



Toxicity impact of butachlor on the development of green toad *Bufo viridis*

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Butachlor is the most commonly used herbicide on rice paddy fields in Asian countries. Paddy fields are habitats commonly used for reproduction by many species of amphibians. We examined the effects of butachlor on *Bufo viridis* development. Amplectant pairs of *B. viridis* were kept in the laboratory in an aquarium overnight, and their spawn collected the next morning. Eggs were exposed to butachlor at different concentrations (0.1, 0.2, 0.8, 1, 2, 3.5, 7, 14 µg/l), all lower than the concentration used in the area (rice paddy fields) by farmers. Eggs were allowed to develop to Gosner stage 24 and their developmental patterns compared with those reared in normal conditions (control). In order to examine whether jelly coats have a significant role in the protection of developing eggs from the toxin, another group of eggs were de-jellied and treated in the same procedure. The LC50 value of butachlor was calculated as 14 µg/l and 7 µg/l after 96h for jellied eggs and de-jellied eggs respectively. Butachlor led to a range of external and internal body malformations. Butachlor concentrations of 2 µg/l, 7 µg/l and 14 µg/l reduced embryonic growth and development. A high mortality rate and both internal and external abnormalities were observed at lower concentrations than used in fields, suggesting that butachlor can have significant negative effects on amphibians where this herbicide is used.

Key words: amphibian; *Bufo viridis*; butachlor; embryo; embryonic development; de-jellied egg

INTRODUCTION

The decline of amphibian populations has become more dramatic in the last three decades (Pechmann & Wilbur, 1994; Beebee & Griffiths, 2005; Hoffman et al., 2010; IUCN, 2015). A recent report indicates that about 40% of amphibian species are threatened with extinction, more so than any other vertebrate class (Hoffman et al., 2010).

There are many factors that are influencing amphibian declines, including habitat loss, predation, competition, increasing doses of UV radiation as a result of atmospheric ozone degradation, infectious diseases, and pollution (Collins & Storfer, 2003; Beebee & Griffiths, 2005; Rohr et al., 2008). About 8% of threatened amphibian species are reported to be as a result of pollution (Alford, 2010). Human activities such as agricultural practices are believed to be among the causes of natural environmental contamination (Vos & Chardon, 1998; Lehtinen et al., 1999; Relyea, 2005; Johansson et al., 2006; Gurkan & Hayretdah, 2012). The high effectiveness of pesticides in controlling pest species both in aquatic and terrestrial environments has had a great role in promoting farm production and therefore improving human health (Jones, et al., 2010). Although the benefits of pesticides are well known and

documented, there is less information about the possible side effects of pesticides on non-target organisms. There are many research projects around the most commonly used pesticides, but our knowledge is far from being complete (Fleeger et al., 2003; Forson & Storfer, 2006; Sparling & Feller, 2009; Egea-Serrano et al., 2012).

During agricultural activities, many chemical components are used that can have a negative impact on aquatic and terrestrial ecosystems (Van Dam et al., 1998; Garcia-Munoz et al., 2009; Emurotu & Anyanwu, 2016). Amphibians are under greater risk of chemical exposure than other vertebrates, as in many cases they use standing, temporary or shallow waters around agricultural fields for their reproductive activities and spawning (Duellman & Trueb, 1994; Tyler, 1994; McDiarmid & Altig, 1999; Rowe et al., 2003). They are highly sensitive and therefore vulnerable to chemical pollutants during their aquatic embryonic and larval stages (DeYoung et al., 1996; Murphy et al., 2000). As most amphibian life cycles encompass both aquatic and terrestrial environments, and amphibians have permeable and sensitive skin, they may be good bio-indicators of changing environments (Wyman, 1990; Lips, 1998). Almost all amphibian species in temperate regions spawn at the time of year when pesticides are being used on agricultural land for controlling fungi,

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weeds, insects or other pests. These applications bring amphibians into high risk and vulnerability to the toxicity of the pesticides (Greulich & Pflugmacher, 2004; Pašková et al., 2011).

Previous studies have shown that some pesticides applied in agricultural activities have had an impact on the decline of amphibian populations (Davidson et al., 2001; Sparling et al., 2000; Davidson, 2004; Sparling & Fellers, 2007; Kang et al., 2008; Gill & Garg, 2014). Amphibians are known to be very sensitive to teratogenic effects by pesticide exposure including myoskeletal malformation, tail deformation and limb mal-differentiation (Fort et al., 2004a, b; Bacchetta et al., 2008). Nervous system and epidermal defects, oedemas, gut malformations (Degitz et al., 2003; Robles-Mendoza et al., 2009), high embryonic and larval mortality rate, extended hatching time, and growth retardation are among the other recognised teratogenic effects (Vismara et al., 2000, 2001a,b). Further studies show that a number of agrochemicals reduce the activity of the immune system of some organisms and cause amphibian decline by facilitating disease emergence (Rohr et al., 2008).

