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Uptake, Metabolism, and Effects on Detoxication Enzymes of Isoproturon in Spawn and Tadpoles of Amphibians

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Using *ring*-^{14}C-labeled isoproturon (1 μg/L), the uptake into spawn and tadpoles of *Bombina bombina* and *Bombina variegata* was investigated. Two percent of the applied radioactivity was found per gram fresh weight in the embryo after 24 h. Results indicate that the jelly mass of the spawn does not act as a sufficient physical barrier for protection against the uptake and influence of isoproturon (IPU) on the embryo. *In vivo* metabolism of *ring*-^{14}C-labeled IPU by the cytochrome *P*-450 system was analyzed in tadpoles. Different metabolites of IPU, such as N-demethylated and C-hydroxylated derivatives, and the olefinic metabolite were detected. In tadpoles of *B. variegata*, the activity of microsomal and soluble glutathione-*S*-transferase (sGSTs) toward different model substrates was measured after treatment with IPU. Activities of sGST increased corresponding to elevated stress by IPU dependent on exposure time and dose. Compared to the pure active ingredient IPU, the commercial phenyl-urea herbicide Tolkan Flo, consisting of IPU and an emulsifier, also caused significantly elevated enzymatic response. © 2002 Elsevier Science (USA)

Key Words: **isoproturon; herbicide; amphibians; spawn; tadpoles; microsomal and soluble glutathione-*S*-transferase.**

INTRODUCTION

Populations of many amphibian species all over the world appear to have undergone declines and range reductions recently (Wake, 1991). A series of conservation measures is urgently required to maintain or restore the natural habitats and the populations of threatened amphibian species. In Annex IV of the European Union Habitat and Species Directive (1992)[2] the fire-bellied toad (*Bombina bombina*) and the yellow-bellied toad (*Bombina variegata*) are described as animal species of common interest in need of strict protection. Also the Red Data Book of Germany classifies *B. bombina* as a critically endangered and *B. variegata* as an endangered species. Both species are closely related, and hybridization occurs at their range boundaries.

The northwest of middle Europe has experienced marked declines in *B. bombina.* In northeastern Germany this species has declined by around 30% since the early 1960s (Schneeweiss, 1993). After the loss of its preferred habitat of riverside meadows, *B. bombina* uses small ponds in agricultural landscapes as summer habitat and for reproduction. *B. bombina* seems to be especially sensitive to environmental pollution (Schiemenz, 1979; Herrmann *et al*., 1988; Schneeweiss, 1996).

In general, amphibian populations are potentially sensitive to aquatic contaminants like acid deposition, nitrogen fertilizers, and pesticides which may have contributed to their decline (Hecnar, 1995; Hall and Henry, 1992). Lower species diversity and density in the agricultural zone indicate that despite the presence of a water course, ditches, and ponds, many anuran species do not tolerate intensive agricultural activity (Bishop *et al*., 1999).

Approximately 30 tons of pesticides reach the surface water in Germany each year with isoproturon (3-(4-isopropylphenyl)-1,1-dimethylene) (*IPU*) contributing 2 tons annually (Federal Environmental Protection Agency, 2000). Isoproturon (*IPU*) is a selective systemic herbicide, which is absorbed by the roots and rapidly transported through the xylem to the leaves. It inhibits photosynthetic electron transport (Berger and Heitefuss, 1991). Phenyl-urea herbicides are used for pre- and postemergence control of annual grasses and many annual broad-leaved weeds in spring and winter wheat, barley, rye, and triticale. Because of its widespread use and its properties of moderate persistence and relatively low adsorption, IPU has become a water contaminant in many agricultural areas.

Sensitivity of aquatic nontarget animals to contamination by IPU has been studied in aquatic invertebrates and fishes.

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[2] Council Directive 92/43/EEC of 21 May 1992 on the Conservation of Natural Habitats and of Wild Fauna and Flora.

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The growth rate of the ciliate *Tetrahymena pyriformis* was slightly inhibited at 700 μg/L IPU, whereas other hydrotrophic organisms (*Daphnia magna* and the nematode *Caenorhabditis elegans*) were not affected by IPU up to concentrations of 1000 μg/L (Traunspurger *et al.*, 1996). LC_{50} values (96 h) of 37 mg/L for *Onchorynchus mykiss* and 9 mg/L for *Ictalurus punctatus* were reported by Perkow (1988) and Leiva *et al.* (1997). However, there are no reports in the literature of lethal or sublethal effects of phenyl-urea herbicides on amphibians.

In principle, organisms are able to detoxicate phenyl-urea herbicidces to some extent. The first phase of metabolism of IPU involves hydroxylation of carbon atoms, resulting in reactive functional groups, and catalyzation by cytochrome P450-dependent monooxygenases (Haas, 1997). These activated electrophilic derivatives can be conjugated to glutathione and catalyzed by glutathione-*S*-transferase (GST) in Phase II (George, 1994). The hydrophilic products of conjugation are excreted in Phase III.

GST is widely distributed in nature and may be implicated in cell line resistance to pesticides (Hayes and Wolf, 1988; Bucciarelli *et al.*, 1999; Glässgen *et al.*, 1999). With the exception of a single microsomal enzyme, GSTs are soluble (George, 1994). Soluble GSTs have been grouped into six distinct classes: α, μ, π, θ, δ, ζ, and β (Bucciarelli *et al.*, 1999). The variety of isoenzymes represents the wide range of different compounds which can be conjugated. Detoxication of aquatic contaminants by conjugation to glutathione is well documented in fish (Donnarumma *et al.*, 1988; George *et al.*, 1989; Wiegand *et al.*, 2000). In contrast, information about detoxication in amphibians is rare. For example, Johnson *et al.* (1995) examined biotransformation and the glutathione antioxidant system after exposure to 2, 4,6,-trinitrotoluene (TNT) in the amphibian species *Ambystoma tigrinum*. The results indicate that *A. tigrinum* possess considerable levels of antioxidant enzymes, but tested tissues were not sensitive indicators of TNT exposure. Investigations of Bucciarelli *et al.* (1999) yielded changes in GST amphibian patterns in embryonic and adult life stages of *Bufo bufo*. A specific GST form of later development stages was found to counteract the toxic effects of reactive metabolites of xenobiotics with higher efficiency.

