

Impacts of the herbicide butachlor on the larvae of a paddy field breeding frog (*Fejervarya limnocharis*) in subtropical Taiwan

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Abstract Butachlor is the most commonly used herbicide on paddy fields in Taiwan and throughout Southeast Asia. Since paddy fields provide habitat for pond breeding amphibians, we examined growth, development, time to metamorphosis, and survival of alpine cricket frog tadpoles (*Fejervarya limnocharis*) exposed to environmentally realistic concentrations of butachlor. We documented negative impacts of butachlor on survival, development, and time to metamorphosis, but not on tadpole growth. The 96 h LC₅₀ for tadpoles was 0.87 mg/l, much lower than the 4.8 mg/l recommended dosage for application to paddy fields. Even given the rapid breakdown of butachlor, tadpoles would be exposed to concentrations in excess of their 96 h LC₅₀ for an estimated 126 h. We also documented DNA damage (genotoxicity) in tadpoles exposed to butachlor at concentrations an order of magnitude less than the 4.8 mg/l recommended application rate. We did not find

that butachlor depressed cholinesterase activity of tadpoles, unlike most organophosphorus insecticides. We conclude that butachlor is likely to have widespread negative impacts on amphibians occupying paddy fields with traditional herbicide application.

Keywords Butachlor · LC₅₀ · *Fejervarya limnocharis* · Paddy field · Genotoxicity · Cholinesterase

Introduction

Rice (*Oryza sativa* L.) is the most important food crop for the majority of the world's population. It was first cultivated in Asia, more than 10,000 years ago (Vaughan et al. 2008), and is typically grown in paddy fields. The conversion of natural habitats to rice growing, like other agricultural practices, undoubtedly results in loss of amphibian diversity (Wells 2007; Zug et al. 2001). However, this agro-ecosystem which is characterized by contiguous aquatic habitats and dry lands harbors a rich biological diversity (Bambaradeniya and Amarasinghe 2003). Some frogs and toads (anurans) have benefited from the creation of paddy fields, which have become an important breeding habitat. For example, in Taiwan, at least 38% (11 of 29 species) of anuran species breed in paddy fields (Shang et al. 2009). The situation is probably similar in many parts of Asia, where anurans readily adapt to man-made bodies of water (Bambaradeniya 2000; Berry 1975).

Pesticides (herbicides and insecticides) are frequently used on paddy fields to enhance rice production. The impacts of pesticide use on the local fauna are largely unknown, and the impacts are highly dependent on toxicity, time of spraying, and the persistence of each pesticide

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and its breakdown products. Butachlor was developed by Monsanto Co. (USA) and is a post-emergence herbicide that is commonly used in Asia and Africa to control a wide variety of grasses and some broadleaf weeds in paddy fields (Senseman 2007). Butachlor is thought to inhibit the synthesis of long chain fatty acids (Senseman 2007). In Taiwan, more than 8,000 tons (active ingredient) of butachlor is applied annually (Taiwan Agrochemical Industrial Association 1996).

During the rice cultivation, paddy fields are plowed and irrigated to a water depth of about 4 cm after rice seedlings are transplanted. Butachlor is typically applied over paddy fields right after rice has been transplanted. This pesticide is known to retard growth and reproduction in earthworms such as *Eisenia fetida* (Muthukaruppan and Gunasekaran 2010) and *Perionyx sansibaricus* (Muthukaruppan et al. 2005), as well as damage epithelial tissue in *E. fetida* (Muthukaruppan and Gunasekaran 2010). Butachlor is neurotoxic to land snails (Rajyalakshmi et al. 1996) and genotoxic to toad and frog tadpoles, flounder, and catfish where it causes DNA strand break induction in erythrocytes (Ateeq et al. 2005; Geng et al. 2005b; Yin et al. 2007; Yin et al. 2008). It is also genotoxic to cultured mammalian cells where it causes DNA strand breaks and both micronucleus and chromosomal aberration inductions (Panneerselvam et al. 1999; Sinha et al. 1995). Butachlor is also an indirect mutagen to hamsters and rats (Hsu et al. 2005). Prolonged exposure to butachlor was toxic to spotted snakehead fish (*Channa punctata*) and has been found to accumulate through the food chain (Tilak et al. 2007).

The irrigation of paddy fields has created large bodies of water that attract breeding anurans. The breeding pattern of *Fejervarya limnocharis* (formerly *Rana limnocharis*, Alpine Cricket Frog) in Taiwan is highly influenced by agricultural activities (Alexander et al. 1979). *Fejervarya limnocharis* breeds during the rainy season, but the immediate stimulus for breeding is primarily the flooding of paddy fields (Alexander et al. 1979). The initiation of breeding coincides with the spraying of rice fields with pesticides, especially herbicides such as butachlor. As a result, *F. limnocharis* eggs and tadpoles are exposed to high concentrations of butachlor, especially compared with anurans that breed later in the season. This makes *F. limnocharis* particularly vulnerable to herbicide use. However, little information is available regarding the ecological and physiological effects of butachlor on anurans. Geng et al. (2005a) compared acute toxicity of butachlor on four species of anurans (*Bufo melanostictus*, *F. multistriata*, *Polypedates megacephalus*, and *Microhyla ornata*) and postulated that the sensitivity to butachlor is related to body size, larval period, and habitat use. Butachlor was genotoxic to *P. megacephalus* and *Bufo gargarizans* tadpoles (Yin et al. 2008). However, given that larval period varies

among species, how butachlor affects the long-term survivorship, development and growth, and metamorphic traits of tadpoles is largely unknown. The larval period of *F. limnocharis* is about 1–2 months (Alexander et al. 1979; Alexander et al. 1963; Wu and Kam 2009), and hence tadpoles are potentially exposed to the herbicide for a long period of time, making it an ideal subject for this study.

