HERPETOLOGICAL JOURNAL, Vol. 9, pp. 73-81 (1999)

ACUTE TOXICITY TESTS ON JAPANESE AMPHIBIAN LARVAE USING THIOBENCARB, A COMPONENT OF RICE PADDY HERBICIDES

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Acute toxicity tests were carried out on five species of Japanese amphibian larvae, at different developmental stages, to assess the risk posed by thiobencarb, a component of rice paddy herbicides. Test substances were four types of commercially formulated herbicide containing mainly thiobencarb, and the 24 h, 48 h, 72 h and 96 h LC_{50} (median lethal concentration) values of these herbicides were calculated by probit analysis. These values ranged from 0.9 to 6.5 mg/l of thiobencarb. Newly hatched larvae seemed to be slightly more resistant to the herbicides than well-developed larvae in all test species. There were no clear interspecific differences in responses. The actual thiobencarb concentration in paddy water was measured with indoor models for two weeks, and it ranged from <0.005 to 3.1 mg/l. Some of the measured concentrations exceeded the LC_{50} values. Thiobencarb residue in paddy water can therefore be lethal to amphibians throughout larval development. Tests with *Xenopus laevis* produced approximately the same LC_{50} values as those of Japanese amphibians. This indicates that experimental frogs such as *Xenopus laevis* can act as a model for these native and wild amphibians when toxicity tests are conducted.

Key words: Japanese amphibians, herbicide, thiobencarb, acute toxicity, risk assessment

INTRODUCTION

One of the most serious problems in wildlife conservation concerns the reasons underlying the decline of amphibians in different parts of the world (Barinaga, 1990; Blaustein & Wake, 1990, 1995; Blaustein, Wake & Sousa, 1994; Tyler, 1994; Stebbins & Cohen, 1995). Some amphibian declines may be caused by environmental contaminants, such as agricultural chemicals and heavy metals (e.g. Power, Clark, Harfenist & Peakall, 1989). In the study by Corn, Stolzenberg & Bury (1989), the effects of acid precipitation were related to the declines of several species of amphibian in the Rocky Mountains. However, there is little evidence that acid precipitation is a widespread cause of amphibian declines (Dunson, Wyman & Corbett, 1992). Recently, increased ultraviolet radiation resulting from ozone layer depletion has been highlighted as a possible cause of amphibian declines (Licht & Grant, 1997).

In Japan, common amphibians such as *Cynops pyrrhogaster*, *Rana nigromaculata* and *Hyla japonica*, as well as hynobiid salamanders have decreased in number (Matsui, 1996). The area of paddy fields has been reduced because of the overproduction of rice. This may be related to the decline of amphibians which inhabit or breed in paddy fields, as described by Matsui (1996). However, agricultural chemicals, in particular those herbicides frequently used in rice paddies, also seem to contribute to the declines of Japanese amphibians, because the recent amount of chemicals used on agricultural land in Japan is much higher (1.77 t/km^2) than that of most other western developed countries (0.09–0.58 t/km^2; Organization for Economic Co-operation and Development - OECD, 1991). Nevertheless, the risk from and the harmful effects of herbicides on Japanese amphibians have been little studied. Recent research by the Japan Ministry of Agriculture, Forestry and Fisheries (1994, 1995, 1996) shows that the production of thiobencarb (*S*-4-chlorobenzyl *N*,*N*-diethylthiocarbamate) has been the highest of all herbicidal chemicals, and herbicides containing thiobencarb have been generally used in rice paddies.

Consequently, this study investigated the toxicity of and the risk from thiobencarb to Japanese amphibians, focusing on the aquatic larval stages which seem to be most vulnerable to contaminants in water. Several species of amphibian were selected which differ phylogenetically from one another, and acute toxicity tests were conducted with larvae of these amphibians at several developmental stages. The actual thiobencarb concentration in paddy water was measured with indoor paddy models. The potential risk from thiobencarb to Japanese amphibians is discussed by comparing lethal concentrations with the actual concentrations in paddy water. The differences in susceptibility to thiobencarb among developmental stages and among species are also described.

MATERIALS AND METHODS

TEST SPECIES

Five species of Japanese amphibian belonging to different families were selected as test species. They were the Japanese fire-bellied newt, *Cynops pyrrhogaster* (Salamandridae), the eastern Japanese common toad, *Bufo japonicus formosus* (Bufonidae), the Japanese tree frog, *Hyla japonica* (Hylidae), the black-spotted pond

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TABLE 1. Developmental stages of amphibian larvae to which acute toxicity tests were applied. [1] Stage no. of test species was based on the tables of normal stages by Kajishima & Eguchi (1989), Ichikawa & Tahara (1989), Iwasawa & Futagami (1992), Iwasawa & Kawasaki (1979) and Nieuwkoop & Faber (1975) for *Cynops pyrrhogaster*, *Bufo japonicus formosus*, *Hyla japonica*, *Rhacophorus arboreus* and *Xenopus laevis*, respectively. Because the table of normal stages of *Rana nigromaculata* has not been published, that of *Rana porosa porosa* (Iwasawa & Morita, 1980), a related species to *Rana nigromaculata*, was used. [2] Each value is a mean of 20–50 individuals from the test populations.