Although pesticide consumption in a number of countries in Europe, North America and Japan is decreasing, being restricted or even banned (EU Pesticides Database, 2016), global use of pesticides has increased 50 times since 1950 (PAN Germany, 2012). Butachlor (N-butoxy-methyl-2-chloro-2, 6'-diethyl-acetanilide) is the most commonly used herbicide on rice paddy fields in tropical and subtropical areas in South America, Africa, and Asia, specially throughout south east Asia. The information available in south and far east Asia shows that in the Philippines, Korea, Thailand, China, Taiwan, Japan and some other countries in the region, farmers use high concentrations of butachlor as an herbicide, either alone or in a mixture with other herbicides (Naylor, 1996). It is used to control a wide range of grasses and some broadleaf weeds in paddy fields (Senseman, 2007; Abigail, 2015) and non-paddy fields/dry-seeded rice fields (Jiang et al., 2014). Butachlor is thought to be an inhibitor for synthesising long chain fatty acids (Senseman, 2007). This pesticide is also genotoxic to toads where it causes DNA strand break induction in erythrocytes.

Paddy fields are one of the typical habitats that Bufonid species commonly use for spawning (Liu et al., 2011). Although there are studies that have investigated the toxic effects of butachlor on the embryos and tadpoles of different anuran species (Geng, et al., 2005b; Liu et al., 2011, Li, et al., 2016), the effects of butachlor on the embryos and tadpoles of the green toad *Bufotes viridis* (Laurenti, 1768) are unknown. Liu et al. (2011) assessed both the acute and chronic effects of butachlor at different concentrations on Gosner (1960) stage G26 tadpoles of a Ranid species *Fejervarya limnocharis*. At concentrations low enough to allow tadpoles to grow ($\leq 0.2\text{mg/l}$), tadpoles grew to metamorphosis at a similar size to controls but took significantly longer. At higher concentrations ($\geq 0.4\text{mg/l}$) survival was zero, with the time to death dependent on the concentration. Li et al. (2016) used *Xenopus laevis* embryos and exposed

them to different butachlor concentrations ranging from 0.313 mg/l to 5 mg/l . They showed that butachlor caused developmental toxicity (e.g. eye malformation, precardial oedema, tail curvature) and thyroid endocrine disruption. Geng et al. (2005b) reported that the acute toxicity of butachlor to *Rana guentheri* tadpoles was high ($\text{LC}_{50} = 0.74\text{ mg/l}$, 96 hrs). Yin et al. (2008) reported high levels of DNA damage in tadpole erythrocytes (*Bufotes bufotes gragarizans*) exposed to sublethal concentration of butachlor ($\text{LC}_{50} = 1.32\text{ mg/l}$, 96 hrs).

The aim of this study was to determine the effects of butachlor on the development and growth of *B. viridis* embryos under laboratory conditions. We concentrated on *B. viridis* as this species is the only widely distributed species among amphibians in our area (southern Iran) where spawning happens solely in temporary pools including paddy fields. *Bufotes viridis* is a species complex with morphologically at least 14 related species widely distributed in many European, Asian and north African countries (Stock et al., 2006; Frost, 2016). The status of the green toad in southern Iran, Fars Province (study area) is unclear, and needing further research. One of the *Bufotes surdus* subspecies (*Bufotes surdus annulatus*) and probably *Bufotes variabilis*, which is retained here as *B. viridis*, are the two members of the *B. viridis* species group found in our study area (Fakharzadeh et al., 2014; Parvaresh, et al., 2016).

We hypothesised that butachlor has negative effects on the development and growth of embryos. In order to examine whether jelly coats have a significant role in development and in the response to butachlor, groups of eggs were de-jellied and treated with the same procedure as intact eggs. It is suggested that with removing the jelly coat, we are actually removing a protective barrier to chemical absorption: therefore, we hypothesised that butachlor would have more of an effect on de-jellied eggs compared to intact jellied eggs.

MATERIALS AND METHODS

Ethics Statement

The work carried out conformed to national legislation and guidance for animal welfare and conservation (BHS Ethics Policy and UK legislation). Our studies of the effects of butachlor on embryonic development did not proceed past the stage of onset of feeding (defined as Gosner stage 25) as required for unlicensed experiments (Animal – Scientific Procedures – Act, UK). We use the term 'embryo' rather than 'larva' for all these stages. Sample sizes were kept to a minimum and the experiments had no impact on the conservation status of wild populations of *B. viridis*.

Egg collection, incubation and hatching assessment

The green toad, *B. viridis*, is included in the conservation category of Least Concern, but populations of this species are decreasing (IUCN, 2015). This species is water-dependent during its reproductive season and stays in water for a long time. This toad generally uses slow-flowing and standing waters, seasonal ponds, and shallow pits filled with water for egg laying (Kinzelbach

& Kasperek, 1992). The eggs and tadpoles of this species can be observed in the seasonal ponds around agricultural fields and inside the fields.

Seven pairs of adult *B. viridis* were caught in the amplexant state in March-May 2013 in a seasonal pond at Bajgah field station in Fars Province, Iran. The toad specimens were brought to the laboratory in plastic containers and transferred to aquaria filled with aerated tap water to a shallow level. Each pair of toads was kept separately in an aquarium in the laboratory overnight. Their spawn was collected the next morning and transferred to polypropylene containers filled with de-chlorinated and aerated tap water. Mixed clutches of fertilised eggs (three clutches in total; mean clutch size = 6643 ± 1855) from different parents were used for all experimental treatments (parents were released back in the wild after spawning). The temperature, pH and dissolved oxygen levels of the water were measured daily with an Elmetron meter.