The purposes of our study were to (1) evaluate the impact of environmentally realistic concentrations of butachlor on the survivorship and growth of *F. limnocharis* tadpoles, and (2) assess neurotoxic and genotoxic effects of butachlor on tadpoles of *F. limnocharis*.

Materials and methods

Study animal and sampling procedures

Fejervarya limnocharis is a medium-sized (30–60 mm) frog that is distributed throughout eastern, southeastern, and southern Asia, including many of the small, isolated islands (Sumida et al. 2007). In Taiwan, *F. limnocharis* is widely distributed up to elevations of 1,000 m (Alexander et al. 1979; Lue et al. 1999; Yang 1998). Although this species has the potential for breeding year round, in central Taiwan it usually breeds only from February to September (Alexander et al. 1979). Breeding of *F. limnocharis* is correlated with rainfall and irrigation, and is restricted by low temperatures (Alexander et al. 1979). *Fejervarya limnocharis* usually breeds in temporary freshwater pools such as rice paddy fields and roadside puddles. Amplexus occurs after midnight followed by spawning at dawn (Alexander et al. 1979). Clutch size is highly variable and ranges from ca. 450–1800 eggs. The larval period is 1–2 months (Alexander et al. 1979; Alexander et al. 1963; Wu and Kam 2009).

We collected *F. limnocharis* egg masses right after deposition in paddy fields in Wuchi (120°33'10"E and 24°14'40"N) in March of 2005. We transported the eggs back to the laboratory in Styrofoam boxes and incubated the eggs in dechlorinated tap water in the laboratory at 26°C. Embryos hatched within 2 days, and tadpoles at Gosner stage 26 (Gosner 1960) were used for subsequent experiments.

Analyses of water samples

For all experiments, we used the commercial formulation of butachlor (60% active ingredient, *N*-butoxy-methyl-2-chloro-2',6'-diethylacetanilide, Monsanto Co., USA) that is typically applied to rice paddy fields in Taiwan. Different solutions of butachlor (ranging from 0.025 to 3.2 mg/l) were made for various experiments. To ensure consistency,

we validated the concentration of butachlor using a HPLC system (Del Buano et al. 2005; Junghans et al. 2003).

LC₅₀ estimation

We randomly assigned Gosner stage 26 tadpoles to one of the eight treatments: 0 (control), 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/l butachlor. The recommended application rate for butachlor in rice paddy water was 4.8 mg/l. Each of our eight treatments had three replicates; each replicate contained 10 tadpoles in 500 ml of test solution. Tadpoles were fed with boiled vegetables ad libitum and the water was changed once every day. All tadpoles were reared at 26°C under a 12/12 h light/dark cycle. Survival rates were recorded daily for 96 h.

Long-term survivorship, growth, and development

Gosner stage 26 tadpoles were randomly assigned to one of the seven concentrations of butachlor: 0 (control), 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 mg/l. There were 15 tadpoles in each treatment. Each tadpole was kept in an individual 100 ml plastic container (10.5 × 7.5 × 4.5 cm, L × W × H) and covered with a transparent, punctured lid to reduce evaporation. We fed tadpoles boiled vegetables ad libitum and changed the water once every 3 days. All tadpoles were reared at 26°C under a 12/12 h light/dark cycle. We monitored tadpole survival and measured growth and development once every week until metamorphosis. Growth was measured by weighing each tadpole to the nearest 0.1 mg. Before weighing, each tadpole was gently blotted to remove excess water. Development was measured as time to metamorphosis; developmental stage was determined using a hand lens. Tadpoles reached metamorphosis at Gosner stage 42, when forelimbs emerge and labial denticles have disappeared. At that time, we recorded the date and weighed each tadpole. Survivorship was compared through day 35 when the first tadpole metamorphosed.