Species	Developmental stage[1]	Time after hatching (day)	Total length[2] (mm)	Body weight[2] (mg)	Morphological characteristics
Cynops pyrrhogaster	Early (41–43)	<1	11	13	Balancers remaining.
	Middle (50–52)	14	16	25	Almost completely developed forelimbs.
	Late (57–59)	42	27	160	Almost completely developed hindlimbs and degenerating external gills.
Bufo japonicus formosus	Early (30)	<1	15	25	External gills remaining.
	Middle (36)	14	26	110	Developed opercula.
	Late (40, 41)	28	28	170	Almost completely developed hindlimbs but forelimbs still invisible.
Hyla japonica	Early (22, 23)	<1	6	2	External gills remaining.
	Middle (27–29)	14	16	61	Developed opercula.
	Mid.-late (31, 32)	28	26	190	Limbs still only buds.
Rana nigromaculata	Early (23, 24)	<1	10	11	External gills remaining.
	Middle (26, 27)	14	18	60	Developed opercula.
	Mid.-late (28, 29)	28	28	180	Limbs still only buds.
Rhacophorus arboreus	Early (30, 31)	<1	19	52	External gills remaining.
	Middle (36, 37)	14	27	160	Limbs still only buds.
Xenopus laevis	Early (35–38)	<1	6	5	External gills remaining.
	Middle (46, 47)	14	16	29	Developed opercula.
	Mid.-late (49)	32	35	210	Appearance of sensory tentacles but limbs still only buds.

frog, *Rana nigromaculata* (Ranidae) and the forest green tree frog, *Rhacophorus arboreus* (Rhacophoridae). These amphibians were collected in and around the paddy fields of mountainous areas in the northern part of Kyoto Prefecture, from April to June. *Bufo japonicus formosus*, *Rana nigromaculata* and *Rhacophorus arboreus* were obtained as egg masses. For *Hyla japonica*, amplectant pairs were captured and allowed to spawn in the laboratory. For *Cynops pyrrhogaster*, only adult females were collected and induced to lay eggs in the laboratory by injection of human chorionic gonadotropin (HCG) (Wako Pure Chemical Industries Ltd., Osaka, Japan). In addition to these Japanese amphibians, *Xenopus laevis* (Pipidae), a common experimental frog, was also used as a test animal. Adult *Xenopus laevis* were obtained from a commercial dealer (Shimizu Laboratory Supplies Co., Kyoto, Japan). Amplexus and egg-laying were induced by HCG injection. After hatching, larvae of the six species were maintained at 20±1°C, in polypropylene aquaria filled to a depth of 10 cm with dechlorinated tap water. Feeding began at one week after hatching. Larval newts were fed brine shrimp (larvae of *Artemia salina*) and frog tadpoles were fed homogenate of boiled spinach daily. The daily diet amount was determined by larval size so as to avoid water deterioration from excess food and cannibalism from insufficient food. The six species of larvae were divided into two or three stage groups on the basis of larval size and morphological characteristics (Table 1). Test species were examined at each developmental stage, except for the late stages of *Hyla japonica*, *Rana nigromaculata*, *Rhacophorus arboreus* and *Xenopus laevis* because the larvae were too large to be examined under proper conditions with the limited experimental facilities. Each test was conducted using larvae from three egg masses (*Bufo japonicus formosus*, *Hyla japonica*, *Rana nigromaculata*, *Rhacophorus arboreus* and *Xenopus laevis*) or those from twenty-four adult females (*Cynops pyrrhogaster*).

The numbers of amphibians collected and larvae used for toxicity tests were minimized in the interest of conservation and ethics. With the exception of *Xenopus laevis*, adult amphibians after egg-laying and the larvae not used for toxicity tests were released at the sites of capture.

TABLE 2. The components of the four types of commercially formulated herbicide based on the data on the herbicide packages, and the recent annual amount of each herbicide on the market published by the Japan Ministry of Agriculture, Forestry and Fisheries (1994, 1995, 1996). [1] Oct. 1993–Sept. 1994; [2]Oct. 1994–Sept. 1995; [3]Oct. 1995–Sept. 1996.

Type	Formulation and thiobencarb content	Herbicidal chemicals other than thiobencarb	Other ingredients	Recent annual amount of each herbicide on the market in Japan		
				1994[1]	1995[2]	1996[3]
Type A	Granules (5%)	Mefenacet (1%) Bensulfuron-methyl (0.17%)	Mineral powder etc.	10 052 t	10 335 t	9167 t
Type B	Granules (7%)	Simetryn (1.5%)	Mineral powder etc.	1093 t	1220 t	668 t
Type C	Granules (10%)	MCPB-ethyl (0.8%) Simetryn (1.5%)	Mineral powder etc.	2231 t	2207 t	1595 t
Type D	Emulsifiable concentrate (50%)	None	Organic solvents (xylene etc.) and emulsifiers etc.	38 kl	45 kl	42 kl

TEST SUBSTANCES

The primary test substance for acute toxicity tests was not standard thiobencarb but four types of commercially formulated herbicide whose main ingredient was thiobencarb (Table 2), because the latter are actually used in rice paddies and the former is used only for chemical analysis. To confirm whether the lethality of the formulated herbicides was caused mainly by thiobencarb, tests of standard thiobencarb (purity 99 %, Wako Pure Chemical Industries Ltd., Osaka, Japan) were conducted with middle stage larvae of *Cynops pyrrhogaster* and *Xenopus laevis* - these two species of amphibians can be easily induced to lay eggs by hormone injection. (If there are large differences in lethality values of the four formulated herbicides among species and among developmental stages, standard thiobencarb tests should be conducted with all test species and all developmental stages of larvae.)