Experimental design

Butachlor (EC 60%, Karkhanejat Giahi Tehran, Iran, Butachlor) used in this study was considered as a toxic substance. A stock solution was prepared by dissolving the butachlor in dechlorinated tap water. A preliminary experiment was carried out with butachlor by using one of the clutches of the eggs. A broad range of butachlor concentrations was used to select the most suitable concentrations, and to avoid testing non-necessary concentrations for the actual experiment. Batches of 150 fertilised *B. viridis* eggs starting from Gosner stage 5 were transferred to plastic containers (25cm W. \times 45cm L. \times 3cm H.), each containing different concentrations of butachlor solutions (0.1, 0.2, 0.8, 1, 2, 3.5, 7, 14 $\mu\text{g/l}$) as well as to the control container, with total volume of 2L water. The selected butachlor concentrations were chosen based on LC50 determination, and were lower than the concentration used in the study area in the paddy fields in Iran (4.8 mg/l). Table 1 presents a list of the treatments with details for each examination carried out in this study.

Water in each container was aerated by an air pump. The developing embryos were kept in identical conditions of light (12h. light, 12h. darkness), temperature ($20^\circ\text{C} \pm 2.0$) and pH (7.71 ± 0.15). *Bufo viridis* embryos were exposed to the different butachlor concentrations from G5 through to stages G23/24, and their developmental patterns were compared with the patterns exhibited by the control group. No pesticides were applied to the eggs in the control group and they were allowed to develop under identical environmental conditions as the eggs in the experimental groups. For the treatment groups, embryos were exposed to Butachlor just once at the beginning of the experiment, to each of three ($n=150$ per replicate).

The time and stage at which hatching occurred in each treatment was noted, and mean hatching time and stage were calculated both for the experimental treatments and the controls according to Gosner's (1960) developmental table. Hatching assessment was started after the first embryo hatched and hatching was observed regularly after that. Hatching time was calculated as the time when 50% of embryos were hatched in experimental and control groups.

In order to examine whether jelly coats have a significant role in development and in the response to butachlor, other groups of eggs ($n=150$ for each treatment) were de-jellied and treated to the same procedures as the intact eggs. The developmental patterns of the de-jellied eggs exposed to the butachlor were compared with the patterns found in normal conditions of de-jellied eggs (control). Eggs were de-jellied using cysteine solution (Viso & Khokha, 2012). To make the solution, 0.24g 20 M Tris buffer was dissolved in 80ml distilled water, and 2g cysteine was added, then the solution was conveyed to pH=8.1. The pH was adjusted to the desired value by using acid (HCl) and base (NaOH) solutions and by using pH probe.

Among the various butachlor concentrations tested in this study, the concentration at which 50% of the embryos in a container died by 96h was determined as the LC50. Those eggs that did not develop to further

Table 1. List of the treatments with details for each examination carried out in this study. Apart from the number of eggs used for experimental treatments, indicated in the table below, another 300 specimens in total were used for control treatments (jellied and de-jellied). G= Gosner stage

No	Type of treatment	Total number of specimens used	Butachlor concentrations ($\mu\text{g/l}$)	Developmental stages exposed to butachlor	Developmental stages examined	Examined characters
1	Embryo growth and developmental progress	2700	2, 7, 14	G5-G24	G10-G24	SVL, TAL, TL against time; Stages against time
2	Biometry of embryos	3600	0.8, 1, 3.5, 7	G5-G24	G24	SVL, TAL, TL
3	Mortality rate	1800 plus specimens reused from treatment number 1,2	0.1, 0.2, 0.8, 1, 2, 3.5, 7, 14	G5-G24	G5-G24	Dead and abnormal specimens
4	Morphological assessment	Specimens reused from treatment number 1	2, 7, 14	G5-G24	G10-G24	External organs including tail, trunk and head morphology
5	Histological assessment (sections)	Specimens reused from treatment number 1	2, 7, 14	G5-G24	G20	Internal organs including digestive tube; kidney and muscles

embryonic stages, that stopped their development and turned from black to white, or became motionless because of movement malformations were counted as dead samples.

Embryo growth and developmental progress analysis

To study the growth and developmental progress with time of embryos, we chose three of the concentration series (2µg/l, 7µg/l, and 14µg/l). For each concentration treatment examined ten developing samples at each stage (G10-24) were fixed in Bouin's solution (n=150). The collecting and fixation were carried out for jellied and de-jellied groups, as well as for the control jellied and de-jellied groups. Observations of developmental progress were made for about 140h in total and the curves for developmental progress with time were then drawn. Total length (TL) of fixed samples both in jellied and de-jellied groups at different times were also measured, and the curves for embryonic growth were drawn and analysed for the different treatments and the control.

