditions are poorly characterized, the amount of food is not specified, post-metamorphic juveniles were fed non-standard diet (pork liver). For the simazine studies, there was a weak treatment response and time to metamorphosis for solvent control frogs exceeded 90 days.

The Sai *et al.* studies of both simazine and atrazine contain multiple confounding effects which based on input from FIFRA SAPs could obscure the ability of the study to evaluate treatment effects. These factors include potentially poor water quality, poor growth, development and survival of test animals, and variability in terms of a clear concentration-response. Also, while there were statistically significant effects on male gonad weight and gonado-somatic index (GSI), apical assessment endpoints were not affected. While there is uncertainty regarding how each of these factors affected the overall study, the limited understanding of water quality and husbandry conditions raise concerns regarding whether conditions were suitable for evaluating effects of triazines on amphibians. As in past assessment, EPA relied on the Nieuwkoop and Faber (1994[13]) publication describing various aspects of *Xenopus* development. This reference indicates that generally *Xenopus* should be completing metamorphosis by Day 58 of development whereas control frogs in the Sai *et al*. study of simazine took 90 days. Additionally, mortality in the simazine study ranged from 11 – 19% (Sai *et al*. 2015[14]) and isn't substantially different between controls and simazine although simazine exposure concentrations span 3 orders of magnitude. Even in terms of gonad weight, while testicular weight was significantly ($p<0.05$) reduced relative to the solvent control by 49% and 57% at simazine treatment levels of 11.1 and 100.9 µg/L, respectively, the percent reductions are relatively similar even though the simazine concentrations differ by 10x. Male GSI further illustrates that the measurement endpoint is not substantially different at the two highest simazine treatment concentrations.

Summary of EDSP Weight-of-Evidence Analysis for Triazines

As an additional line of evidence, both simazine and atrazine have been evaluated as part of the EPA Endocrine Disruptor Screening Program (EDSP) and have Tier 1 *in vitro* and *in vivo* data with which to assess potential impacts to the estrogen, androgen, and thyroid signaling pathways. In 2015, EPA completed the simazine EDSP weight-of-evidence analysis[15] indicating that GSI was marginally affected but only at concentrations of 3.5 mg ai/L, which is well above the concentrations at which Sai *et al*. 2015 and 2016 report effects. The EDSP Tier 1 weight-of-evidence analysis on simazine concluded that the data did not provide convincing evidence of potential interaction with the thyroid pathway and that amphibian development was not affected at concentrations up to 1.9 mg ai/L based on the results of the

[13] Nieuwkoop, P.D., and J. Faber. 1994. Normal Table of *Xenopus laevis* (Daudin) A Systematical and Chronological Survey of the Development From Fertilized Egg Till the End of Metamorphosis. Garland Publishing, Inc., New York and London 1994. ISBN 0-8153-1896-0

[14] Sai L., Y. Liu, B. Qu G. Yu; Q. Guo, C. Bo, L. Xie, Q. Jia, Y. Li, X. Li, J.C. Ng, C. Peng. 2015. The Effects of Simazine, a Chlorotriazine Herbicide, on the Expression of Genes in Developing Male *Xenopus laevis* Bull. Environ. Contam. Toxicol. 95(2): 157-163.

[15] USEPA 2015. Simazine Weight of Evidence Analysis. https://www.regulations.gov/document/EPA-HQ-OPP-2013-0251-0033

Amphibian Metamorphosis Assay with *Xenopus laevis* (Schneider *et al*. 2012[16]). The weight-of-evidence analysis for atrazine[17] indicated that the sporadic effects observed in the Tier 1 battery of standardized test and other scientifically relevant information (OSRI) was consistent with SAP conclusion that the chlorotriazines (including atrazine and its degradates) function through a neuroendocrine mode of action (MoA) that suppresses the hypothalamic release of GnRH and therefore LH. However, the WoE concluded that EDSP Tier 2 testing with mammals, fish, amphibians, or birds is not recommended for atrazine at this time because it is not expected to impact current EPA-established regulatory endpoints for ecological risk assessment, which relied on freshwater fish as surrogates for aquatic-phase amphibians. In other words, current assessment endpoints were considered protective for these effect thresholds. While the Sai et al. 2016b study of atrazine is more consistent with effect thresholds identified in the EDSP Tier 2 analysis, the uncertainties associated with the Sai *et al.* study would not support the use of the study as a line of evidence in assessing potential impacts to endocrine signaling pathways.

Conclusion that Fish are Appropriate Surrogates for Amphibians Exposed to Triazines

For simazine, the regulatory endpoint for freshwater fish is based on an early life stage toxicity study with Common Carp (*Cyprinus carpio*; Velisek *et al*. 2012[18]) with a NOAEC, maximum acceptable toxic concentration (MATC) and lowest observed adverse effect concentration (LOAEC) values of 0.060, 0.190 and 0.600 mg ai/L, respectively, based on a 29% reduction in body weight at the LOAEC. These values are more sensitive than the LOAEC identified in for simazine the Amphibian Metamorphosis Assay using *X. laevis* at 1.9 mg ai/L under standardized test conditions.

Similarly and based on the information contained within a third study by Sai *et al.* 2016b of atrazine, there is evidence to suggest that husbandry conditions for the test animals were not suitable although time to metamorphosis is not reported. While the NOAEC, MATC and LOAEC values of 0.0097, 0.0308 and 0.0977 mg ai/L, respectively, are relatively consistent with those for Atlantic Salmon (*Salmo salar*; Nieves-Puigdoller *et al*. 2007[19]) of 0.0085, 0.0367 and 0.0843 mg ai/L, they differ from the DCI studies with a NOAEC of 0.100 mg ai/L. As noted by the WoE analysis for atrazine, the fish endpoint is protective of amphibians.

Overall, the weight-of-evidence indicates that the open literature studies by Sai *et al.* used in support of the simazine BE have low relevance, reliability and strength and that consistent with past evaluations,

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[16] Schneider, S.Z., T.Z. Kendall, H.O. Krueger. 2012. Simazine- Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances. Unpublished study performed by Wildlife International, Ltd., Easton, Maryland. Laboratory report number 528A-227. Study sponsored by Syngenta Crop Protection, LLC, Greensboro, North Carolina. Study completed November 8, 2012. https://www.regulations.gov/document/EPA-HQ-OPP-2013-0251-0034

[17] USEPA 2015b. Atrazine Weight of Evidence Analysis. https://www.regulations.gov/document/EPA-HQ-OPP-2013-0266-0313

[18] Velisek, J., A. Stara, J. Machova, Z. Svobodava. 2012. Effects of long-term exposure to simazine in real concentrations on common carp (*Cyprinus carpio* L.). Ecotoxicology and Environmental Safety 76: 79 – 86. https://doi.org/10.1016/j.ecoenv.2011.10.013

[19] Nieves-Puigdoller, K., B. T Björnsson, S. D. McCormick. 2007. Effects of hexaxinone and atrazine on the physiology and endocrinology of smolt development in Atlantic Salmon. Aquatic Toxicology 84(1): 27 – 37. https://doi.org/10.1016/j.aquatox.2007.05.011

the freshwater fish is the most sensitive indicator of toxicity and serves as a suitable endpoint for aquatic vertebrates (*i.e.*, freshwater fish and aquatic-phase amphibians).