In general, concerning the development stages of amphibians and their differences in sensitivity to pesticide contamination, contradictory results were reported in the literature. Berrill *et al.* (1995) found that within each tested ranid species, 1- and 8-day-old tadpoles are equally sensitive to treatment with the insecticide fenitrothion. However, Pauli *et al.* (1999) described that different aquatic stages of four investigated ranid amphibians exhibited differing degrees of sensitivity to the tested insecticide tebufenozide. Susceptibility of different developmental stages of amphibian tadpoles to pesticide contamination appears to be strongly dependent on both the species and the chemicals tested.

In order to assess the environmentally relevant effects of xenobiotics on aquatic life, the active compounds of pesticides as well as the formulated commercial products should be investigated (Liess, 1993; Mann and Bidwell, 1999; Plötner and Günther, 1987). The mechanisms of chemical interaction in mixtures are not fully understood (Howe *et al.*, 1998). Some theories include increase in rate of uptake, formation of toxic metabolites, reduction of excretion, alteration of distribution, and inhibition of detoxication systems, which appears to be the most popular theory (Marking, 1977). Field-grade herbicide formulations were found to cause more than just additive toxicity to amphibians. This "chemical synergy" suggests a mechanisms that is based on a severe inhibition of detoxication (Howe *et al.*, 1998).

The aim of the present study was to investigate the influence of environmentally relevant concentrations of IPU to the early developmental stages of *B. bombina* and *B. variegata*. At first it was necessary to check whether IPU was taken up by spawn and tadpoles of these amphibian species and if they are able to metabolize IPU. Furthermore, the physical and behavioral changes after treatment with IPU were checked. The effects of the pure ingredient IPU on the detoxication enzyme system of phase II were compared to effects of a formulated product consisting of IPU and an emulsifier.

MATERIALS AND METHODS

Study Area

The field investigations were carried out in a pond without buffer strip, situated in an intensively used arable area within the younger Pleistocene landscape of Northeast Germany. These regions are distinguished by a lot of natural potholes and ponds with high diversity and density of amphibians.

The catchment area of the investigated pond comprises approx. 3 ha of slightly sloping field. Summer barley was grown here in 2000 and once, in the spring (5/4/2000), the field was sprayed with the herbicide Tolkan Flo, a commercial phenyl-urea herbicide consisting of IPU (500 g/L) and an emulsifier. Application of Tolkan Flo was carried out strictly in conformance with the rules of agricultural procedure; the spraying distance to the pond was 15 m. The investigated pond is a very important habitat for eight species of amphibians, including the endangered *B. bombina.*

Embryo and Tadpole Culture

Studies of uptake and metabolism of IPU in amphibian tadpoles were carried out with *B. bombina* as well as *B. variegata.* Experiments to clarify the effects on detoxication enzymes were studied in *B. variegata*, because this species can be handled more easily than *B. bombina* in captivity.

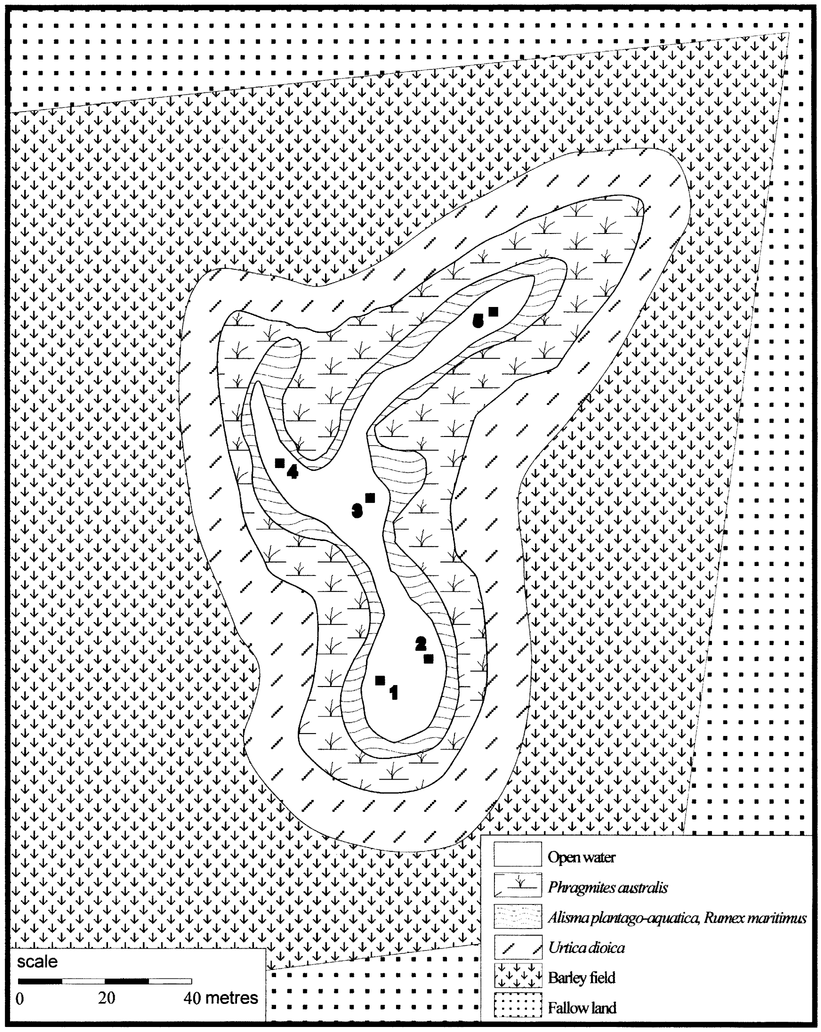

FIG. 1. Study pond showing the five sampling sites.