In addition to the tests with four types of herbicide and standard thiobencarb, tests with pentachlorophenol sodium salt (PCP-Na; purity 90 %, Wako Pure Chemical Industries Ltd., Osaka, Japan), a reference substance, were also conducted for two reasons: (1) the toxicity of PCP-Na has been studied well and it is recommended as a reference substance for acute toxicity tests with aquatic animals, in order to confirm whether or not experimental conditions are appropriate and the response of test animals is normal (the Japan Environment Agency, 1990); and (2) past effects of herbicides on Japanese amphibians can be also estimated, as pentachlorophenol (PCP) and its salts were generally used as herbicides in Japanese rice paddies in the 1960's until thiobencarb replaced them (Kobayashi, 1979).

ASSAY PROCEDURE

Various toxicity tests on aquatic organisms other than amphibians have been validated by OECD to evaluate the effects of chemicals on biotic systems. In the present study, toxicity tests with amphibians were performed in accordance with OECD *Guidelines for Testing of Chemicals* No. 203: "Fish, acute toxicity test" (OECD, 1993).

Dechlorinated tap water (hardness: 40 mg/l as $CaCO_3$) was used for exposure water and control water. For one test substance, one blank (control) and at least five concentrations in a geometric series with a factor of $10^{1/4}$ (=1.8) were prepared. The test solutions of each concentration including the control were each poured into a set of ten glass beakers (200 ml-beakers for early stage larvae of all test species and middle stage larvae of *Cynops pyrrhogaster*, *Hyla japonica*, *Rana nigromaculata* and *Xenopus laevis*, and 500 ml-beakers for the others). Solution volume was 100 ml per 200 ml-beaker and 300 ml per 500 ml-beaker. Standard thiobencarb was first dissolved in a small amount of acetone, because it is nearly insoluble in water. Accordingly, in the test of standard thiobencarb, all of the test solutions were adjusted to contain the same volume of acetone (0.01 %). Each beaker held one individual to prevent cannibalism and was kept in an environmental chamber maintained at 20±1°C on a 12 h L: 12 h D photoperiod with white fluorescent lamps. Handling of larvae was performed with a pipette or a net of 0.2 mm mesh depending on larval size. Exposure period was 96 h. All tests were conducted without feeding or aeration. The test solutions were renewed every day to prevent degradation of water quality. Both used and renewed

solutions were examined for pH and dissolved oxygen (DO) with a pH meter and a DO meter.

In the tests with PCP-Na and standard thiobencarb, three beakers of each concentration were picked out at random and the used solutions in them were examined for the concentration of the test substance. PCP-Na is easily absorbed by aquatic animals (Kobayashi, 1979) and the PCP-Na concentration of the test solutions may drift during the test. Thiobencarb may also be absorbed by larvae or broken down during the test period, and the thiobencarb concentration of used solutions may be significantly decreased. However, used solutions of the four formulated herbicides, types A–D, were not examined for the thiobencarb concentration because of low recoveries (<60 %) of standard thiobencarb from the test solutions of types A–D (The test solutions of types A–D were suspensions or emulsions of the formulated herbicides, and this may account for the low recoveries). The PCP-Na concentration was measured with a spectrophotometer by the chloroform extraction method (American Public Health Association, American Water Works Association and Water Pollution Control Federation, 1975). The thiobencarb concentration was measured with a gas chromatograph connected to a mass spectrometer (GC/MS) after extraction with dichloromethane, as described in the subsequent section.

Dead larvae were counted at the time of daily changes of the test solutions. Larvae were considered dead if there was no visible movement and if touching the caudal peduncle produced no reaction. The mortality at each test concentration was calculated, and the 24 h, 48 h, 72 h and 96 h LC_{50} (median lethal concentration) values were calculated by probit analysis following Finney (1952) and Yoshimura & Ohashi (1992).

To choose the appropriate test concentration range, a range-finding test was properly conducted before the definitive test. There was no replication in the definitive test unless its results contradicted those of the range-finding test.

Measuring Thiobencarb in Paddy Water

The actual thiobencarb concentration in paddy water was measured with simple indoor paddy models. Although measuring in the field may be a better indication of exposure levels in natural amphibian populations, the results would vary according to prevailing weather conditions and be difficult to standardize. Thiobencarb concentration was therefore measured using the following indoor models.

Five polypropylene containers (60 cm × 40 cm × 20 cm) were prepared. They were filled with paddy soil to a depth of 10 cm and dechlorinated tap water to a depth of 3 cm from the top of the paddy soil. The paddy soil (clay-based soil; carbon, 1.57 %; nitrogen, 0.12 %) was collected from an experimental paddy area where no herbicides had been used for over a year, in the Kyoto Prefectural Institute of Agriculture. The herbicides of types A–D were spread over four of the containers separately at the rate of 3 kg/10 a (types A–C) or 800 ml/10 a (type D) based on the directions for use of each herbicide printed on its package. The last container was used as a control and no herbicides were applied to it. The containers were put in an environmental chamber under the same conditions as the acute toxicity tests, and analysed after two weeks. When the water level in a container dropped because of evaporation, water was added up to the original level.

TABLE 3. GC/MS operating conditions.

Gas chromatograph	Hewlett Packard 5890
Column	J & W DB-1 (length 30 cm, i.d. 0.25 mm, film 0.25 μm)
Oven temperature	60 °C for 2 min, then rising at 20 °C/min to 180 °C, 4 °C/min to 240 °C, and 10 °C/min to 280 °C
Injection mode	Splitless (purge off 1 min)
Mass spectrometer	JEOL JMS-AX505WA
Monitor ion (m/z)	257

Water from each paddy sample was carefully decanted so as not to disturb the mud, at intervals of 6 h, 24 h, 48 h, 72 h, 96 h, 7 d and 14 d after applying the herbicides. The water samples were extracted by liquid-liquid partitioning with dichloromethane, and the extract was analysed with a GC/MS. The operating conditions are shown in Table 3. The lower detection limit for thiobencarb was 0.005 mg/l. The recovery of standard thiobencarb through the chemical analysis was more than 90 %.