Biometry analyses of embryos

Four embryos at stage G24 reared in each of four additional experimental tanks with concentrations 0.8, 1, 3.5, and 7µg/l of butachlor, as well as the control group, were randomly selected three times per treatment. After fixing the embryos in Bouin's solution, biometric measurements of the embryos (Altig & McDiarmid, 1999), were carried out immediately after the samples were stored in 70% alcohol. Snout-vent length (SVL), tail length (TAL) and total length (TL) of the embryos were measured to the nearest 0.01 mm with a digital calliper. Histograms for each character in both jellied and de-jellied groups for different concentrations were then drawn.

Mortality percentage of embryos

During the developmental pattern experiments a number of embryos in different groups (0.1, 0.2, 0.8, 1, 2, 3.5, 7, 14µg/l) became abnormal and did not continue to develop: embryos turned white in colour and showed abnormalities in body shape. To determine the embryonic mortality percentage, after each stage of development in each treatment, embryonic mortality was estimated. The final mortality percentage was calculated by adding together all mortality from all stages in each treatment. The mortality percentage in each treatment was estimated up to stage G24.

Morphological and histological assessment of embryos for abnormalities

During the developmental pattern experiments, fixed embryos at different stages (G10-24) were examined separately for a selection of treatment groups (2µg/l, 7µg/l, and 14µg/l) under a binocular microscope and any morphological abnormalities were recorded. Photographs of the embryos at different stages were taken under a binocular microscope with a digital camera. In addition, five surviving embryos were randomly selected at stage G20 from the control and each treatment group. Specimens were preserved in 70%

alcohol after having been fixed in Bouin's solution for 10h. Afterwards, these five embryos were processed through a series of alcohol concentrations (i.e. dehydration), xylene, and paraffin. Serial cross-sections (5-7µm) of whole embryos were obtained from the samples and stained with haematoxylin and eosin (H&E). Microscopic slides were examined under a light microscope and over a range of magnifications, with images being recorded using a camera attached to the microscope (h550L, Nikon, Japan). Any abnormality observed in the internal organs of the treatment samples including digestive tube, kidney and muscles were recorded and compared to the control.

Statistical analyses

All statistical analysis was performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). Differences between the control and treatments were compared by analyses of variance (ANOVA) and regression. Comparisons between the control and experimental groups were performed using Tukey tests. Statistical significance was accepted as $\alpha < 0.05$ (the acceptance level was set at $P < 0.05$).

RESULTS

Hatching time and hatching stage

Hatching of 50% of the embryos occurred by stage G17-18 and 72h (three days) in the control group at lab temperature ($20^{\circ}\text{C} \pm 2.0$). Hatching of 50% of the embryos occurred by stage G19 and 96h (four days) in a selection of experimental groups (2µg/l, 7µg/l, and 14µg/l butachlor), all with intact jelly coats and at the same temperature. Hatching time and stage were not assessed at the lower butachlor concentrations ($\leq 1\mu\text{g/l}$) used in this study.

Embryonic development: response to different concentrations of butachlor

The results for the development of the embryos from stage G5 (the stage at the start of the experiment) in three different butachlor concentrations (2µg/l, 7µg/l, and 14µg/l) showed that as the concentration of the toxin increased, most of the embryos stopped their development at early developmental stages. The rate of development in de-jellied groups was slower than in jellied groups when they were exposed to the same toxin concentration (Fig. 1). Figure 1 also shows that the embryos exposed to the 2µg/l, 7µg/l, and 14µg/l concentrations of toxin mostly stopped developing at stage G19/20 (only de-jellied group), G19 and G18 respectively in both jellied and de-jellied groups. There was no stop in development in the 2µg/l treatment jellied group. Figure 1 shows data only for embryos that survived for the total treatment period. Jellied and de-jellied eggs in the control group continued their growth and development to stage 25 without any problem.

The results for the growth in length of embryos from stage G10 in three different butachlor concentrations (2µg/l, 7µg/l, and 14µg/l) show that with the increasing concentration of the toxin, an increased number of embryos stopped their growth at a shorter length and

the embryos were always shorter in length in the de-jellied group than the jellied group (not shown).

Figure 2 shows the mean sizes of the embryos at different treatments and in both jellied and de-jellied groups. The data are shown only for embryos that survived for the total treatment period. The growth

rate and development of the embryos in the control group were normal, indicating that a density of 150 eggs presented no overcrowding issues. Removing the jelly coat from embryos did not affect the normal pattern of embryonic development in laboratory conditions.