Determination of the developmental stages of both *Bombina* species was carried out according to Gosner (1960).

Spawn and tadpoles were kept in tanks with artificially salted (100 mg/L sodium chloride, 200 mg/L calcium chloride dihydrate, 103 mg/L sodium hydrogen carbonate), demineralized water. Twenty larvae, or one clutch of eggs, were placed in each aquarium containing 10 L water on a 14:10-h light:dark cycle. Tadpoles were fed with powdered dry food (Tetramin AZ 40). Larvae were placed into water to acclimate 24 h prior to testing. The water temperature was maintained at 20°C.

Experimental Procedures

Field sampling. To quantify IPU contamination in the pond, water samples were take at five different sites (Fig. 1), following a time schedule: on the day of spraying the sampling was carried out 0.5, 1, 3, 6, and 12 h after application. In order to determine the background contamination the first sample was taken 2 weeks prior to herbicide application. Samples were also taken after the first precipitation (a thunderstorm) and 10 weeks after application. At this time four of the sampling sites were found dry.

IPU analysis. Target compounds were enriched by solid-phase extraction (Sep-Pak, Waters). Subsequently, 1 L of water was introduced into the cartridges and the sorbent was eluted with 1 mL of methanol for measuring by HPLC.

Analysis of the nonlabeled IPU and its metabolites was performed with a liquid chromatograph (Waters) equipped with an injection valve with a 100-μl loop and a 996 diode array UV detector, set at 240 nm, containing a 10-mm flow cell. To separate IPU and its metabolites, a LiChrospher 100 RP-18 column (250 × 4 mm, 5 μm, Merck) and an adequate precolumn (4 × 4 mm) were used. The separation was carried out at 40°C at a flow rate of 1 mL/min. The liquid chromatography high-pressure gradient was prepared by mixing acetonitrile and water according to Haas (1997).

Uptake and metabolism of ring-^{14}C-labeled IPU. For the investigation of uptake of IPU into spawn and todpoles of the amphibians, *ring*-^{14}C-labeled IPU (sp. act. 3.965 MBq/mg; purity $>97\%$; Intl. Isotope, Germany) was used. Exposure of the eggs of *B. bombina* to 1 μg/L IPU lasted 24 h. Embryos exposed to the herbicide remained within their jelly capsules. After the treatment, the clutches of spawn were rinsed thoroughly. Then the jelly capsules and the embryos were separated carefully, frozen in liquid nitrogen, and homogenized in ethyl acetate. The radioactivity in the jelly mass and embryo was measured by liquid scintillation counting (Wallac 1409) in optima gold scintillator liquid. For determining the recovery at the beginning and the end of the investigation, the radioactive residues were analyzed in the exposure medium and also in the rinsing liquid of spawn and beakers.

Tadpoles of *B. variegata* at stage 25 were exposed to 1 μg/L *ring*-^{14}C-labeled IPU at 0.5, 1, 2, 4, 6, 12, and 48 h and investigated in the same manner as the spawn. *In vivo* metabolism of *ring*-^{14}C-labeled IPU was investigated in *B. bombina* and *B. variegata* tadpoles after 48 h exposure time. Whole-body extracts were analyzed for the detection of IPU metabolites by HPLC.

HPLC analysis of ring- ^{14}C-labeled IPU. The analysis of the *ring*-^{14}C labeled IPU was performed with a high-performance liquid chromatograph (Shimadzu) equipped with ^{14}C Yttrium–glass scintillator (Berthold). A 250×4.6-mm i.d. analytical column with a precolumn packed with Spherisorb ODSII 3 μm was used for separation at a flow rate of 0.6 mL/min. The liquid chromatography high-pressure gradient was prepared by mixing acetonitrile and water according to Hoque (1998).

Effects of IPU exposure on tadpoles. Eight tadpoles of *B. variegata* (stage 25) were exposed to 0, 0.1, 1, 10, and 100 μg/L IPU for 24 h at 20°C. The tadpoles were examined for mortality and prodded for assessment of their avoidance response. All experiments were run in triplicate.

Eight developmental stages of amphibians (Gosner stages 20–27) were chosen to check the most susceptible level. The examination covered the developmental phases from newly hatched tadpoles, with detectable heartbeat (stage 20), to the development of external gills (stages 21–23), followed by the development of the operculum and the disappearance of external gills (stages 23–25), up to the appearance of the hind limbs (stages 26–27). Eight tadpoles of *B. variegata* per stage were exposed to 0.01 μg/L IPU for 24 h at 20°C. The enzymatic response was determined by measuring the microsomal GST (mGST) and soluble GST (sGST) to model substrate CDNB (cf. detoxication enzyme system).

Detoxication enzyme system. In order to measure the enzymatic response of amphibians to contamination of IPU herbicides, larvae of *B. variegata* were exposed to solutions of the active ingredient, pure IPU, and also to the commercial herbicide Tolkan Flo.