Genotoxicity (DNA damage)

The effect of butachlor on DNA was analyzed using an alkaline comet assay; this technique had been successfully used for many eukaryotic cells, including tadpole erythrocytes (Ralph and Petras 1997; Ralph et al. 1996). Tadpoles were exposed to 0.4 and 0.8 mg/l butachlor for 96 h; the higher concentration is the 96 h LC₅₀ of butachlor for *F. limnocharis* tadpoles. Our procedure followed Ralph et al. (1996) and Chen et al. (2004) with some modifications. After treatment, eight tadpoles per treatment were decapitated and immediately placed in PBS for 2 min. Erythrocytes released into PBS were collected by

centrifugation at 500 g for 10 min. After washing twice with PBS, the isolated erythrocytes were subjected to an alkaline comet assay to assess butachlor-caused DNA damage. In a typical assay, 100 µl of 1% agarose at 65°C was dropped onto a glass microscope slide placed on a hot plate at 60°C. Cover slips were applied immediately, and slides were placed on ice to allow the agarose to form a gel. After 5 min, cover slips were removed by dipping slides into water containing ice. A second layer of agarose containing cells was then added. A 250 µl aliquot of 1.2% low melting-point agarose at 40°C was mixed with 50 µl of a suspension containing 2–3 × 10⁵ cells in phosphate buffered saline. From this mixture, 65 µl was added to the first layer of agarose. Cover slips were then added and removed as described above. In a similar manner, 70 µl of 1% low-melting-point agarose was applied as the third layer of agarose. After removing the cover slips, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, with the pH adjusted to 10.0 with NaOH and 1% N-laurylsarcosine, 1% Triton X-100, and 10% dimethyl sulfoxide) at room temperature for 1 h. Slides were transferred to an electrophoresis tank containing freshly prepared alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH 13.4) at room temperature and soaked for 20 min to allow unwinding of the DNA. Electrophoresis was carried out at 23 V, 300 mA for 25 min. Slides were washed briefly in distilled water, blotted, then transferred to 0.4 M Tris-HCl, pH 7.5. DNA was stained by adding 70 µl of ethidium bromide (20 µg/ml water), and covered with a cover slip. For each sample, comet images were observed at 400× magnification with a fluorescence microscope (Nikon E400, Tokyo, Japan), and 50 cells per slide were randomly scored for tail moment using the comet III software (Perceptive Instruments, Haverhill, UK).

Cholinesterase (AChE) activity

Tadpoles at stages 26–28 were treated with 0 (control) or 0.8 mg/l butachlor, which is the 96 h LC₅₀ of butachlor for *F. limnocharis* tadpoles. We conducted a 5 × 6 AChE assessments with a total of 30 tadpoles per treatment; five tadpoles were sampled at 0, 3, 6, 9, 12 and 24 h to evaluate AChE activity. For comparison, we also exposed tadpoles to 0.12 mg/l chlorpyrifos, an insecticide with known AChE inhibition (Senseman 2007). A 20 mg sample of tadpole body was washed with de-ionized water, wiped dry, and then powdered in liquid nitrogen before homogenizing in 1 ml of 0.1 M phosphate buffer (pH 8.0). A homogenate of enzyme extract was centrifuged at 1,000 g for 5 min at 4°C, and an aliquot of 400 µl supernatant was added to a 350 µl 0.1 M phosphate buffer solution (pH 8.0) in cuvette, and mixed with 25 µl DTNB reagent and 5 µl substrate

(i.e., homogenized body tissue). For the control blank, a 400 μ l phosphate buffer solution was added instead of enzyme extract. Changes in absorbance of reaction solution were recorded at 412 nm for the first and second minutes, and the average of the change in absorbance per min was calculated. AChE activity was calculated as:

$$r = \frac{\Delta A}{1.36 \times (10^4)} \times \frac{1}{(400/780) C_0}$$

where R rate in moles substrate hydrolyzed per min per g tissue, A average change in absorbance per min, and C₀ original concentration of tissue (mg/ml).

Statistical analyses

Tadpole 96 h survivorship, and the growth and development during the first 4 weeks were evaluated using two-way repeated measure analysis of variance (MANOVA). The 96 h LC₅₀ was estimated using the log-logistic model (Seefeldt et al. 1995). We used the Kaplan–Meier survival analysis (Kaplan and Meier 1958) to compare the 35 day survival curves of tadpoles at various concentrations. If a significant difference was found, we further examined the survival curves between paired concentration treatments. We used ANOVA to compare the AChE activities at each time point, tail moment of erythrocytes (comet assay), and metamorphic traits of tadpoles between treatments. Post-hoc comparisons were done using the Tukey method. The significance level was set at 0.05. All data were analyzed with SPSS 11.0 software programs (SPSS, Chicago, IL, USA).

Results

Acute effects of butachlor

The 96 h survivorship of *F. limnocharis* tadpoles was significantly affected by butachlor (MANOVA, treatment \times time: Wilk's $\lambda = 0.10$, $F_{15,28} = 2.49$, $P = 0.0018$; Fig. 1). All tadpoles died within 72 h when exposed to 1.60 mg/l, and all tadpoles died within 48 h when exposed to 3.21 mg/l butachlor. Tadpoles in concentrations of 0–0.8 mg/l survived at a rate of 53.3 to 100% (Fig. 1). Using a log-logistic model, we calculated the 96 h LC₅₀ of butachlor as 0.87 mg/l for *F. limnocharis* tadpoles.