RESULTS AND DISCUSSION

Lethality Values of PCP-Na as a Reference Substance

Throughout all tests of PCP-Na, no abnormal responses of the larvae were observed in the control solutions, and the values of pH and DO in the test solutions were normal (pH, 6.7–7.9; DO, 72–120 % of the air saturation value at 20 °C). Consequently, death of larvae throughout the tests seemed to be caused only by the test substance. The PCP-Na concentration of the used test solutions was more than 80 % of the nominal concentration, even though it was possible that the PCP-Na concentration was somewhat reduced due to absorption by test individuals. Therefore, the results of PCP-Na tests were accepted and the LC_{50} values were calculated.

The LC_{50} values of PCP-Na are shown in Fig. 1. These values ranged from 0.070 to 0.31 mg/l (24 h

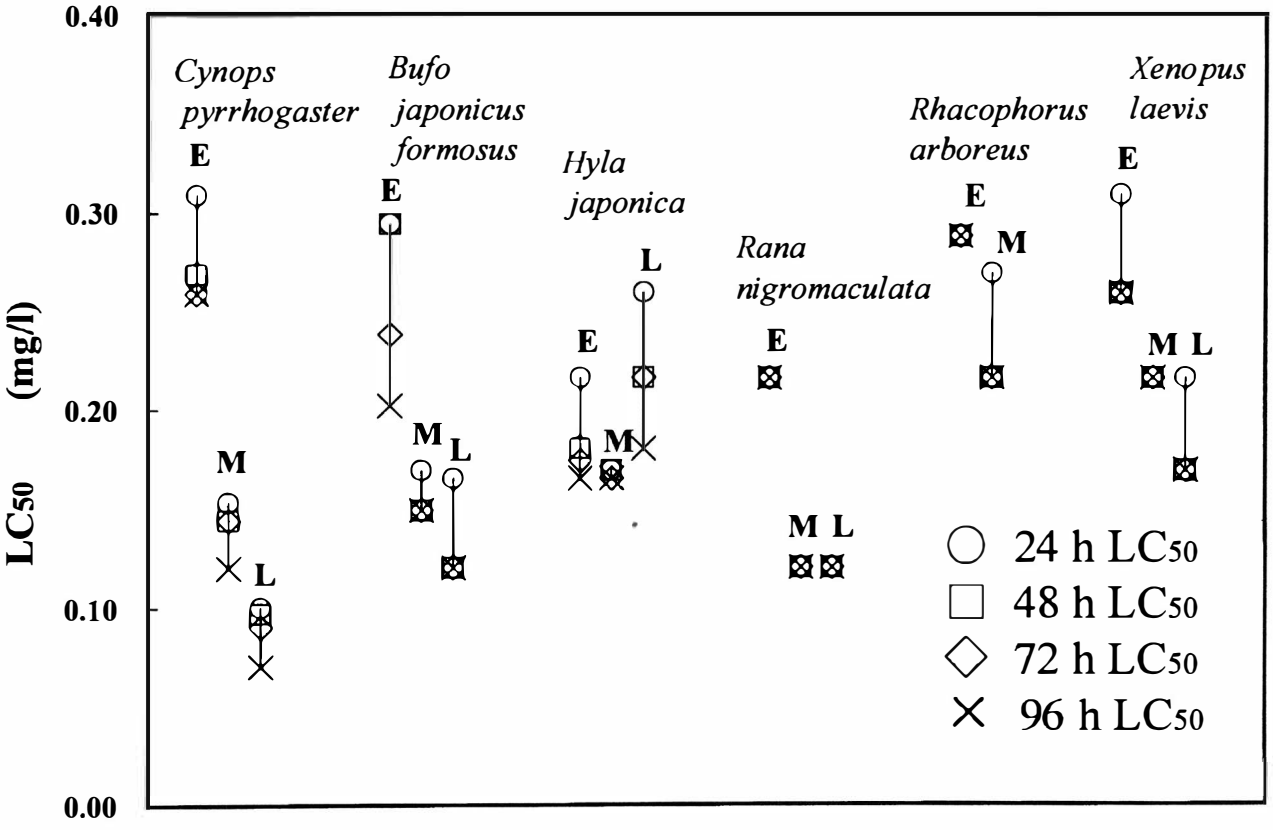

FIG. 1. The LC_{50} values of PCP-Na for the larvae of six species of amphibian in various developmental stages. E, M and L represent early, middle and mid-late or late stages, respectively. Characteristics of each stage are described in Table 1.

LC_{50}, 0.10–0.31 mg/l; 48 h LC_{50}, 0.097–0.29 mg/l; 72 h LC_{50}, 0.090–0.29 mg/l; 96 h LC_{50}, 0.070–0.29 mg/l). All the LC_{50} values, except those of late stage *Cynops pyrrhogaster* larvae, were distributed between 0.1 and 0.4 mg/l. There were no obvious interspecific differences in the LC_{50} values. In all species except *Hyla japonica*, the LC_{50} values became lower as developmental stage proceeded. This tendency was clearest in *Cynops pyrrhogaster*. A similar tendency was observed by Sanders (1970) who reported that susceptibility of *Bufo woodhousii fowleri* to DDT increases with age of the tadpoles. In my tests, late stage larvae of *Cynops pyrrhogaster* seemed to be highly susceptible to PCP-Na. This high susceptibility may be related to developmental changes associated with metamorphosis as suggested by Sanders (1970). Hall & Swineford (1980, 1981), however, reported the opposite trend, i.e. a positive correlation between age and resistance to chemicals, in toxicity tests of toxaphene with the larvae of seven species of amphibian. There may be different patterns in the relationships between age and susceptibility to chemicals in different amphibian larvae.

