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Chronic toxicity of 1,3,5-triazine herbicides in the postembryonic development of the western clawed frog *Silurana tropicalis*

Masahiro Saka[a,*], Noriko Tada[a], Yoichi Kamata[b]

[a] Division of Aquatic Environment, Kyoto Prefectural Institute of Public Health and Environment, Murakamicho 395, Fushimi-ku, Kyoto 612-8369, Japan
[b] Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550, Japan

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ABSTRACT

Seven 1,3,5- triazine (*s*-triazine) herbicides (ametryn, prometryn, dimethametryn, simazine, atrazine, propazine, and cyanazine) were tested using an amphibian (*Silurana tropicalis*) metamorphosis assay focusing on morphometric, gravimetric, and thyroid-histological endpoints. Premetamorphic tadpoles were exposed to each *s*-triazine at 2 concentrations between 1/1000 and 1/10 of the 96-h acute toxicity values, until all tadpoles in the control group reached either the late prometamorphosic stages or the initial stage of metamorphic climax. All *s*-triazines tested induced significant retardation in growth and development at the higher concentrations (0.2–1.0 mg/L), and some of them induced similar effects even at the lower concentrations (0.02–0.1 mg/L) while each showing a linear dose-response. Total size of the thyroid glands tended to be reduced corresponding to the delayed development, but without showing histomorphological lesions typical of anti-thyroid chemicals. These consistent results suggest that the *s*-triazines can act as a chemical stressor inhibiting tadpole growth and development, possibly without disrupting the thyroid axis. In addition, tadpoles exhibiting spinal curvatures appeared in either one or both of the lower and higher concentration groups for each *s*-triazine tested. The incidence rate in the *s*-triazine exposure groups where tadpoles with scoliosis were observed ranged from 3.3% to 63.3%, some of which were significantly higher than that in the respective control groups (0–6.7%). It is speculated that the *s*-triazines may promote to occur axial malformations in developing tadpoles.

1. Introduction

Triazine chemicals, which are photosynthetic inhibitors and effective for the selective control of grassy weeds, have been used as herbicides worldwide (Trebst, 2008). The most common type of triazine herbicides is a group of 1,3,5-triazines, i.e., symmetrical triazines (*s*-triazines) that have 3 carbon and 3 nitrogen atoms placed alternately on a planar 6-membered ring (Müller, 2008). In general, *s*-triazines exert low acute toxicity to aquatic animals (Giddings and Hall, 1998; Solomon and Cooper, 2008), including amphibians (Bishop et al., 2010), and it is therefore unlikely that *s*-triazines directly cause mass die-offs of wild aquatic animals at environmentally relevant concentrations. However, in recent amphibian ecotoxicology, sublethal chronic effects caused by low-dose exposure of atrazine, one of the *s*-triazines, have attracted particular attention with great concern regarding worldwide amphibian population declines (Bishop et al., 2010). Globally, atrazine is one of the most widely used herbicides and commonly detected in the surface and ground water in agricultural areas and their outskirts (Bishop et al., 2010). This fact has promoted amphibian ecotoxicology of atrazine (Bishop et al., 2010; Sparling et al., 2010). The sublethal effects suspected for atrazine include gonadal abnormalities in sexual development (Hayes et al., 2002, 2006; Tavera-Mendoza et al., 2002a, 2002b), delayed timing of metamorphosis (Larson et al., 1998), reduced size and weight at metamorphosis (Diana et al., 2000; Larson et al., 1998), alterations in tadpole growth rates (Diana et al., 2000; Forson and Storfer, 2006; Larson et al., 1998), and various malformations (Allran and Karasov, 2001; Lenkowski et al., 2008). In particular, the potential of atrazine to induce gonadal abnormalities in amphibians was extensively examined in the 2000s in the United States (Bishop et al., 2010). Based on these examinations, the U.S. Environmental Protection Agency concluded that atrazine alone did not induce gonadal abnormalities of amphibians (Bishop et al., 2010). However, other sublethal chronic effects of atrazine, such as developmental retardation and growth inhibition, remain ongoing scientific debates because of conflicting results reported in several different studies (Carr et al., 2003; Coady et al., 2004; Diana et al., 2000; Forson and Storfer, 2006; Kloas et al., 2009; Larson et al., 1998; Oka et al., 2008).