Chronic effects of butachlor

The 35 day survival for *F. limnocharis* tadpoles differed among treatments (Kaplan–Meier analysis, $X^2 = 115$, $P = 0.0001$, df = 6, Fig. 2). Pair-wise comparisons showed that the survival of tadpoles in 0.2, 0.4 and 0.8 mg/l

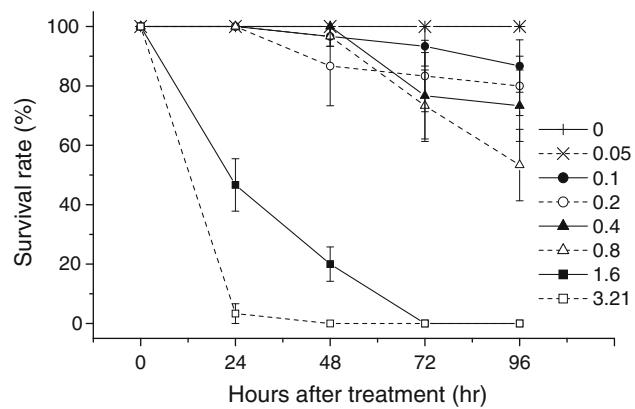


Fig. 1 *Fejervarya limnocharis* tadpole survivorship over time when exposed to different concentrations (mg/l) of butachlor. Values are means \pm SE

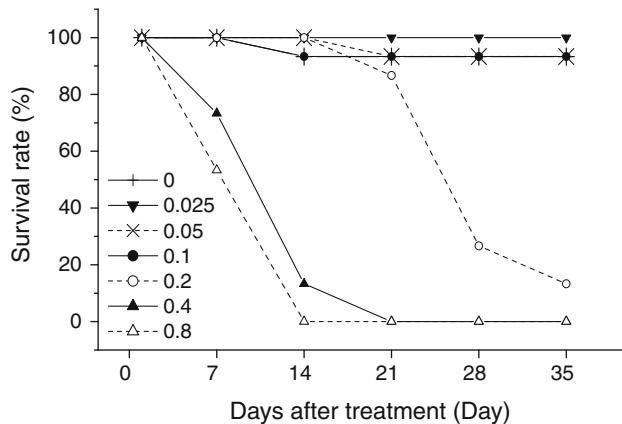


Fig. 2 Long-term tadpole survivorship in different concentrations (mg/l) of butachlor

butachlor was significantly lower than those in all other treatments (all $P < 0.0001$). The 0.4 and 0.8 mg/l curves were statistically similar and all tadpoles died after 14 days of exposure. The survival curve for tadpoles in 0.2 mg/l butachlor differed from those in lower concentrations of butachlor (all $P < 0.05$). Survival of tadpoles in 0.1 mg/l or less was statistically similar (Fig. 2).

Tadpole growth was similar among the five lower treatments (0–0.2 mg/l) (MANOVA; treatment \times time, Wilks' $\lambda = 0.62$, $F_{12,77} = 1.28$, $P = 0.250$), whereas tadpole development was statistically different (Wilks' $\lambda = 0.50$, $F_{12,77} = 1.93$, $P = 0.043$, Fig. 3). However, post-hoc comparisons showed that only tadpole development in 0.2 mg/l treatment differed from the control ($P = 0.028$) because tadpoles in 0.2 mg/l had delayed metamorphosis.

The percent of tadpoles that reached metamorphosis differed among treatments ($X^2 = 206.81$, df = 6, $P < 0.0001$, Table 1). Only those tadpoles reared in 0.1 mg/l or lower concentrations of butachlor survived to metamorphosis; at

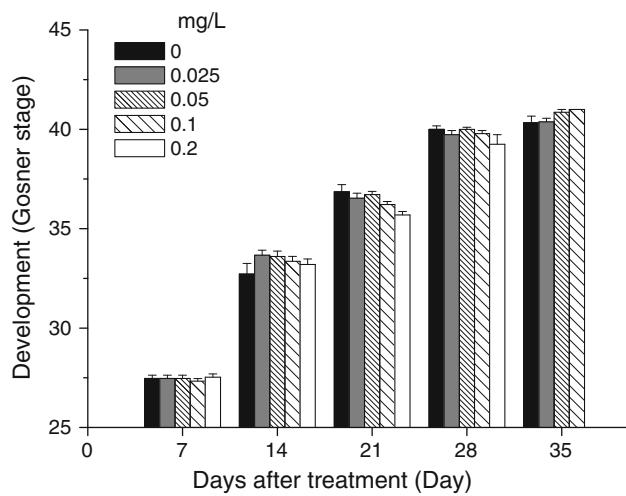


Fig. 3 Tadpole development in different concentrations (mg/l) of butachlor. Values are means \pm SE

those concentrations, the percent that metamorphosed ranged from 80–100%. For tadpoles that survived, time to reach metamorphosis differed among treatments (ANOVA, $F_{3,51} = 6.34$, $P < 0.01$), and post-hoc comparisons showed that tadpoles in the 0.025 and 0.1 mg/l treatments took a significantly longer time to reach metamorphosis (Table 1). In contrast, size at metamorphosis was not significantly different among treatments ($F_{3,51} = 0.154$, $P = 0.2148$, Table 1).