PCP and its salts were common rice paddy herbicides in the 1960's in Japan, and their application to rice paddies often caused mass mortality of freshwater fishes and shellfish living near paddy fields (Kobayashi, 1979). The 48 h LC_{50} values of PCP for Japanese freshwater fishes such as carp and trout, and shellfish such as setashijimi are 0.056–0.38 mg/l (Kanazawa, 1979). In the six amphibian species, the 48 h LC_{50} values of PCP-Na were 0.097–0.29 mg/l, corresponding to 0.090–0.27 mg/l of PCP. Therefore, PCP is as lethal to amphibians as it is to Japanese freshwater fishes and shellfish, and it is possible that in the past, PCP residue in paddy water had a lethal influence on amphibians as well as on freshwater fishes and shellfish.

ACUTE TOXICITY OF THIOBENCARB

In all tests of thiobencarb (types A–D and standard thiobencarb), no stressed or weakened individuals were observed in the control solutions, and there were no abnormal values of pH or DO in the test solutions (pH, 6.7–7.5; DO, 76–110 % of the air saturation value at 20 °C). In the tests with standard thiobencarb, the thiobencarb concentration of used solutions was more than 80 % of the nominal concentration.

The LC_{50} values of types A–D are shown in Fig. 2. The LC_{50} values of each type did not differ obviously among species. Large decreases in LC_{50} values with increased larval development were not observed, unlike the results of the PCP-Na tests. However, among the 24 h LC_{50} values within each species, the 24 h LC_{50} value of early stage larvae was always the highest. Consequently, early stage larvae seemed to be slightly more resistant to the herbicides than well-developed larvae when they were exposed for only 24 h. In the report by Licht (1985), a jelly coat seems to protect embryos

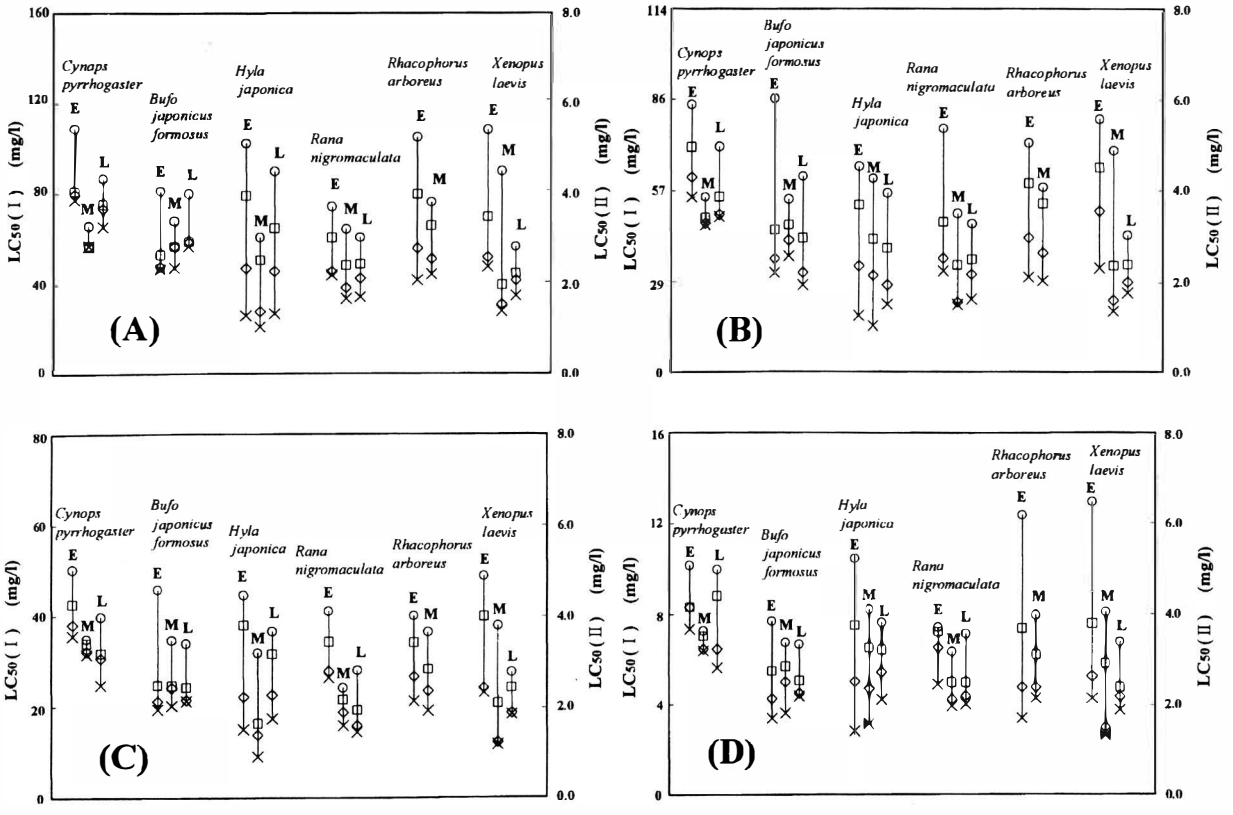

FIG. 2. The LC_{50} values of the four types of herbicide shown in Table 2 for the larvae of six species of amphibian in various developmental stages. (A), (B), (C) and (D) represent the LC_{50} values of types A, B, C and D, respectively. LC_{50} (I) is shown as the concentration of a whole formulated herbicide and LC_{50} (II) is shown as the thiobencarb concentration on the basis of the thiobencarb content. Symbols and abbreviations are the same as in Fig. 1.

from absorbing pesticides. The short term resistance of early stage larvae may be caused by jelly coats remaining around the bodies of newly hatched larvae.