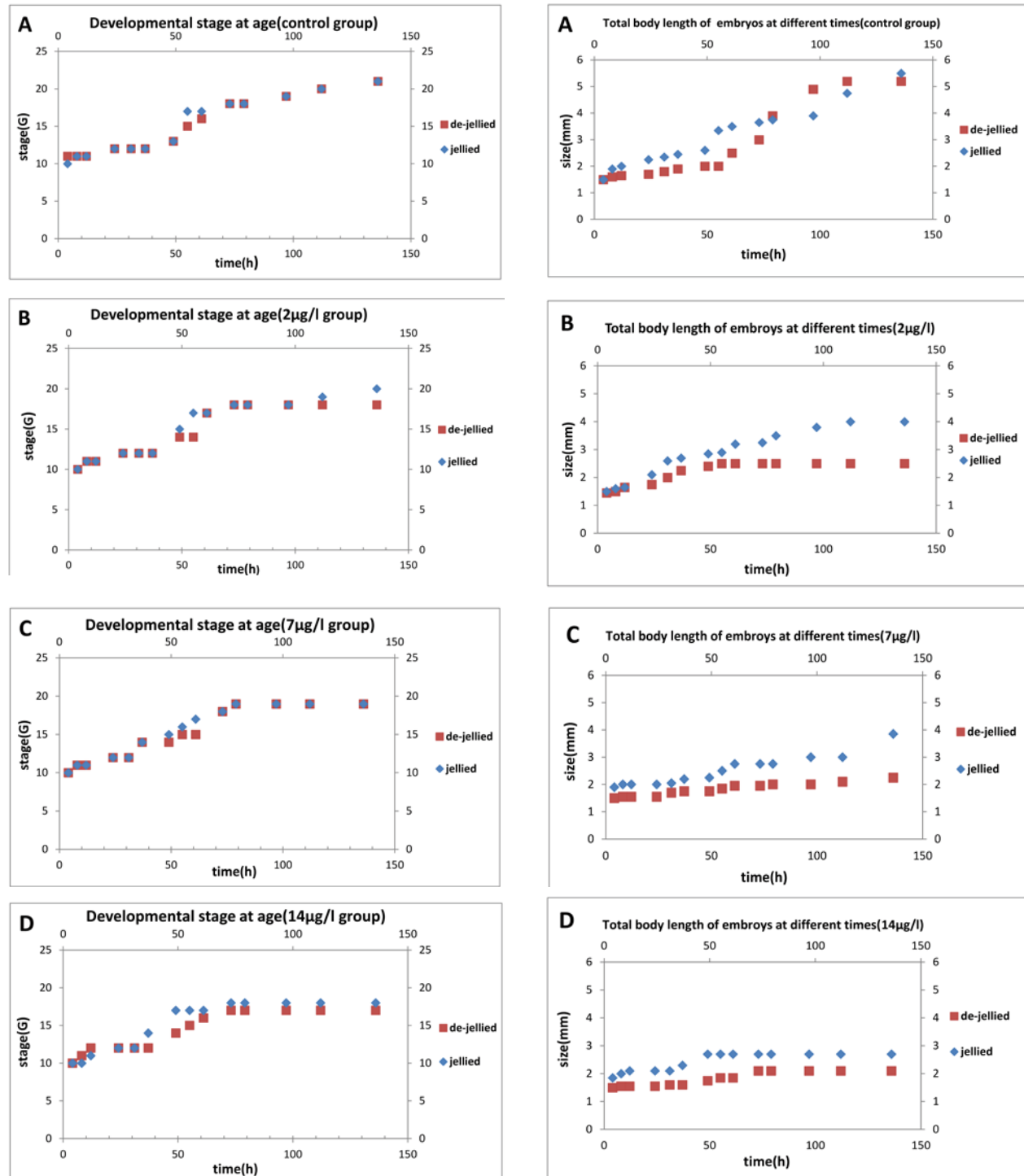


Figure 1. The level of developmental progress with time in control (A) and in different concentrations of butachlor 2 µg/l, 7 µg/l, and 14 µg/l in (B), (C) and (D) respectively and between jellied and de-jellied groups (10 samples were assessed at each sample point, but no variation in stage was observed amongst them).

Figure 2. The relationship between total embryo length and age in control (A) and in different concentrations of butachlor 2 µg/l, 7 µg/l, and 14 µg/l in (B), (C) and (D) respectively as well as between jellied and de-jellied groups ($n=4$ at each sample point).

Biometry of embryos that successfully reached stage G24
Biometric results of embryos at stage G24 after exposure to different concentrations of butachlor (0.8, 1, 3.5µg/l) and of very few survived embryos at stage G24 exposed to 7µg/l (according to the sensitivity of embryos in initial development to this concentration), are shown in figures 3A and 3B in jellied and de-jellied groups respectively.

The Tukey test showed that there were some significant differences ($p < 0.05$) in total length (TL), snout-vent length (SVL) and tail length (TAL) between the control and the four different concentrations of butachlor in both jellied and de-jellied groups in the embryos at stage G24. Details are as follows and the results are simplified in Table 2.

According to the data analysis, as the toxin concentration increased, TL, SVL and TAL were significantly lower in the treatment groups compared to the control group ($p < 0.05$) in both jellied and de-jellied groups. There were no significant differences in TL, SVL and TAL between the jellied and de-jellied groups amongst the experimental groups or compared to the control groups. Results indicated some significant differences ($p < 0.05$) in TL, SVL, TAL of the embryos in the experimental group and between the experimental and the control group in the de-jellied group (Table 2). For every 1µg/l butachlor concentration increased, 0.1mm of the overall length was reduced. The results showed that TL and TAL of the embryos amongst the experimental groups and between the control and the experimental groups were significantly different ($p < 0.05$) in the jellied group (Table 2). For every 1µg/l butachlor increased, 0.6mm SVL and 0.9mm TAL were reduced.