Genotoxicity (DNA damage)

The tail moment of erythrocytes differed significantly among treatments ($F_{2,57} = 103.8$, $P < 0.0001$, Fig. 4). Erythrocytes of the control group did not exhibit a comet tail, indicating that there were no detectable DNA strand breaks. However, cells treated with 0.4 or 0.8 mg/l butachlor showed a comet formation, indicating that DNA strand breaks had migrated in the electric field. Post-hoc comparisons showed that the extent of butachlor-induced DNA strand breaks increased significantly with dosage ($P < 0.05$).

AChE activity

The AChE activity of control and butachlor-treated tadpoles remained relatively constant over 24 h, whereas chlorpyrifos-treated tadpoles exhibited a steady decline in

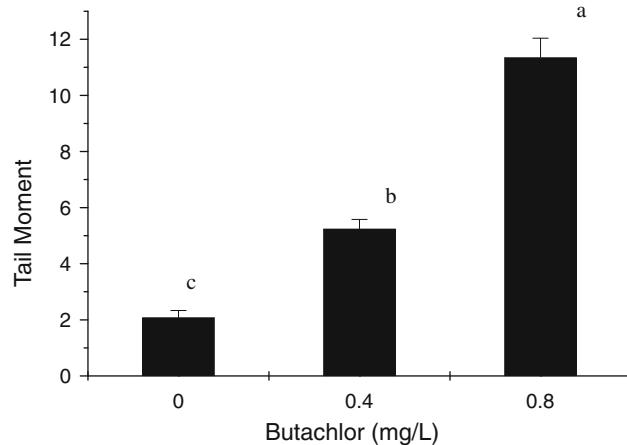


Fig. 4 Tail moment of erythrocytes of tadpoles subjected to different concentrations of butachlor. All three treatments were significantly different from each other. Values are means \pm SE

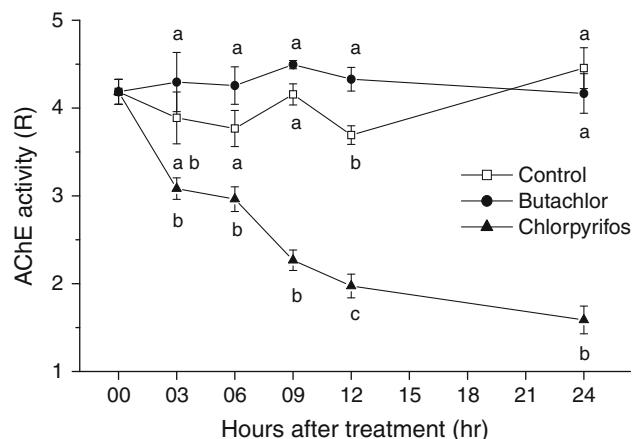


Fig. 5 AChE activity in control, butachlor-treated, and chlorpyrifos-treatment tadpoles for 24 h. Different letters represent significant differences between treatments. Values are means \pm SE

AChE activity (Fig. 5). By 3 h, tadpoles exposed to chlorpyrifos had significantly depressed AChE activity compared with tadpoles exposed to butachlor.

Discussion

In our experiments, butachlor had no impact on *F. limnocharis* tadpole growth, but it negatively affected survival,

Table 1 Metamorphic success and traits of *Fejervarya limnocharis* tadpoles in different butachlor concentrations

Concentration (mg/l)	Control	0.025	0.05	0.1	0.2	0.4	0.8	Test	P-value
Number metamorphosed ($n = 15$)	14	15	14	12	0	0	0	$X^2 = 206.8$	<0.0001
Time to metamorphosis (days)	38.43 ± 1.74^a	40.87 ± 4.29^b	39.86 ± 2.76^a	43.92 ± 3.77^b	–	–	–	$F_{3,51} = 6.34$	0.01
Size at metamorphosis (mg)	402.1 ± 52.9	420.1 ± 54.8	421.4 ± 80.8	453.5 ± 50.7	–	–	–	$F_{3,51} = 1.54$	0.2148

No tadpoles at concentrations of 0.2 mg/l or higher survived to metamorphosis. Different letters represent significant different between treatments

development, and time to metamorphosis. Most importantly, the 96 h LC₅₀ (0.87 mg/l) was well below the recommended concentration in paddy water (4.8 mg/l; <http://www.tactri.gov.tw/htdocs/ppmtable/ri-05.pdf>). The half-life of butachlor is fairly short in aquatic ecosystems. Yu et al. (1993) took field measurements of butachlor in the water and calculated a half-life of 1.7–2.5 days. The concentration had diminished to 0.3 mg/l by day 8, and butachlor was almost undetectable in the water after 20 days. If we use the mean half-life estimate (2.1 days), it would take 126 h (5.25 days) for butachlor to drop below a concentration of 0.87 mg/l (range 102–149 h), which is the 96 h LC₅₀ for *F. limnocharis* tadpoles. Presumably this would result in more than 50% of the tadpoles succumbing to the herbicide. In addition, our exposure experiments found that less than 5% of *F. limnocharis* tadpoles survived 24 h when exposed to 3.21 mg/l of butachlor (Fig. 1). These data suggest that *F. limnocharis* tadpoles do not survive well in fields where butachlor is used, however, the *F. limnocharis* can be found in most of the paddy fields throughout Taiwan, indicating frogs may adapt to this agro-ecosystem.