There were apparent differences in lethality values among the four types of herbicides: as the concentration of a whole formulated herbicide, the 24–96 h LC_{50} (I) values of types A, B, C and D were 21–110 mg/l, 14–86 mg/l, 9.0–50 mg/l and 2.6–13 mg/l, respectively. These values decreased in alphabetical order corresponding to the increase of the thiobencarb content. When these values were expressed as thiobencarb concentration on the basis of the thiobencarb content, there were no distinct differences among the four types: the 24–96 h LC_{50} (II) values of types A, B, C and D were 1.0–5.4 mg/l, 1.0–6.0 mg/l, 0.9–5.0 mg/l, and 1.3–6.5 mg/l, respectively. These were approximately the same as the LC_{50} values of standard thiobencarb for middle stage larvae of *Cynops pyrrhogaster* and *Xenopus laevis* (Fig. 3). The results suggest that the lethal effects of the four types of herbicide were caused mainly by

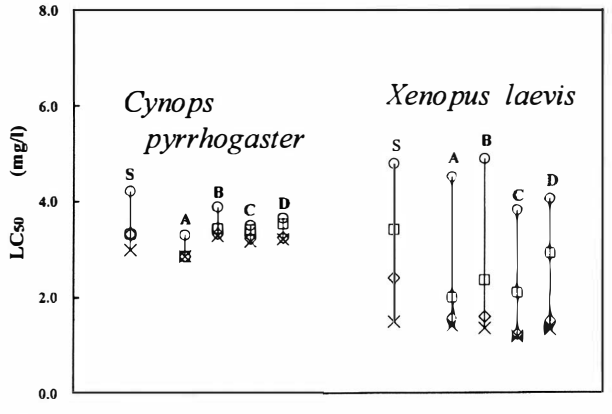

FIG. 3. Comparison of the LC_{50} values of types A–D with those of standard thiobencarb for middle stage larvae of *Cynops pyrrhogaster* and *Xenopus laevis*. The LC_{50} values of types A–D are shown as the thiobencarb concentration. S, A, B, C and D represent standard thiobencarb, type A, type B, type C and type D, respectively. Symbols are the same as in Fig. 1.

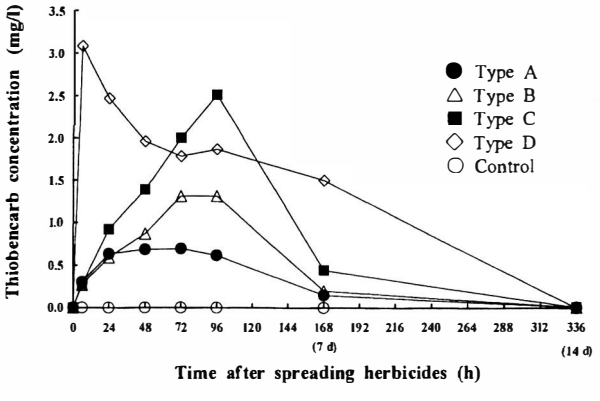

FIG. 4. Temporal changes in the measured thiobencarb concentration in model paddy water for two weeks. Each paddy model was treated with types A–D herbicides separately.

thiobencarb, although these herbicides contain other chemicals such as mefenacet, bensulfuron-methyl, simetryn, MCPB-ethyl and xylene.

THIOBENCARB CONCENTRATION IN PADDY WATER AND RISK ASSESSMENT OF THIOBENCARB FOR JAPANESE AMPHIBIANS

Thiobencarb concentrations in paddy water of the indoor models are shown in Fig. 4. In types A–C, the thiobencarb concentration increased gradually in the beginning, and attained maximum levels after 72 or 96 h. This was because they were in granular form and took a long time to dissolve in water. The thiobencarb concentration then decreased rapidly and dropped to approximately 0.005 mg/l, the lower limit of detectable thiobencarb, two weeks after applying the herbicides. In type D, the thiobencarb concentration increased rapidly and reached its maximum soon after applying the herbicide, because type D was an emulsifiable concentrate which mixed with water easily. Subsequently, the thiobencarb concentration decreased gradually and dropped to lower than 0.005 mg/l at the end of the experiment. In the control paddy, thiobencarb was undetectable during the experiment. The maximum thiobencarb concentrations were 0.70 mg/l, 1.3 mg/l, 2.5 mg/l and 3.1 mg/l for types A, B, C and D, respectively, and they increased in proportion to the increase of the thiobencarb content.