Eight tadpoles of *B. variegata* (stage 25) were transferred to the tanks containing treatment solutions with concentrations of 0, 0.1, 1, 10, 100, and 1,000 μg/L IPU for 24 h at 20°C. In order to investigate the time-dependent response of the detoxication system to IPU, the larvae were exposed to 1 μg/L IPU for 0.5, 2, 6, 24, and 48 h at 20°C.

Enzyme extracts were prepared according to Pflugmacher and Steinberg (1997) and Wiegand *et al.* (1999) with modifications in volume. In the present experiments, samples were homogenized on ice in 25 mL sodium phosphate buffer (0.1 M, pH 6.5) containing 20% glycerol, 1.4 mM dithioerythritol (DTE), 1 mM EDTA. Cell debris was removed by centrifugation at 10,000 *g* (10 min). The supernatant was centrifugaed again at 40,000 *g* (60 min) to obtain the microsomal fraction which was resuspended in sodium phosphate buffer (20 mM, pH 7.0), 20% glycerol, 1.4 mM DTE. Ammonium sulfate precipitation cut between 35 and 80% saturation, followed by centrifugation at 30,000 *g* (30 min) and desalting in sodium phosphate buffer (20 mM, pH 7.0), was used to obtain soluble proteins.

Substrate specificities of mGST and sGST were quantified colorimetrically using the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) (Habig *et al.*, 1974) and the diphenylether herbicide fluorodifen (Schröder *et al.*, 1992). Enzyme activity was calculated in terms of the protein content of the sample (Bradford, 1976).

Analysis of Data

In order to test the significance of increasing or decreasing enzyme activities between control and exposure, a statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Newman–Keuls test, $P < 0.05$ (SPSS 9.0 for Windows).

RESULTS

Field Investigations

Before herbicide application on the field, IPU was not proved in any water-sample of the investigated pond. Thirty minutes after spraying, IPU was detectable at sampling sites 1 and 2 and after 3 h at all the others. Twelve hours after the application the input ranged from 3 to 9 μg/L IPU with the higher concentrations measured in deeper water in the southern sector of the pond. Presumably drainage water enters the pond here. At the time of the first precipitation—2 weeks after spraying—a clear increase in IPU concentration

(5–22 μg/L) was noted at four sampling sites; one sampling point was found dry. Ten weeks after the herbicide application all sampling sites except site 1 (containing 0.2 μg/L IPU) were dried out due to lack of precipitation (Fig. 2). For *in vitro* tests the concentration range was based on the results of the field experiments in order to clarify the effects of IPU on embryos and larvae.

Uptake and Metabolism of IPU

Using *ring*-^{14}C-labeled IPU to expose the spawn of *B. bombina*, radioactive residues were analyzed in the jelly capsules as well as in the embryos. In the jelly 0.7%/g fresh weight of the applied radioactivity were detected, while in the embryo about 2%/g fresh weight was found after 24 h (Fig. 3).

Measuring the time-dependent uptake of *ring*-^{14}C-labeled IPU into the tadpoles, 2% of the applied radioactive substance was found in the larvae after 30 min (Fig. 4). The radioactive amount increased to 4.8%/g fresh weight after 48 h, which differed significantly from all other results. During the first 30 min the uptake of IPU into the tadpoles began quickly, increased slightly, and after 4 h reached a steady state which continued till the end of the exposure time. The recovery of radioactivity in the experiment was 84.5%.

In tests of both *Bombina* species, different metabolites of IPU, such as the N-demethylated derivative monodesmethyl-IPU, the C-hydroxylated derivatives 1-hydroxy-IPU/2-hydroxy-IPU, 1-hydroxy-monodesmethyl-IPU/2-hydroxy-monodesmethyl-IPU, and the olefinic metabolite isopropenyl-IPU were detected (Table 1).

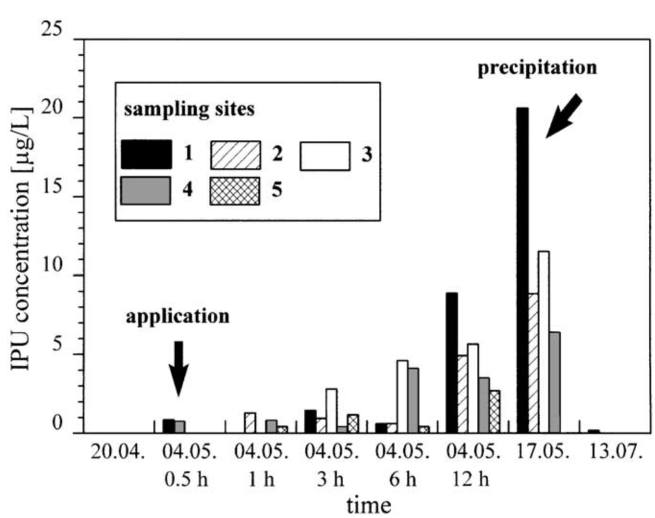

FIG. 2. Concentrations of isoproturon at the five sampling sites before and after application of the commerical herbicide Tolkan Flo.

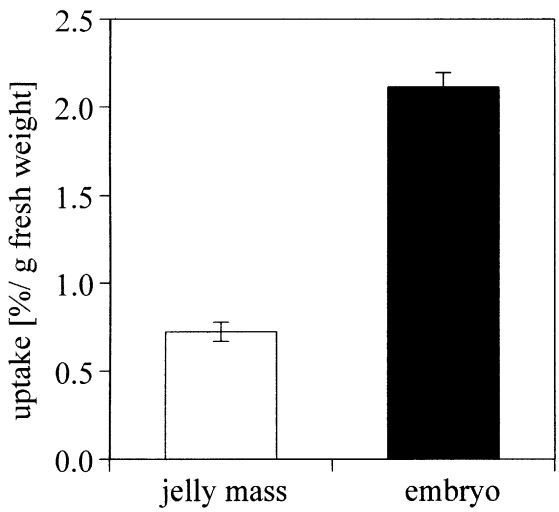

FIG. 3. Uptake of *ring*-^{14}C-labeled isoproturon into jelly capsules and embryo of *Bombina bombina* after 24 h. Error bars indicate standard deviations of triplicate experiment.