* Corresponding author.
E-mail address: m-saka66@pref.kyoto.lg.jp (M. Saka).

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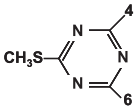
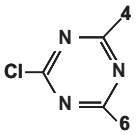
Table 1
Physical/chemical properties of simetryn and 7 symmetrical triazine (*s*-triazine) herbicides tested in the present study, with the market information on the herbicides in Japan.

Chemical group and the basic structure	Common name	Substitution on the triazine ring at positions		Molecular weight	Solubility in water at 20 °C (mg/L)[a]	Chemical Abstracts Service No.	Annual amount of herbicide production in Japan (1990–2014)[b]
		4th position	6th position				
Methylthio-*s*-triazines	Simetryn	–NH–C_2H_5	–NH–C_2H_5	213.3	400	1014–70–6	91.1 t/yr
	Ametryn	–NH–C_2H_5	–NH–$CH(CH_3)_2$	227.3	200	834–12–8	9.4 t/yr
	Prometryn	–NH–$CH(CH_3)_2$	–NH–$CH(CH_3)_2$	241.4	33	7287–19–6	25.0 t/yr
	Dimethametryn	–NH–C_2H_5	–NH–$CH(CH_3)$–$CH(CH_3)_2$	255.4	50	22936–75–0	16.3 t/yr
Chloro-*s*-triazines	Simazine	–NH–C_2H_5	–NH–C_2H_5	201.7	6.2	122–34–9	82.4 t/yr
	Atrazine	–NH–C_2H_5	–NH–$CH(CH_3)_2$	215.7	33	1912–24–9	69.7 t/yr
	Propazine	–NH–$CH(CH_3)_2$	–NH–$CH(CH_3)_2$	229.7	5	139–40–2	(Withheld since 1977)
	Cyanazine	–NH–C_2H_5	–NH–$C(CH_3)_2$–CN	240.7	171	21725–46–2	22.4 t/yr

[a] Water solubility data were obtained from a chemical list of triazine herbicides presented by LeBaron et al. (2008).
[b] Information on herbicide production in Japan was based on a database of agrochemicals released by the Japan National Institute for Environmental Studies(2015).

In contrast to an extensive literature on the toxicity of atrazine, available toxicity data of other *s*-triazines have been quite limited in amphibian ecotoxicology. In Japan, simetryn and simazine are more commonly and widely used as *s*-triazine herbicides than atrazine (Table 1). Considering the homology in the chemical structure, it is conceivable that like atrazine, other *s*-triazines may also have similar toxic effects on amphibians. This hypothesis may be supported by our previous study that reported growth inhibition, developmental retardation, and axial malformations observed in frog tadpoles exposed to simetryn (Saka et al., 2013). These sublethal effects of simetryn are quite similar to those suspected for atrazine.