In the field, thiobencarb concentrations may be lower than these results because thiobencarb can be absorbed by soil or vegetation in rice paddy, and can be decomposed by microorganisms or ultraviolet radiation. According to Kanazawa (1992), in Japan, thiobencarb concentrations in paddy water in the field attained a peak of 0.908 mg/l on the day following the herbicide application and dropped to 0.26 mg/l after seven days. Ross & Sava (1986) reported that when thiobencarb was applied in granular form to a rice field in California, the thiobencarb concentration in paddy water was highest (0.576 mg/l) four days after the herbicide application and dropped to 0.056 mg/l after sixteen days. There were no great differences in the pattern of temporal change of the thiobencarb concentration and its maximum level between the results of these two field experiments and those of the indoor experiment, considering the differences in experimental conditions such as temperature and water depth of rice paddies. The results of the indoor experiment were therefore used for the risk assessment of thiobencarb for Japanese amphibians.

As described above, the 24–96 h LC_{50} values for amphibian larvae ranged from 0.9 to 6.5 mg/l. In all four types, the thiobencarb concentrations remained lower than 6.5 mg/l for two weeks after the herbicides were applied. However, in types B–D, there was a period when the thiobencarb concentration exceeded 0.9 mg/l. In type A, although the thiobencarb concentration did not exceed 0.9 mg/l, there was a period when it came very close to this level. Thus, the thiobencarb concentration in paddy water can be lethal to amphibian larvae for two weeks after applying herbicides.

Although there were no clear differences in the LC_{50} values of thiobencarb among the amphibian species, the risk of thiobencarb to them in nature should be discussed with due regard to not only the LC_{50} values but also to their natural histories. The risk of thiobencarb to Japanese amphibians was assessed with reference to the reports by Nakamura & Ueno (1963) and Maeda & Matsui (1989). Herbicides are spread within a month of rice-planting, between April to June in Japan (Japan Plant Protection Association, 1994). Because several species of Japanese amphibian such as *Cynops pyrrhogaster*, *Hyla japonica*, *Rana nigromaculata* and *Rhacophorus arboreus* spawn in rice paddies in the rice-planting season, spawning of these amphibians can coincide with herbicide applications. As Fig. 4 shows, thiobencarb concentrations in paddy water were detected for less than two weeks after applying herbicides. Therefore, amphibians such as *Rana nigromaculata*, which usually spawn in rice paddies for a comparatively short period, would be affected by thiobencarb. If their spawning occurs when the thiobencarb concentration in paddy water attains its peak, many individuals would be affected by thiobencarb. On the other hand, although *Cynops pyrrhogaster*, *Hyla japonica* and *Rhacophorus arboreus* also spawn in rice paddies, entire local populations would not be damaged by thiobencarb as much as *Rana nigromaculata*, because their breeding seasons continue for a long period and their risk of encountering the peak of the thiobencarb concentration in paddy water would be attenuated overall. In comparison with the above amphibians, *Bufo japonicus formosus* would be hardly affected at all by thiobencarb, because it usually spawns in mountain roadside ditches or temporary pools in mountainous areas, rather than in rice paddies.

In the present study, all five species of Japanese amphibian used to investigate interspecific differences in susceptibility to herbicides showed approximately the same lethality values to PCP-Na and thiobencarb. Moreover, there was no remarkable difference in lethality values between these Japanese amphibians and *Xenopus laevis*, a common experimental frog. Because there was no replication in each definitive test, the result of each test is not entirely representative of the species and the developmental stages which were tested. However, the consistency in susceptibility among species and among developmental stages suggests that the results are representative of amphibians in general. Hall & Swineford (1981) also observed that acute lethality values of endrin and toxaphene tested on the larvae of six species of amphibian differed from those of *Rana sphenocephala*, a standard test species, by less than one order of magnitude. They concluded that a water quality standard based on tests with *Rana*

sphenocephala would protect many species of amphibian if a safety factor of 0.1 is used. Therefore, a variety of amphibian species are not always necessary for toxicity tests. When toxicity tests are conducted, an experimental frog such as *Xenopus laevis* can be used as a substitute for many species of amphibian, including both widespread and locally restricted species.

ACKNOWLEDGMENTS

I thank A. Mori for his assistance in collecting amphibians and his critical reading of an early draft of this paper. I also thank S. Hatakeyama for his valuable comments of the manuscript. I am indebted to M. Noda and A. Nishimura for their help in operating the GC/MS. The collection of paddy soil was done with permission from the Kyoto Prefectural Institute of Agriculture. This study was partially supported by a grant-in-aid for the investigation of ecotoxicological effects of chemicals in the environment from the Japan Environment Agency.

REFERENCES

American Public Health Association (APHA), American Water Works Association (AWWA) & Water Pollution Control Federation (WPCF) (1975). Chloroform extraction method. In *Standard Methods for the Examination of Water and Wastewater, 14th Edition*, 557–580. Rand, M. C., Greenberg, A. E. & Taras, M. J. (Eds). Washington: APHA.

Barinaga, M. (1990). Where have all the froggies gone ? *Science* **247**, 1033–1034.

Blaustein, A. R. & Wake, D. B. (1990). Declining amphibian populations: A global phenomenon ? *Trends Ecol. Evol.* **5**, 203–204.

Blaustein, A. R. & Wake, D. B. (1995). The puzzle of declining amphibian populations. *Sci. Am.* **272**, 52–63.

Blaustein, A. R., Wake, D. B. & Sousa, W. P. (1994). Amphibian declines: Judging stability, persistence and susceptibility of populations to local and global extinctions. *Conserv. Biol.* **8**, 60–71.

Corn, P. S., Stolzenburg, W. & Bury, R. B. (1989). Acid precipitation studies in Colorado and Wyoming: Interim report of surveys of montane amphibians and water chemistry. *U. S. Fish Wildl. Serv. Biol. Rep.* **80 (40. 26)**.