Effects of IPU Exposure on Tadpoles

Physical and behavioral abnormalities developed at concentrations of 0.1 μg/L in 24 h exposure (Fig. 5). In addition to reduced mobility (lateral swimming), developmental deformities including bent tails, body swelling and bulging, head deformities, and digestive system deformities occurred. At concentrations of 1, 10, and 100 μg/L IPU up to 50% of exposed tadpoles were paralyzed or died after 24 h. Compared to the control the number of impaired and dead tadpoles increased significantly. The tadpoles exhibited mortality up to 25% at 1 and 100 μg/L IPU and clear indications of diminished avoidance response when prodded.

At developmental stages 24 and 25 the sGST activities, tested with the model substrate CDNB, increased to 15 and

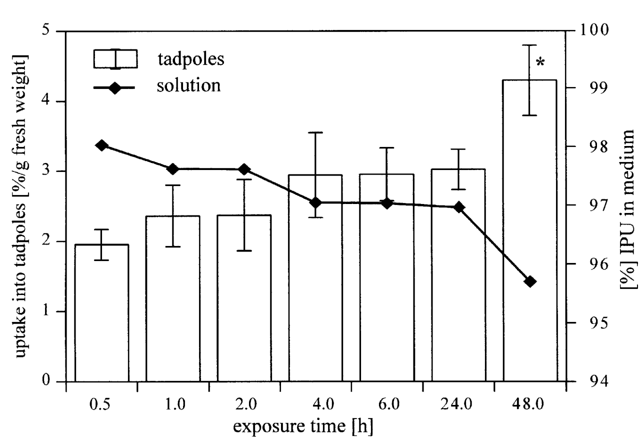

FIG. 4. Uptake of *ring*-^{14}C-labelled isoproturon by tadpoles of *Bombina variegata*. Mean of three independent samples and standard deviation. * Significantly different compared to control.

TABLE 1

Identities of Metabolites of [*ring*-^{14}C] IPU Established by Comparing the Retention Times (t_R, min) of the *ring*-^{14}C-Labeled Metabolites in *B. bombina* and *B. variegata* with Authentic Standards

Metabolite	Standard (t_R)	*B. bombina* (t_R)	*B. variegata* (t_R)
2-OH-MDM-IPU	16.4	—	—
1-OH-MDM-IPU	16.7	17.1[a]	16.7
2-OH-IPU	17.8	17.8	—
1-OH-IPU	17.9	—	17.9
DDM-IPU	24.5	—	—
MDM-IPU	26.6	26.6	26.6
Isoprenyl-IPU	28.3	28.3	—
IPU	28.4	28.4	28.4

Note. 2-OH-MDM-IPU, 2-hydroxy-monodesmethyl-IPU; 1-OH-MDM-IPU, 1-hydroxy-monodesmethyl-IPU; 2-OH-IPU, 2-hydroxy-IPU; 1-OH-IPU 1-hydroxy-IPU; DDM-IPU, didesmethyl-IPU; MDM-IPU, monodesmethyl-IPU.

[a] 1-OH-MDM-IPU-like metabolite.

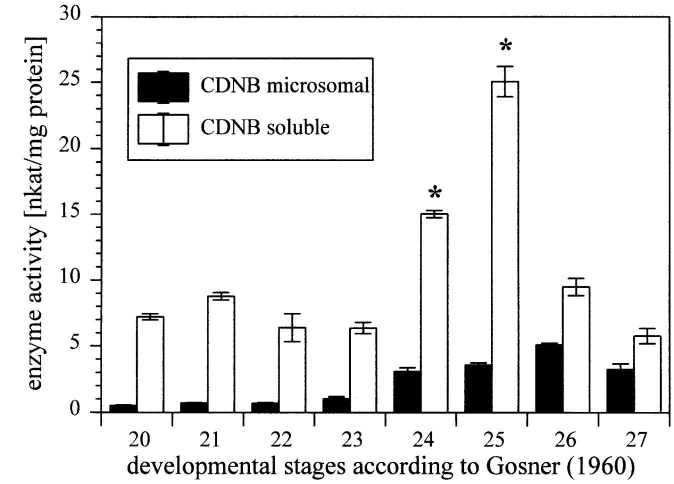

FIG. 6. Activities of soluble and microsomal glutathione-*S*-transferase (GST) toward CDNB in different developmental stages (Gosner, 1960) of *Bombina variegata* at 0.01 μg/L isoproturon. Mean of three independent samples and standard deviation. * Significantly different compared to all the other stages.

25 nkat/mg protein, respectively, and therefore differ significantly compared to all other stages (Fig. 6).

Effects on the GST Enzyme System

The enzymatic activity of the GST system was detectable in microsomal as well as in soluble enzyme fractions on all tested substrates. After exposure to IPU the activity of GST toward CDNB was always higher in the soluble fraction than in the microsomal one (Fig. 7). Soluble GST activity increased significantly up to 23 nkat/mg protein in response to concentrations ≥ 1 μg/L IPU, whereas the activity of the mGST decreased significantly (3 nkat/mg protein) compared to control (5 nkat/mg protein). Measured activities toward DCNB were clearly smaller in comparison to the other substrates. No mGST activity was measurable in the control, whereas a rapid increase at 0.1 μg/L IPU to 0.7 nkat/mg protein followed by a decline was observed. In the soluble fraction, the activity of the GST was significantly elevated at 1 and 10 μg/L IPU. The activity of mGST toward fluorodifen increased up to 22 nkat/mg protein at 0.1 μg/L IPU and then significantly decreased below the control value. The sGST levels did not changed significantly.