LC50 value

LC50 values for a 96h exposure period in jellied and de-jellied groups of *B. viridis* embryos were found at butachlor concentrations of 14µg/l and 7µg/l respectively.

Table 2. Tukey test results in jellied and de-jellied groups showing only significant differences amongst experiments and between control and experimental groups for total length (TL), tail length (TAL) and snout-vent length (SVL) in the embryos at stage G24.

Character		Groups		Sig
De-jellied	TL	3.5µg/l	0.8µg/l	0.001
		3.5µg/l	1µg/l	0.006
		Control	3.5µg/l	0.0001
	TAL	Control	7µg/l	0.014
		3.5µg/l	0.8µg/l	0.0001
		3.5µg/l	1µg/l	0.007
Jellied	SVL	Control	0.8µg/l	0.05
	TL	Control	0.8µg/l	0.001
		Control	3.5µg/l	0.0001
		Control	7µg/l	0.0001
		1µg/l	7µg/l	0.013
	TAL	Control	0.8µg/l	0.013
		Control	3.5µg/l	0.0001
		Control	7µg/l	0.0001

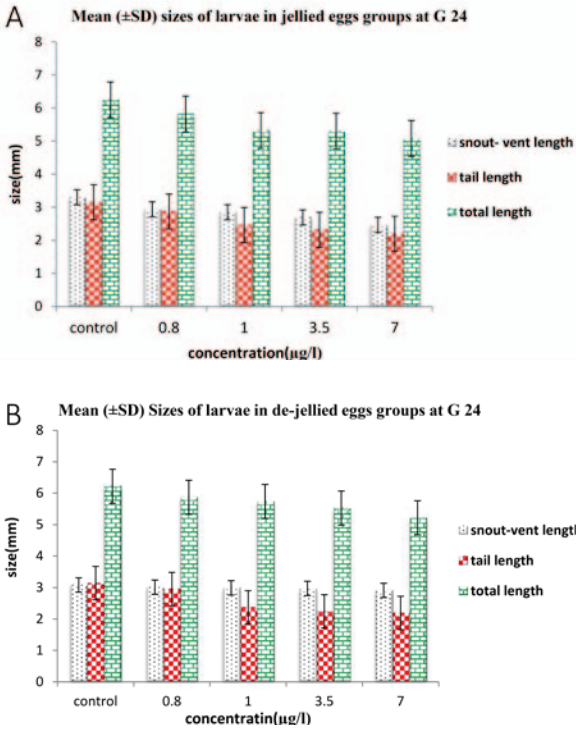


Figure 3. Sizes of embryos (snout-vent length, tail length, total length) at stage G24 after eggs were exposed to different concentrations of butachlor (0.8µg/l, 1µg/l, 3.5µg/l, 7µg/l) and compared to control in (A) jellied and (B) de-jellied groups ($n = 10$ for each treatment).

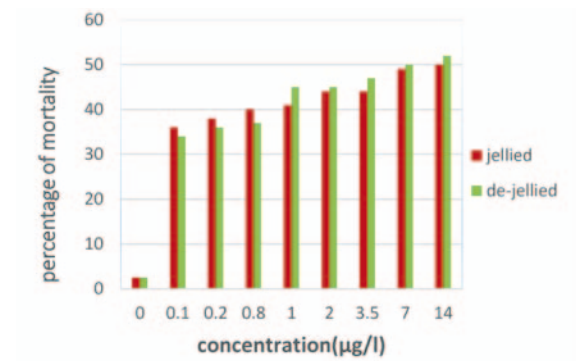


Figure 4. Mortality rate comparison in between controls and eggs exposed to different concentrations of butachlor and between jellied and de-jellied groups. Mortality rate was assessed in each developmental stage from stage G5 to stage G24.

Mortality rate of embryos

In general, embryos at early stages exhibited higher mortality rates than later stages. The mortality percentages of embryos at low concentrations of butachlor (0.1, 0.2, and 0.8µg/l) in the jellied group were slightly greater than in the de-jellied group; but at higher concentrations of butachlor (1, 2, 3.5, 7 and 14µg/l), the mortality percentage of embryos was higher in the de-jellied group than in the jellied group (Fig. 4).