Dunson, W. A., Wyman, R. L. & Corbett, E. S. (1992). A symposium on amphibian declines and habitat acidification. *J. Herpetol.* **26**, 349–352.

Finney, D. J. (1952). *Probit Analysis.* Cambridge: Cambridge University Press.

Hall, R. J. & Swineford, D. (1980). Toxic effects of endrin and toxaphene on the southern leopard frog *Rana sphenocephala*. *Environ. Pollut. Ser. A* **23**, 53–65.

Hall, R. J. & Swineford, D. M. (1981). Acute toxicities of toxaphene and endrin to larvae of seven species of amphibians. *Toxicol. Lett.* **8**, 331–336.

Ichikawa, M. & Tahara, Y. (1989). Normal stages of development of *Bufo japonicus*. In *Development of Vertebrates Vol. 1*, 357–373. Okada, T. (Eds). Tokyo: Baifukan Co., LTD.

Iwasawa, H. & Futagami, J. (1992). Normal stages of development of a tree frog, *Hyla japonica* (Günther). *Jpn. J. Herpetol.* **14**, 129–142.

Iwasawa, H. & Kawasaki, N. (1979). Normal stages of development of the Japanese green frog *Rhacophorus arboreus* (Okada et Kawano). *Jpn. J. Herpetol.* **8**, 22–35.

Iwasawa, H. & Morita, Y. (1980). Normal stages of development in the frog, *Rana porosa porosa* (Cope). *Zoological Magazine* **89**, 65–75.

Japan Environment Agency (1990). Guidelines for ecotoxicological tests. In *Chemicals in the Environment 1990*, 252–261. Tokyo: The Japan Environment Agency.

Japan Ministry of Agriculture, Forestry and Fisheries (1994). *Annual White Book on Agricultural Chemicals.* Tokyo: Japan Plant Protection Association.

Japan Ministry of Agriculture, Forestry and Fisheries (1995). *Annual White Book on Agricultural Chemicals.* Tokyo: Japan Plant Protection Association.

Japan Ministry of Agriculture, Forestry and Fisheries (1996). *Annual White Book on Agricultural Chemicals.* Tokyo: Japan Plant Protection Association.

Japan Plant Protection Association (1994). *Handbook on Agricultural Chemicals.* Tokyo: Japan Plant Protection Association.

Kajishima, T. & Eguchi, Y. (1989). Normal stages of development of *Cynops pyrrhogaster*. In *Development of Vertebrates Vol. 1*, 333–355. Okada, T. (Eds). Tokyo: Baifukan Co., LTD.

Kanazawa, J. (1979). Damage done to fisheries by agricultural chemicals. In *Aquatic Organisms and Agricultural Chemicals–Theories and Applications*, 61–63. Kanazawa, J. & Tanaka, J. (Eds). Tokyo: Scientist-sha Co., LTD.

Kanazawa, J. (1992).*Environmental Science on Agricultural Chemicals*,. Tokyo: Godo Shuppan Co., LTD.

Kobayashi, K. (1979). Absorption and excretion of agricultural chemicals in fish and shellfish. In *Aquatic Organisms and Agricultural Chemicals–Theories and Applications*, 20–37. Kanazawa, J. & Tanaka, J. (Eds). Tokyo: Scientist-sha Co., LTD.

Licht, L. E. (1985). Uptake of ^{14}C DDT by wood frog embryos after short term exposure. *Comp. Biochem. Physiol. C.* **81**, 117–119.

Licht, L. E. & Grant, K. P. (1997). The effects of ultraviolet radiation on the biology of amphibians. *Amer. Zool.* **37**, 137–145.

Maeda, N. & Matsui, M. (1989). *Frogs and Toads of Japan.* Tokyo: Bun-ichi Sogo Shuppan Co., Ltd.

Matsui, M. (1996). *Natural History of the Amphibia.* Tokyo: University of Tokyo Press.

Nakamura, K. & Ueno, S. (1963). *Japanese Reptiles and Amphibians in Colour.* Osaka: Hoikusha Publishing Co., Ltd.

Nieuwkoop, P. D. & Faber, J. (1975). *Normal Table of Xenopus laevis (Daudin), 2nd Edition.* Amsterdam: North-Holland Publishing Co.

Organization for Economic Co-operation and Development (OECD) (1991). *OECD The State of the Environment 1991.* Paris: OECD Publications Service.

OECD (1993). Fish, acute toxicity test. In *OECD Guidelines for the Testing of Chemicals*, No. 203. Paris: OECD Publications Service.

Power, T., Clark, K. L., Harfenist, A. & Peakall, D. B. (1989). A review and evaluation of the amphibian toxicological literature. *Canadian Wildlife Service Technical Report Series* **61**, 1–44.

Ross, L. J. & Sava, R. J. (1986). Fate of thiobencarb and molinate in rice fields. *J. Environ. Qual.* **15**, 220–225.

Sanders, H. O. (1970). Pesticide toxicities to tadpoles of the western chorus frog *Pseudacris triseriata* and Fowler's toad *Bufo woodhousii fowleri. Copeia* **1970**, 246–251.

Stebbins, R. C. & Cohen, N. W. (1995). *A Natural History of Amphibians.* Princeton: Princeton University Press.

Tyler, M. (1994). *Australian Frogs–A Natural History.* Chatswood: Reed Books.

Yoshimura, I. & Ohashi, Y. (1992). *Statistical Analysis for Toxicology Data.* Tokyo: Chijinshokan Co., Ltd.

Accepted: 22.12.98