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Application of an amphibian (*Silurana tropicalis*) metamorphosis assay to the testing of the chronic toxicity of three rice paddy herbicides: Simetryn, mefenacet, and thiobencarb

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ABSTRACT

We examined the chronic toxicity of three rice paddy herbicides (simetryn, mefenacet, and thiobencarb) using an amphibian (*Silurana tropicalis*) metamorphosis assay (a 28-day semistatic test under an individual-separated exposure system). Each herbicide was tested at two concentrations (1/100 and 1/10 of the 96-h LC50 value reported previously) with morphometric, gravimetric, and thyroid-histological endpoints. Simetryn caused significant retardation in growth and development at both test concentrations (0.04 and 0.40 mg/L), as indicated by significantly shorter total body lengths and hind limb lengths, smaller wet body masses, and delayed developmental stages compared to those observed in the control tadpoles. However, no clear histopathology was observed in the thyroid glands of the tadpoles exposed to simetryn. These results suggest that simetryn can act as a chemical stressor retarding tadpole growth and development without disrupting thyroid functions, even at 1/100 of the 96-h LC50 value. In addition, scoliosis near the tail base was observed in the tadpoles exposed to 0.40 mg/L of simetryn at a significantly high incidence (7/30=23.3%). Therefore, simetryn can also act as a teratogen inducing axial malformations at 1/10 of the 96-h LC50 value. During the 28 days of exposure, neither mefenacet (0.03 and 0.30 mg/L) nor thiobencarb (0.008 and 0.080 mg/L) induced any abnormalities, although the test concentrations measured immediately before the solution renewals decreased to nearly 50 percent of the nominal concentrations since day 14. Because the concentrations tested for simetryn are likely to occur in paddy water, wild anuran tadpoles in paddy water may therefore be adversely impacted by simetryn.

1. Introduction

Recent progress made in amphibian ecotoxicology reflects heightened concern regarding amphibian ecology related to environmental contaminants (Sparling et al., 2010). The increased interest in amphibian ecotoxicology doubtlessly originates from awareness of globally occurring amphibian population declines. As summarized by Linder et al. (2001), agents proposed to be causative of worldwide amphibian population declines can be classified into four categories: physical alterations (habitat destruction, climate change, and increasing UV-B radiation), biotic stressors (e.g., pathogens and invasive animals), chemical contaminants (pesticides, heavy metals, nitrates, and other man-made chemicals), and synergism of these factors. In the 2000s, emerging infectious diseases (mostly chytridiomycosis) were a focus of special interest because the chytrid fungus *Batrachochytrium dendrobatidis*, which is highly virulent for amphibians, was found to account for sudden and relatively synchronous declines of amphibian populations in pristine environments (e.g., Bosch et al., 2001; Ron et al., 2003; Lips et al., 2006; Kriger and Hero, 2009; Voyles et al., 2009). However, infectious diseases do not completely replace other factors as the major causes of amphibian population declines, and chemical contaminants have been suggested to be one of the primary potential factors (Alford, 2010). In fact, some field studies have reported landscape-scale data suggesting that windborne or surface–water-borne chemical contaminants may act as one of multiple stressors and may therefore be correlative to population declines of several amphibian species; for example, in California (Fellers et al., 2004; Sparling and Fellers, 2009), Illinois (Reeder et al., 2005), and Maine (Bank et al., 2006), USA.

In Japan, *B. dendrobatidis* was first identified in 2006 in imported pet frogs (Une et al., 2008) and a countrywide field survey was conducted in the following year. This survey revealed that the overall incidence of chytridiomycosis in free-living native amphibians was extremely low, although a naturalized alien anuran species, *Rana*

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catesbeiana, was found to be infected by the fungus at various sites (Goka et al., 2009). One possible explanation for this result is that the chytrid fungus is endemic in Japan and many Japanese amphibians are innately resistant to *B. dendrobatidis* (Goka et al., 2009). This hypothesis agrees with the fact that no mass die-offs of wild Japanese amphibians exhibiting chytridiomycosis have been reported (Ota, 2000; Garner et al., 2006). Therefore, the amphibian population declines occurring in Japan may be attributable to other factors. In particular, pesticides applied in rice paddy fields have attracted attention as a key factor contributing to amphibian population declines (Saka, 1999, 2010; Konno, 2003) because Japanese amphibians frequently utilize rice paddy fields during their breeding season (Maeda and Matsui, 1989).