Teratology of butachlor (external morphology and histological section examination)

Figures 5 and 6 show the results for the effects of butachlor toxicity at different concentrations and in both

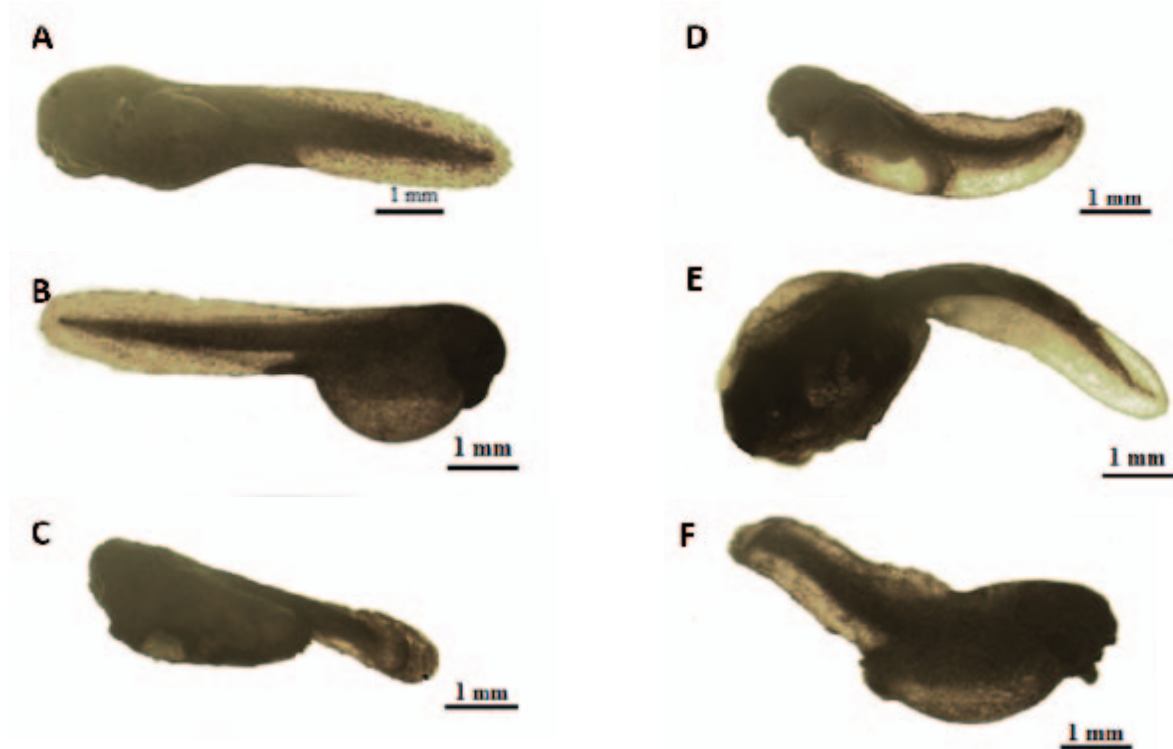


Figure 5. External morphology of embryos selected at stage G24 in the comparison between control (A) and the eggs exposed to different concentrations of butachlor (2 μ g/l, 7 μ g/l, and 14 μ g/l) showing various abnormalities (B) abdominal oedema, (C) Tail malformation, (D) tail and trunk curvature upward, (E) tail curvature sideward, (F) mouth and tail malformations ($n=5$ for each treatment).

jellied and de-jellied groups in *B. viridis* from external (at stage G24) and internal (at stage G20) views respectively. Different types of malformations including abdominal oedema (55%), axial malformations (trunk and tail curves, 20% and 25% respectively) and deformities in mouth parts were observed at stage G24 exposed to 2 μ g/l, 7 μ g/l, and 14 μ g/l concentrations of butachlor (Fig. 5). The results from tissue examination for the impact of 7 μ g/l butachlor on the internal organs at stage 20 showed that the intestine and digestive component's quantity including digestive content in the experimental group was about 50% smaller than in the control group, and a significant part of the trunk space appeared empty of tissue because of trunk inflammation and reduction in the digestive component. Reduction of the number of renal tubules in one side of the trunk was also observed (Fig. 6A, 6B). Considerable reduction of muscle (50%) as well as curvature in dorsal and ventral fins of the tail (25%) was also observed in the experimental group in comparison to the control group (Fig. 6C, 6D).

DISCUSSION

Among the different kinds of pollution, pesticides are partially responsible for amphibian declines (Lehman & Williams, 2010). Although the data for global consumption of pesticides in general are available (PAN Germany, 2012), the quantitative data for each individual pesticide, including butachlor, are incomplete.

Butachlor is applied to rice paddy fields at the time of rice transplantation, as a means of controlling weeds. This

is also the time when the fields are flooded, which can act as a stimulus for breeding in local amphibian populations (Liu et al., 2011). Previous work on the effects of butachlor on amphibians has demonstrated different types of toxic effects on several species (Geng et al., 2005 a,b; Liu et al., 2011). Liu et al. (2011) examined the acute and chronic effects of butachlor at different concentrations on stage G26 tadpoles of the Ranid *Fejervarya limnocharis*. Liu et al. (2011) did not, however, examine effects on pre-hatching stages. In our experiments, we exposed the spawn of the *Bufo viridis* to butachlor from the start of development, and also assessed the potential role of the jelly coat in limiting the effects of butachlor.

A preliminary experiment was carried out with butachlor at higher concentrations than were ecologically relevant, similar to those used by Liu et al. (2011), but all specimens died soon after treatment. Our results with lower concentrations showed that the butachlor treated embryos took longer to hatch and that embryos hatched at stage G19 (about two stages later than the normal stage), whereas embryos in the control group hatched at stage G17. The delay in hatching may be due to the late development of the hatching gland cells, which are responsible for secreting enzymes and lysing the vitelline membrane and jelly coat (Nokhbatolfoghahai & Downie, 2007). Andrews & George (1994) reported that increasing concentrations of diazinon (an insecticide) cause a decrease in the hatching rate of the eggs of *Polypedates maculatus*.