The present study was undertaken to verify the hypothesis and addressed the chronic toxicity of 7 *s*-triazines: ametryn, prometryn, dimethametryn, simazine, atrazine, propazine, and cyanazine. As shown in Table 1, the initial 3 herbicides (ending in "-etryn") belong to the methylthio-*s*-triazine group including simetryn, whereas the others (ending in "-azine") fall under the chloro-*s*-triazine group. Except that simazine, atrazine, and propazine have a chloro group instead of a methylthio group, their chemical structures are the same as those of simetryn, ametryn, and prometryn, respectively. The 7 *s*-triazines were tested using a modified amphibian metamorphosis assay that we previously used to examine the chronic toxicity of simetryn (Saka et al., 2013). When conducted only one or two researchers, traditional amphibian metamorphosis assays (Mitsui et al., 2006; Opitz et al., 2005; Organization for Economic Cooperation and Development, 2009) require much time to examine a large number of tadpoles for morphological endpoints. The modified assay employed an individual-separated exposure system that could minimize interindividual variability in tadpole growth/development and thereby allow the number of tadpoles to be reduced (Saka et al., 2012). Furthermore, the modified assay replaced the African clawed frog (*Xenopus laevis*, a traditional experimental anuran species) with the western clawed frog (*Silurana tropicalis*, also called *X. tropicalis*) as a test species, due to its superiority in experimental use over *X. laevis*: a shorter life cycle and a diploid genome feasible for further studies on multi-generational effects and genetic impairment (Kashiwagi et al., 2010). As well as traditional assays, the modified assay was originally established for screening of thyroid-disrupting chemicals by utilizing a rationale that amphibian metamorphosis (tadpole-to-frog transformation) occurs with drastic alteration in morphology under the control of the thyroid axis. However, this assay can also be a useful tool to test chronic toxicity of chemicals with focuses on adverse effects on tadpole growth and development, as were observed in our previous study that tested simetryn (Saka et al., 2013). The main objective of our present study was to evaluate whether the 7 *s*-triazines induced chronic effects similar to those of simetryn in the modified amphibian metamorphosis assay. By comparing the test results, we attempted to characterize the chronic toxicity common to the *s*-triazines that were structurally related to one another. In this study, acute toxicity tests were also performed to determine the test concentrations for the chronic toxicity tests. Accordingly, we also examined the comparative toxicology based on the acute toxicity data of the 7 *s*-triazines.

2. Materials and methods

2.1. Animal husbandry

Adult pairs of *S. tropicalis* (Nigerian-H line) were supplied from the Institute of Amphibian Biology at Hiroshima University. The breeding of the frogs, care of the spawned eggs and hatchlings, and rearing of the tadpoles until experimental use all conformed to the protocols reported previously (Saka et al., 2012). Each toxicity test started using tadpoles that were derived from 3 different pairs and had developed to Nieuwkoop and Faber stage (NF stage) 49 or 50 (mid-premetamorphic phase) (Nieuwkoop and Faber, 1994) with total body length of ca. 20 mm. The care and treatment of all frogs and tadpoles, including euthanasia of surviving tadpoles after the experiments, complied with the current laws of Japan and contemporary guidelines presented by responsible academic societies.

2.2. Test chemicals and preparations of test solutions

The 7 *s*-triazines were tested using standard materials of 98.0–99.9% grade (Wako Pure Chemical Industries, Osaka, Japan). Due to low solubility in water, each chemical was first dissolved in a small volume of acetone at a concentration as high as possible: 400, 240, 500, 1.32, 25.0, 14.0, and 180 mg/mL for ametryn, prometryn, dimethametryn, simazine, atrazine, propazine, and cyanazine, respectively. The solution in acetone was used as a stock solution for each toxicity test. Test solutions were prepared by adding the stock solutions into a diluent (dechlorinated and air-saturated tap water: hardness = ca. 40 mg/L as $CaCO_3$; iodine = ca. 0.004 mg/L; pH = 6.5–7.5). According to our previous studies, acetone below 0.01% (v/v) does not affect the mortality of *S. tropicalis* tadpoles within 96 h of exposure (Saka, 2010) or the tadpole growth/development within 28 d of exposure unless exceeding 0.002% (v/v) (Saka et al., 2013). Therefore, all test solutions, including a control solution, were adjusted to contain the same volume of acetone within 0.01% (v/v, for 96-h acute toxicity tests) and 0.002% (v/v, for 28-d chronic toxicity tests). If the acetone concentration exceeded these upper limit values, the test solutions were gently stirred to