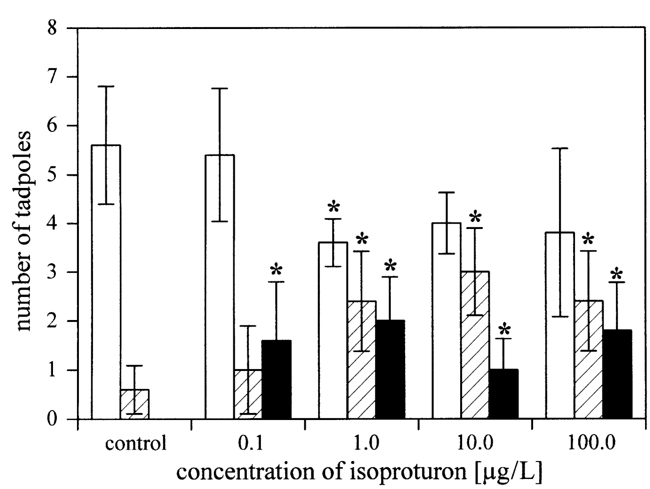

FIG. 5. Response of *Bombina variegata* tadpoles to different concentrations of isoproturon after 24 h exposure. Open bars indicate the mean number of tadpoles per treatment able to dart away with a normal avoidance response when prodded. Hatched bars indicate the mean number of paralyzed tadpoles per treatment or tadpoles twitched in place. Solid bars indicate the mean number of tadpoles per treatment that were completely unresponsive. Error bars indicate standard deviations of triplicate experiment. * Significantly different compared to control.

The study of time-dependent response demonstrated that toward CDNB the enzymatic response of the sGST fraction to IPU was elevated continuously with time up to 44 nkat/mg protein at 48 h (Fig. 8). The mGST activity increased slowly to 9 nkat/mg protein after 24 h IPU exposure. The range of the enzymatic response toward DCNB was clearly weak in comparison to the other substrates. Microsomal GST activity increased up to the maximum 0.4 nkat/mg protein after 6 h and then decreased. All activities differed significantly from the control. No activity of sGST in control toward DCNB was measured. After 48 h, an increase up to 0.6 nkat/mg protein was found. The activity of the mGST toward fluorodifen was always higher than that of the sGST at same concentrations. After 24 h a continuous increase up to 119 nkat/mg protein was observed, which remained at this level for the next 24 h. In addition,

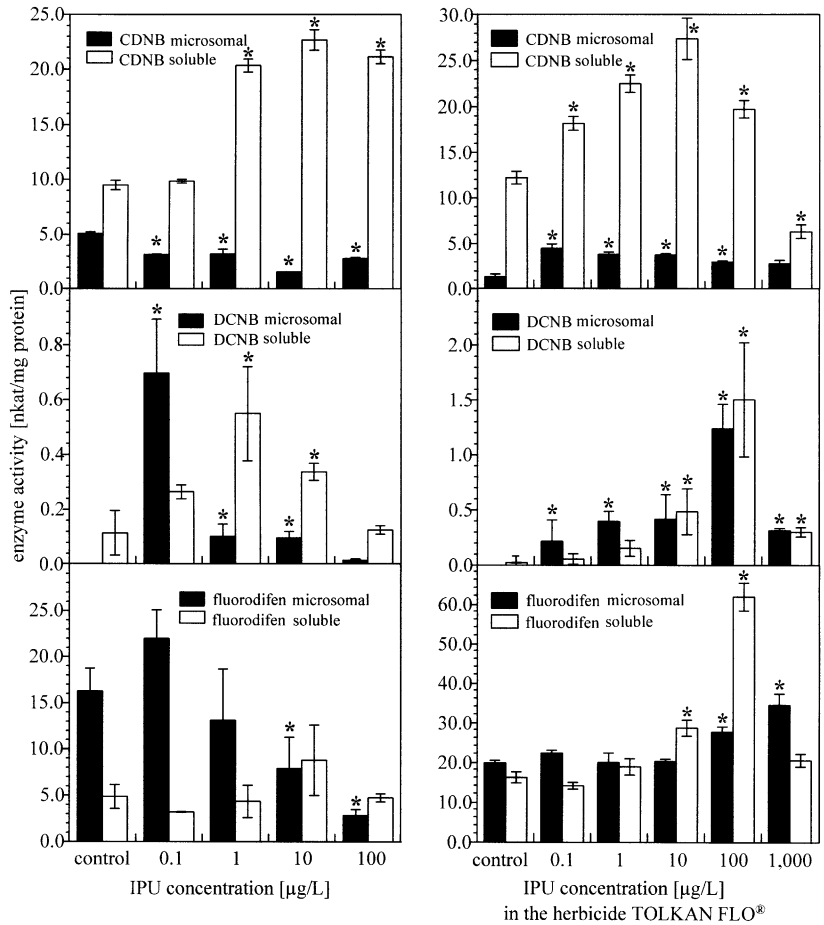

FIG. 7. Activities of soluble and microsomal glutathione-*S*-transferase (GST) toward CDNB, DCNB, and fluorodifen in different concentrations of isoproturon and the commercial herbicide Tolkan Flo in *Bombina variegata*. Mean of three independent samples and standard deviation. * Significantly different compared to control.

the activity of the sGST exhibited an increase up to 46 nkat/mg protein after 48 h.