We suggest that the prolonged breeding season of frogs coupled with fine scale variations in the timing of rice growing would ameliorate the toxic effects of butachlor on frogs. *Fejervarya limnocharis* starts to breed at the time of irrigation, which is also when butachlor is sprayed on rice paddies; however, breeding activity continues throughout spring and summer as long as warm temperatures and standing water in the paddy fields are available. Egg masses that are laid at least a few days after the spraying of butachlor would be less affected by butachlor due to its short half-life. In Taiwan, farmers generally own small pieces of land that do not exceed 1–2 ha. While all the farmers typically grow rice in the spring, the timing of plowing, irrigation, spraying of pesticides, and planting varies by 1 or 2 weeks among nearby fields. These differences in the fine-scale timing of rice growing create a mosaic of water bodies that contain varying concentrations of pesticides. Frogs that oviposit in high concentration of butachlor suffer high embryonic and tadpole mortality, but eggs and tadpoles may survive if frogs oviposit in fields with low concentrations of butachlor. Vonesh and Buck (2007) reported that gray treefrogs (*Hyla chrysoscelis*) consistently oviposited in uncontaminated pools, suggesting that frogs are able to discriminate between uncontaminated and contaminated pools.

Other Asian anurans that breed in paddy fields have even lower 96 h LC₅₀s for butachlor, 0.53 mg/l for *Microhyla ornata* and 0.74 mg/l for *Rana guentheri* (Geng et al. 2005a, 2005b). However, *F. multistriata* has a somewhat higher 96 h LC₅₀ of 1.30 mg/l. This suggests that butachlor is likely to have widespread impacts on

anurans that breed in rice fields, but that the impact will vary by species. This also suggests that *F. limnocharis* tadpoles could be sensitive indicators of butachlor in paddy fields.

Butachlor is known to bioaccumulate in fish. Wang et al. (1992) looked at six species of fish and found butachlor bioconcentration from 2.4 to 220 times the concentrations to which they were exposed for 3–5 days. Mozambique tilapia (*Oreochromis mossambicus*) held in water with 10 µg/l butachlor accumulated to a concentration of 24 µg/l, and bighead carp (*Aristichthys nobilis*) held in water with 0.4 µg/l butachlor accumulated 88 µg/l. Tissue concentrations of butachlor ranged from 7 µg/l for the tilapia, and up to 255 µg/l for the Japanese eel (*Anguilla japonica*; Wang et al. 1992). While these concentrations were below the 48 h LC₅₀ for the fish (880 µg/l for tilapia, 290 µg/l for the eel), the results were almost certainly within the experimental error for the Japanese eel. Hence, eels exposed to 5 µg/l butachlor for 3–5 days would bio-accumulate butachlor to a concentration that would likely kill 50% of them during 48 h of exposure. If *F. limnocharis* bioaccumulates butachlor in a fashion similar to fish, butachlor could reach tissue concentrations that are lethal. At sublethal doses, bioaccumulation would probably make *F. limnocharis* even more susceptible to low concentrations of the herbicide.

Genotoxic compounds interfere with the replication of DNA by forming strong covalent bonds that disrupt meiotic or mitotic processes. Damaged DNA can lead to direct mortality from mutations, tumors, or cancers. *R. megacephalus* is a common frog in agricultural areas of southern China (Geng et al. 2005b). Butachlor is genotoxic to *R. megacephalus* tadpole erythrocytes at concentrations as low as 0.41 mg/l (Geng et al. 2005b). Our study showed that butachlor damaged the DNA of *F. limnocharis* tadpole erythrocytes at 0.4 and 0.8 mg/l. This demonstrates that butachlor-induced genotoxicity is a significant concern for these two species of anurans, and perhaps other anurans that lay their eggs where butachlor is being applied to paddy fields. Furthermore, butachlor is genotoxic at notably low concentrations compared with other two broad-spectrum herbicides such as Roundup (6.75 mg/l, active ingredient: glyphosate) and AAtrex Nine_O (4.81 mg/l, active gradient: Atrazine: 2-chloro-4-ethylamino-6-isopropylamino-s-triazine), when tested on larval *Lithobates* (=*Rana*) *catesbeianus*, a ranid frog native to central and eastern North America (Clements et al. 1997).

Butachlor genotoxicity is not limited to amphibians; Ateeq et al. (2005) and Yin et al. (2007) reported genotoxicity in catfish and flounder erythrocytes. Since only a few species of fish and amphibians have been examined, these findings raise the possibility that butachlor is genotoxic to many other species of fish and amphibians. Because of this, the mechanism of butachlor-induced

genotoxicity in tadpole erythrocytes needs further investigation. Butachlor is genotoxic to mammalian cell cultures (Panneerselvam et al. 1999; Sinha et al. 1995), but butachlor exhibits only limited mutagenic or genotoxic impacts on whole mammals (Brusick et al. 1992; Lohman et al. 1992; Wilson and Takei 2000). Based on these findings, it is likely that butachlor genotoxicity is species-specific, at least within mammals.