The current work concerns the chronic effects on amphibians of three herbicides: simetryn (N^2,N^4-diethyl-6-methylthio-1,3,5-triazine-2,4-diamine), mefenacet (2-benzothiazol-2-yloxy-*N*-methylacetanilide), and thiobencarb (*S*-4-chlorobenzyl diethylthiocarbamate), which are classified into three structurally different chemical groups (triazine, acid-amide, and carbamate herbicides). The Japan National Institute for Environmental Studies has released a database of agrochemicals used from 1987 to 2010 in Japan (http://db-out.nies.go.jp/kis-plus/index_3.html, available in Japanese only). According to this database, the herbicide the production of which has been the highest is thiobencarb (755,447 kg/yr) followed by mefenacet (485,328 kg/yr) of all rice paddy herbicides in Japan. Although the production of simetryn (121,003 kg/yr) is several times lower than that of thiobencarb and mefenacet, simetryn has been frequently used with these two herbicides in rice paddy fields in Japan (Japan Plant Protection Association, 2011). Recently, Saka (2010) conducted acute toxicity (96-h of exposure) tests of these three herbicides using tadpoles of *Silurana tropicalis* (sometimes called *Xenopus tropicalis*), a highlighted model species of experimental amphibians (Song et al., 2003; Fort et al., 2004a, 2004b; Kashiwagi et al., 2010). On the basis of median lethal concentration (LC50) values, Saka (2010) concluded that the three herbicides could exert lethal effects on wild amphibian larvae, even at the concentrations likely to occur in rice paddy water within a few days after herbicide application. However, the three herbicides have not been tested in terms of chronic toxicity to amphibian larvae, such as developmental retardation, growth inhibition, and postembryonic teratogenicity that may also result in decreases in amphibian populations by affecting the survival of each individual (Cowman and Mazanti, 2000). We therefore examined adverse but non-lethal effects on amphibian larvae following laboratory exposure to the three herbicides for a relatively long period and at much lower concentrations than the acute toxicity values. For this purpose, we employed a modified amphibian metamorphosis assay using *S. tropicalis* tadpoles established originally to detect thyroid-disrupting chemicals (Saka et al., 2012). This 28-day assay can also be used to test the chronic toxicity of chemicals on amphibian larvae during postembryonic development because the assay monitors tadpole development with morphometric and gravimetric endpoints from premetamorphosis to prometamorphosis or the initial stage of metamorphic climax. We herein describe the amphibian toxicology of the three herbicides, focusing on the following aspects: (1) the chronic effects of the herbicides detected using the amphibian metamorphosis assay; (2) the concentration levels at which the herbicides exert the above effects on the tadpoles; and (3) the potential risk from the herbicides to wild anuran tadpoles in rice paddy fields.

2. Materials and Methods

2.1. Animal husbandry

Adult pairs of *S. tropicalis* were provided by the Institute of Amphibian Biology at Hiroshima University. Rearing and breeding of the frogs, care of the spawned eggs and hatchlings, and keeping of the tadpoles until experimental use were performed following the protocols reported previously (Saka et al., 2012). The tadpoles derived from three different adult pairs were kept separately by clutch during the pre-experiment period. Healthy tadpoles that had developed to stages 49 and 50 (Nieuwkoop and Faber, 1994) with a total body length of ca. 20 mm were selected from each clutch and pooled together in a large flat pan. Immediately after the tadpole selection, each test started using tadpoles picked up randomly from the pooled population.

The treatment of all frogs and tadpoles complied with the current laws of Japan and the guidelines presented by the American Society of Ichthyologists and Herpetologists (ASIH, 2004).

2.2. Test chemicals and preparation of test solutions

Simetryn (99.9% grade, Wako Pure Chemical Industries, Osaka, Japan) was directly dissolved at 100 mg/L in a diluent (dechlorinated tap water: hardness=ca. 40 mg/L as $CaCO_3$; iodine=ca. 4 μg/L; pH=6.5–7.5; and dissolved oxygen [DO]=95–105 percent air saturation at 25 °C). Mefenacet and thiobencarb (99.6% and 98.0% grade, respectively, Wako Pure Chemical Industries, Osaka, Japan), both of which are hardly soluble in water, were dissolved in acetone at 15,000 mg/L and 4000 mg/L, respectively. These three solutions were used as stock solutions.