Effects of the Commercial Herbicide Tolkan Flo on GST Activity

In most cases, the enzyme activities after exposure to the herbicide Tolkan Flo were elevated compared to the response of GST activities in the exposure using pure IPU, especially toward fluorodifen and DCNB at concentrations of 100 μg/L (Fig. 7). At concentrations from 0.1 to 10 μg/L IPU in Tolkan Flo the sGST toward CDNB increased significantly to 27 nkat/mg protein and decreased in the following at 100 and 1000 μg/L, where the measured activity remained below the control value. Microsomal GST activities were characterized by smaller variations, but differed significantly from the control. No mGST activity toward DCNB was measured in the control. The activities of mGST and sGST fractions toward this substrate were elevated with increasing Tolkan Flo concentrations up to 100 μg/L followed by a rapid decline. In contrast to the results obtained with the pure active substance, the mGST activity toward fluorodifen increased slightly. Variations were significant only at concentrations of 100 and 1000 μg/L Tolkan Flo. The sGST activity increased rapidly to 62 nkat/mg protein at 100 μg/L.

DISCUSSION

Field Studies

The concentration of IPU can be elevated in surface runoff to 110 μg/L dependent on precipitation, soil conditions, and draining (Johnson *et al.*, 1995; Patty and Gril, 1995) and in drainflow up to 465 (Harris, 1995) or 500 μg/L (Johnson *et al.*, 1996). In view of these results, measured levels of IPU in the investigated pond (0.2–22 μg/L) can be explained. Despite compliance of the spraying distance to reduce drift, up to 22 μg/L IPU were found, and other

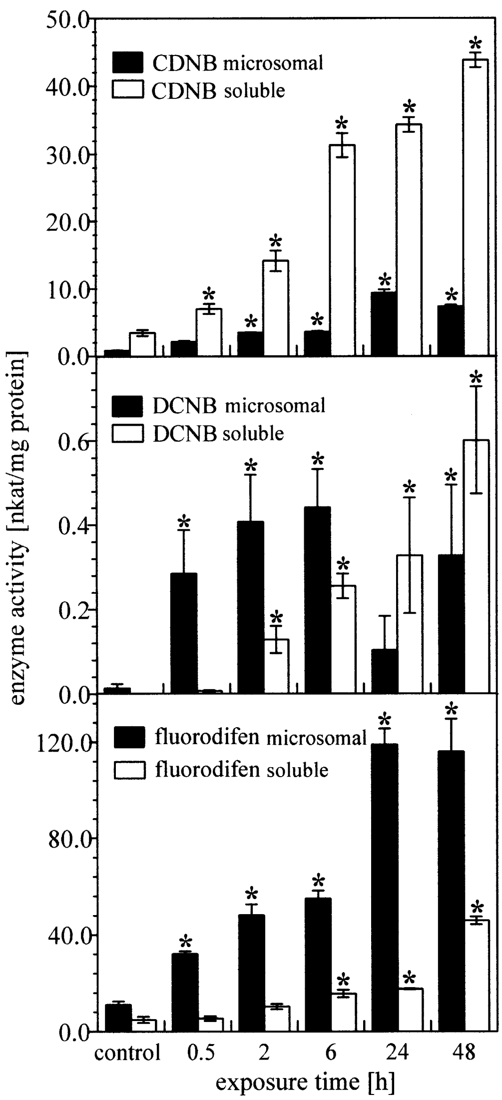

FIG. 8. Activities of soluble and microsomal glutathione-*S*-transferase (GST) toward CDNB, DCNB, and fluorodifen in *Bombina variegata* at different exposure times. Mean of three independent samples and standard deviation. * Significantly different compared to control.

authors detected concentrations in the same range, for example, up to 24 µg/L (Spiteller, 2000) in surface water adjacent to agricultural treatment. Even if farmers follow good agricultural practice on a drained clay soil, IPU applied to winter cereals will reach nearby streams and ditches at levels of concern (Johnson *et al.*, 1996). Many samples of the present study exceeded the limit for total pesticides approved by the federal water quality guidelines for aquatic life, which is 0.3 µg/L. Leaching of IPU was particularly important in the first flushes (thunderstorm on 5/17/2000) after application of the herbicide. Brown *et al.* (1995) described similar results of IPU movement in a heavy clay soil.

In the arable landscape, many stressor compounds can affect the growth and development of the anuran larvae (Bishop *et al.*, 1999); thus, it is necessary to combine experiments in the field and in the laboratory to separate the effects. The observed physical and behavioral abnormities caused by concentrations as low as 0.1 µg/L indicate that the entry of IPU into the reproduction pond could be relevant for the development of spawn and tadpoles of *Bombina* species.

In order to mitigate the entry of IPU into reproduction ponds of sensitive species of amphibians, establishment of grassed buffer strips around the ponds should be considered. Patty and Gril (1995) observed that an 11-m-wide grassed buffer strip removed 98% of IPU from wheat plots during a 1-year cropping period.

Uptake and Metabolism of IPU

The results of the exposure with *ring*-^{14}C-labeled IPU indicate uptake into the spawn of *B. bombina* in relevant ranges of environmental concentrations (1 µg/L IPU). The jelly capsules adsorbed a small amount of IPU and, therefore, cannot protect the embryo from effects of the herbicide. This insufficient physical barrier of protection is reported for other pesticides for amphibians and fish (Hashimoto *et al.*, 1982; Wiegand *et al.*, 2000). In contrast, Berrill *et al.* (1998) and Pauli *et al.* (1999) reported that for amphibians the jelly coat surrounding the embryo provided a protective layer during growth and development. The protection of the forming embryo from pesticide contamination appears to be strongly dependent on tested species and investigated pesticides.