Organophosphorus insecticides, such as chlorpyrifos, malathion, and diazinon, function by binding with acetylcholinesterase (AChE), an enzyme essential for normal nerve function, whereas butachlor is thought to inhibit the synthesis of long chain fatty acids (Senseman 2007). Our finding of depressed AChE levels in *F. limnocharis* tadpoles exposed to chlorpyrifos demonstrated that our experimental protocol was appropriate for evaluating AChE. The lack of any change in AChE levels in butachlor-exposed tadpoles indicates that the toxic effects of butachlor do not include AChE depression. This contradicts an earlier study on snails (Rajyalakshmi et al. 1996). These authors treated the snail *Pila globosa* with 26 mg/l of butachlor in vivo and found that AChE activity was depressed after 12 h, but returned to the control level by 48 h. We believe the different response of *F. limnocharis* versus *P. globosa* may be due to (1) taxaspecific differences in the effects of butachlor or (2) the unusually high dose of butachlor used in the snail study (26 mg/l). That dosage is more than five times the recommended application rate, and more than 30 times the dosage used in our experiments. Even at such extreme concentrations, snail AChE activity was only depressed initially, and then returned to the control level after 48 h, suggesting the effect of butachlor on AChE activity may be a pharmaceutical artifact rather than physiological response. We expect that the AChE activity of snails would not be inhibited if they were treated with the recommended concentration of butachlor in paddy fields.

Overall, the broad-spectrum herbicide butachlor depresses survival, development, and time to metamorphosis in *F. limnocharis*. Additionally, the 96 h LC₅₀ (0.87 mg/l) is well below the recommended concentration for application of butachlor (4.8 mg/l). *Fejervarya limnocharis* is able to survive in paddy fields because (1) butachlor is typically applied to adjacent paddy fields at different times, (2) frogs are probably able to select fields with low concentrations of butachlor, and (3) butachlor has a short half-life so it remains at toxic concentrations for only a short period of time.

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Geologic Survey, Amphibian Research and Monitoring Initiative (ARM).

References

- Alexander PS, Chang C-M, Yang C-H (1963) Reproductive variation in the female rice frog *Rana limnocharis* during the spring season in Taiwan. *Tunghai J* 5(2):19–32
- Alexander PS, Alcala AC, Wu DY (1979) Annual reproductive pattern in the rice frog *Rana L. limnocharis* in Taiwan. *J Asian Ecol* 1:68–78
- Ateeq B, Farsh MA, Ahmad W (2005) Detection of DNA damage by alkaline single cell gel electrophoresis in 2, 4-dichlorophenoxy-acetic-acid- and butachlor-exposed erythrocytes of *Clarias batrachus*. *Ecotoxicol Environ Saf* 62:348–354
- Bambaradeniya CNB (2000) Rice field: an important man-made habitat of herpetofauna. Paper presented at the proceeding of the 4th Asian Herpetological Congress, Chengdu, China
- Bambaradeniya CNB, Amarasinghe FP (2003) Biodiversity associated with the rice field agroecosystem in Asian countries: a brief review. International Water Management Institute, Columbus
- Berry PY (1975) The amphibian fauna of peninsular Malaysia. Tropical Press, Kuala Lumpur
- Brusick DJ, Ashby J, de Serres FJ, Lohman PHM, Matsushima T, Matter BE, Mendelsohn ML, Moore DH II, Nesnow S, Waters MD (1992) A method for combining and comparing short-term genotoxicity test data: preface: a report from ICPEMC committee 1. *Mutat Res* 266:1–6
- Chen HW, Chien ML, Chaung YH, Lii CK, Wang TS (2004) Extracts from cigarette smoke induce DNA damage cell adhesion molecule expression through different pathway. *Chem Biol Interact* 150: 233–241
- Clements C, Ralph S, Petras M (1997) Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. *Environ Mol Mutagen* 29:277–288
- Del Buano D, Scarpone L, Amato RD (2005) An analytical method for the simultaneous determination of butachlor and benoxacor in wheat and soil. *J Agric Food Chem* 53:4326–4330
- Geng BR, Yao D, Xue QQ (2005a) Acute toxicity of the pesticide dichlorvos and the herbicide butachlor of tadpoles of four anuran species. *Bull Environ Contam Toxicol* 75:343–349
- Geng BR, Yao D, Xue QQ (2005b) Genotoxicity of pesticide dichlorvos and herbicide butachlor in *Rhacophorus megacephalus* tadpoles. *Acta Zool Sin* 51:447–454
- Gosner K (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190
- Hsu KY, Lin HJ, Lin JK, Kuo WS, Ou YH (2005) Mutagenicity study of butachlor and its metabolites using *Salmonella typhimurium*. *J Microbiol Immunol Infect* 38:409–416
- Junghans M, Backhaus T, Faust M, Scholze M, Grimme LH (2003) Predictability of combined effects of eight chloroacetanilide herbicides on algal reproduction. *Pest Manag Sci* 59:1101–1110
- Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observation. *J Am Stat Assoc* 53:457–481
- Lohman PHM, Mendelsohn ML, Moore DH II, Waters MD, Brusick DJ, Ashby J, Lohman WJA (1992) A method for comparing and combining short-term genotoxicity test data: the basic system. *Mutat Res* 266:7–25
- Lue KY, Tu MC, Shang G (1999) A field guide of the amphibians and reptiles of Taiwan. Great Nature Magazine Publisher, Taipei
- Muthukaruppan G, Gunasekaran P (2010) Effect of butachlor herbicide on earthworm *Eisenia fetida*—its histology and perspicuity. *Appl Environ Soil Sci* 2010:1–4