As described later, test solutions were prepared at two test concentrations for each test chemical. For the test of simetryn, 4 mL and 40 mL of the stock solution (100 mg/L in the diluent) were added into 9996 mL and 9960 mL of the diluent, respectively. Thus the test solutions were prepared at 0.04 mg/L and 0.40 mg/L. For the test of mefenacet, 20 μL and 200 μL of the stock solution (15,000 mg/L in acetone) were separately added into 10 L of the diluent, and thus the test solutions were prepared at 0.03 mg/L and 0.30 mg/L. Then 180 μL of acetone was added into the test solution containing mefenacet at 0.03 mg/L. The test solutions of thiobencarb were also similarly prepared: 20 μL of the stock solution (4000 mg/L in acetone)+180 μL of acetone and 200 μL of the stock solution alone were separately added into 10 L of the diluent, and thus the test solutions prepared at 0.008 mg/L and 0.080 mg/L. Consequently, the test solutions of mefenacet and thiobencarb were adjusted to contain the same volume of acetone (0.002%, v/v).

2.3. Chronic toxicity tests

2.3.1. Assay protocol and endpoints

The current work consisted of four tests performed following the protocol for the amphibian metamorphosis assay (a 28-day semistatic test under an individual-separated exposure system) established by Saka et al. (2012). In brief, 30 tadpoles held individually in a 500-mL glass beaker with 330 mL of the test solution were used for each treatment group. This group size (n=30) might be considerably large to detect significant differences in morphometric data of tadpoles but was needed for statistic analyses of frequency data such as malformations that might occur at relatively low percentages. The tadpoles were kept at 25 ± 1 °C under a consistent photoperiod (12-h light/12-h dark) with white fluorescent lamps during 28 days of exposure. Feeding was performed daily by adding a 10% (w/v) Sera Micron® (Sera GmbH, Heinsberg, Germany) solution into each beaker according to the following schedule: 0.5 mL/day/beaker (i.e., 0.5 mL/day/individual) during the initial seven days of exposure and at 1 mL/day/beaker thereafter. This feeding regime, originally prepared for the *Xenopus laevis* metamorphosis assay (Kloas et al., 2003; Opitz et al., 2005), was sufficient to maintain adequate water quality (i.e., the tadpoles could eat up the given food within a day) and has been found to ensure proper growth and development of *S. tropicalis* tadpoles (Mitsui et al., 2006; Saka et al., 2012). All test solutions were renewed three times per week (Monday, Wednesday, and Friday).

At seven-day intervals, each tadpole was inspected alive for developmental stage, hind limb length, and total body length from the ventral direction using an inverted digital microscope (CCD camera and controller, VH-6300; zoom lens, VH-Z05; Keyence, Osaka, Japan). The developmental stage was determined by consulting the normal table of *X. laevis* (Nieuwkoop and Faber, 1994). The hind limb length and total body length were measured to the nearest 0.1 mm. In addition, observations without a microscope were made daily regarding mortality, abnormal behavior, and grossly visible malformations. At the termination of each test, all tadpoles were euthanized in a 200 mg/L tricaine methanesulfonate (Kanto Chemical, Tokyo, Japan) solution neutralized with a 0.1% (w/v) $NaHCO_3$ solution and then fixed in Mildform® 10 N (Wako Pure Chemical Industries, Osaka, Japan). Each fixed tadpole was weighed to the nearest 1 mg after removing adherent water with a dry paper towel. For thyroid gland histopathology, eight tadpole specimens were randomly selected from each treatment group. The thyroid gland tissues of the tadpoles were prepared following the procedures reported previously (Saka et al., 2012) and examined while consulting the thyroid histology atlas presented by OECD (2007).

2.3.2. Solvent toxicity test

Because a solvent (acetone: 0.002%, v/v) would be used for the tests of mefenacet and thiobencarb, we first examined whether the solvent could affect the tadpoles regarding the four morphometric and gravimetric endpoints (i.e.,