In addition, *B. bombina* and *B. variegata* were able to metabolize the phenyl-urea herbicide by cytochrome P450-dependent monooxygenases (Phase I of detoxication), because the typical metabolites (Haas, 1997; Glässgen *et al.*, 1999) were detected in both species.

Effects of IPU Exposure on Tadpoles

Up to 50% of the *B. variegata* tadpoles were paralyzed and died on exposure at IPU levels corresponding to environmentally relevant concentrations in agricultural ponds. Paralysis is likely to render tadpoles more vulnerable to predation (Berrill *et al.*, 1994). Entry of IPU into the reproduction ponds seems to be a decisive factor for adverse development of *B. variegata* tadpoles.

In contrast to Plötner and Günther (1987), who stated that newly hatched tadpoles with external gills were most sensitive to contamination of the aquatic habitats, current results revealed that tadpoles at developmental stage 25 were the most susceptible ones. Therefore, amphibians of these stage with complete operculum were used in exposure investigations. Pauli *et al.* (1999) confirmed that tadpoles at stage 25 (about 2 weeks old) were the most sensitive in the four investigated amphibian species. However, older amphibian larvae at stage 40 appeared to be more sensitive than larvae at stage 29 for two species and the chemicals tested by Howe *et al.* (1998). The sensitivity of certain developmental stages of amphibians appear to differ dependent on chemical substances and tested species.

Effects on GST System

In plants, the GSTs transform the reactive metabolites of IPU to more hydrophilic compounds by conjugation of glutathione (Haas, 1997). In amphibian tadpoles, this detoxication pathway could also occur *via* the GST system, but an IPU–glutathione conjugate has not yet been proven. For each level of treatment the mGSTs and sGSTs of *B. variegata* were influenced by dose and exposure time of IPU. The changes of the activities were strongest toward CDNB and fluorodifen. With exception of the θ-class enzymes, all other GSTs are involved in conjugation of CDNB (George, 1994). Pflugmacher and Steinberg (1997) reported that the ability to conjugate fluorodifen seemed to be limited to the soluble fraction, using various macrophythes and limnic algae. In contrast, in *B. variegata* the activity of the mGST as well as the sGST for the model substrate fluorodifen seemed to be constitutive. By comparison of the substrates, the low levels of enzyme activities toward DCNB might indicate the weakness of corresponding isoenzymes.

Compared to the exposure to the pure active IPU the levels of detoxication enzymes after treatment with the commercial herbicide Tolkan Flo were significantly enhanced—twice in case of DCNB and fluorodifen. Thus, Tolkan Flo provoked a stronger enzymatic response in the tadpoles than pure IPU. This could be caused by the presence of an emulsifier in the Tolkan Flo formulation which enhanced the availability of IPU and/or the interaction between IPU and emulsifier. Bidwell and Tyler (1997) investigated the toxicity of the commercial herbicide Roundup 360 and its active ingredient glyphosate to larvae of *Litoria moorei*. Compared to the pure glyphosate, Roundup 360 proved to be the more toxic mixture. Adverse effects of formulation and detergents on amphibians have been described by other researchers (Mann and Bidwell, 1999; Plötner and Günther, 1987). The described effects of formulation and mixtures require further investigations using additional common herbicides in combination with other formulations and detergents.

Here the treatment with the commercial herbicide Tolkan Flo caused an increase of the enzymatic response for all tested isoenzymes up to maximum activities at 10 or 100 μg/L IPU. At higher concentrations (1000 μg/L), the detoxication was limited. *Bombina* tadpoles are not able to tolerate such high concentrations, which are rarely observed in surface waters. In contrast, the maximum enzyme activity after exposure to pure IPU was reached at concentrations as low as 0.1 or 1 μg/L IPU, especially in the microsomal fraction. Obviously, the effects of the treatment were triggered at these environmentally relevant concentrations, but with reduced enzymatic response.

Data indicate that the glutathione system constitutes a sensitive biochemical indicator of pollution by IPU to tested species. In order to regain physiological balance, the amphibians must spend energy for detoxication. This may cause delays in growth, development, and mobility, and lead to a reduction in physiological fitness (Allran and Karasov, 2000; Bridges, 1997; Diana *et al.*, 2000).

In the past the effects of pesticides on metamorphosis, growth, behavior, and mortality of amphibians were investigated intensively (Bridges, 1997; Fioramonti *et al.*, 1997; Howe *et al.*, 1998; Pauli *et al.*, 1999). In contrast to current investigations, significant effects occurred mostly at concentrations exceeding the environmentally relevant range. These discrepancies may be interpreted on the basis of differences in species sensitivity or differences in substrates used among the studies. Experimental exposure of amphibians to other pesticides clearly indicated that there exist differences in sensitivity among anuran species and life stages (Berrill *et al.*, 1994; Pauli *et al.*, 1999; Allran and Karasov, 2000).

CONCLUSIONS

The aim of this study was to investigate the combination of morphological effects and enzymatic response of sensitive amphibian tadpoles on contamination with IPU. Data suggest that, according to the rules of agriculture, the use of herbicides containing IPU has adverse effects on the development of early larval stages of *Bombina* species. The results of these studies indicate that spawn and tadpoles of *B. variegata* seemed to be much more susceptible to pollution by IPU as predicted by data on fish and invertebrates.

The investigation of xenobiotic metabolism is important in order to advance understanding of chemical toxicity at the individual, population, or community levels in aquatic ecosystems. In future studies involving amphibians of different species the combinations of IPU with other formulations should be investigated in order to underline the fact that the toxicity of IPU is modulated by the emulsifier. Based on these investigations it will be possible to make suggestions for sustainable agricultural management in ranges of distribution of sensitive amphibian species.

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