- Muthukaruppan G, Janardhanan S, Vijayalakshmi G (2005) Sublethal Toxicity of the herbicide butachlor on the earthworm *Perionyx sansibaricus* and its histological changes. *J Soils Sediments* 5:82–86
- Panneerselvam N, Sinha S, Shanmugam G (1999) Butachlor is cytotoxic and clastogenic and induces apoptosis in mammalian cells. *Indian J Exp Biol* 37:888–892
- Rajyalakshmi T, Srinivas T, Swamy KV, Prasad NS, Mohan PM (1996) Action of the herbicide butachlor on cholinesterases in the freshwater snail *Pila globosa* (Swainson). *Drug Chem Toxicol* 19:325–331
- Ralph S, Petras M (1997) Genotoxicity monitoring of small bodies of water using two species of tadpoles and the alkaline single cell gel (comet) assay. *Environ Mol Mutagen* 29:418–430
- Ralph S, Petras M, Pandrangi R, Vrzoc M (1996) Alkaline single cell gel (comet) assay and genotoxicity monitoring using two species of tadpoles. *Environ Mol Mutagen* 28:112–120
- Seefeldt SS, Jensen JE, Fuerst EP (1995) Log-logistic analysis of herbicide dose-response relationships. *Weed Technol* 9:218–227
- Senseman SA (2007) Herbicide Handbook. Weed Science Society of America, Lawrence
- Shang KS, Yang YJ, Li PH (2009) Field guide to amphibians and reptiles in Taiwan. Owl Publishing House Co., Ltd, Taipei
- Sinha S, Panneerselvam N, Shanmugam G (1995) Genotoxicity of the herbicide butachlor in cultured human lymphocytes. *Mut Res Genet Toxicol* 344:63–67
- Sumida M, Kotaki M, Islam MM, Djong TH, Igawa T, Kondo Y, Matsui M, Anslem DS, Khonsue W, Nishioka M (2007) Evolutionary relationships and reproductive isolating mechanisms in the rice frog (*Fejervarya limnocharis*) species complex from Sri Lanka, Thailand, Taiwan and Japan, inferred from mtDNA gene sequences, allozymes, and crossing experiments. *Zool Sci* 24:547–562
- Taiwan Agrochemical Industrial Association (1996) Domestic manufacturers production and sale of pesticides in 1995. Taipei, Taiwan
- Tilak KS, Veeraiah K, Bhaskara Thathaji P, Butchiram MS (2007) Toxicity studies of butachlor to the freshwater fish *Channa punctata* (Bloch). *J Environ Biol* 28:485–487
- Vaughan DA, Lu BR, Tomooka N (2008) The evolving story of rice evolution. *Plant Sci* 174:394–408
- Vonesh JR, Buck J (2007) Pesticide alters habitat selection in gray treefrogs. *Oecologia (Berl)* 154:219–226
- Wang YS, Jaw CG, Tang HC, Lin TS, Chen YL (1992) Accumulation and release of herbicides butachlor, thiobencarb, and chlomethoxyfen by fish, clam, and shrimp. *Bull Environ Contam Toxicol* 48:474–480
- Wells KD (2007) The ecology and behaviour of amphibians. The University of Chicago Press, Chicago
- Wilson AGE, Takei AS (2000) Summary of toxicology studies with butachlor. *J Pestic Sci* 25:75–83
- Wu CS, Kam YC (2009) Effects of salinity on the survival, growth, development, and metamorphosis of *Fejervarya limnocharis* tadpoles living in brackish water. *Zool Sci (Tokyo)* 26:476–482
- Yang YR (1998) A field guide to the frogs and toads of Taiwan. Chinese Photography Association, Taipei (in Chinese)
- Yin LC, guo HR, Zhang SC, Wang J (2007) Study on the acute toxicity and genotoxicity of herbicide butachlor in flounder, *Paralichthys olivaceus*, and flounder gill (FG) cells. *J Ocean Univ Qingdao* 37:167–171
- Yin XH, Li SN, Zhang L, Zhu GN (2008) Evaluation of DNA damage in Chinese toad (*Bufo gargarizans*) after in vivo exposure to sublethal concentration of four herbicides using the comet assay. *Ecotoxicology* 17:280–286
- Yu KN, Qi CJ, Tang K (1993) Relationship between degradation rate of butachlor and conditions of paddy fields. *Acta Sci Circumstantiae* 13:169–173
- Zug GR, Vitt LJ, Caldwell JP (2001) Herpetology. Academic Press, New York