In our experiments, the toxicity of butachlor had less impact on the jellied groups than de-jellied groups, and

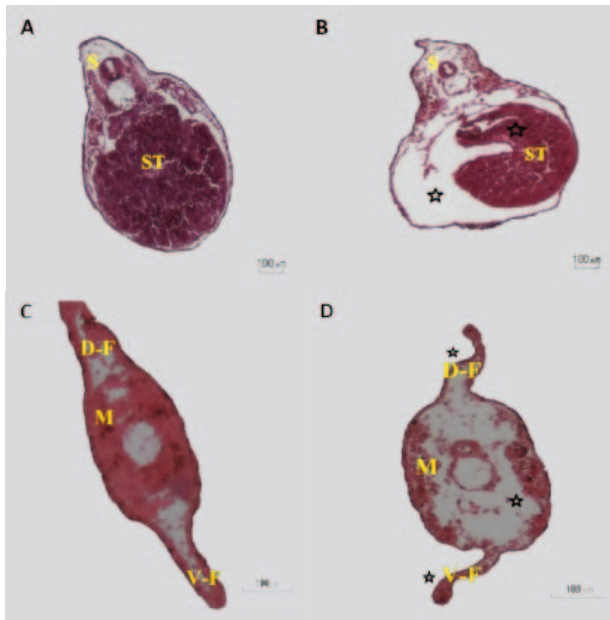


Figure 6. Transverse sections of embryos at stage G20 (A) trunk region, jellied control- group, (B) trunk region, embryo treated with 7µg/l butachlor (jellied), (C) tail region, jellied control group, (D) tail region, embryo treated with 7µg/l butachlor. DF= dorsal fin, M= muscle, RT= renal tubules, S= spinal cord, ST= stomach, VF= ventral fin (deformation of body and tails of embryos showing by asterisks), H&E staining.

LC50 occurred at a higher concentration (14µg/l) in jellied groups compared to de-jellied groups (7µg/l). Pauli et al. (1999) exposed embryos and newly hatched embryos of four Ranid species (*R. pipiens*, *R. clamitans*, *R. catesbeiana* and *R. sylvatica*) to an insecticide (Mimict 240 LV) and concluded that egg jelly increases resistance and protects embryos from the insecticide in high concentrations. We showed that the jelly coat can protect the embryos from the toxic effects of low levels of butachlor, but has only a limited effect when higher concentrations of butachlor are applied. Although there is no report of the presence of eggs without a jelly coat in nature, the thickness and compositions of jelly coats are reported to differ among amphibian species (Duellman & Trueb, 1994), and this may give species different levels of protection against pollution (Marquis, 2006).

Liu et al. (2011) examined the impact of butachlor on an Asian frog *Fejervarya limnocharis* and reported LC50 as 0.87mg/l after 96h exposure to the toxin, which was much lower than the 4.8mg/l recommended application rate. Liu et al. (2011) used a range of butachlor concentrations (0.025, 0.05, 0.1, 0.2, 0.4, 0.8mg/l). The concentrations they used are much higher than in our experiment. Li et al. (2016) examined the impact of butachlor on *Xenopus laevis* and reported LC50 as 0.96 mg/l after 96h exposure to the toxin. One possible explanation for the different values of LC50 is that there is high variation in toxicity resistance between different anuran species (Geng et al., 2005a). In this comparison, the larvae of the species (*F. limnocharis*) that Liu et al. (2011) used and the embryos of the species (*X. laevis*) that Li et al. (2016) used have a higher resistance to butachlor than *B. viridis*.

Another suggestion for the difference may be because of differences in the butachlor manufacturer we used.

There was a negative relationship between butachlor concentration and the size of the embryos (Fig. 3). We also found that where significant differences in TL occurred, there were also significant differences in TAL but not SVL. Jung & Jagoe (1995) found that maximal swimming speed is positively correlated with a tadpole's total length. They concluded that swimming performance is reduced by small body size. Parichy & Kaplan (1995) examined morphology-performance in *Bombina orientalis* and concluded that larval amphibian vulnerability has a negative correlation to the length of the tail. Our data showed that the length of the tail contributes significantly to the total size of the embryos, which means that a longer tail is likely to have a significant effect on increasing swimming speed. Butachlor, in the higher concentrations that we used, inhibited embryonic growth probably because of its extensive side effects on tail structure and function. According to our results on tail structure, butachlor did not only affect tail length, but also tail morphology and internal tail structure. As we showed in Figure 6, butachlor has impacted on tail muscle development and has reduced muscle formation significantly (by about 50%), which would probably have a high impact on embryonic speed and performance.

The abnormality of the tail, especially curvature, was amongst the highest rate of abnormalities found. The reason may be because the tail in amphibians, including *B. viridis*, has a simple structure and the vertebral column does not extend to the tail. Therefore, the tails are more susceptible in the face of environmental change than other embryonic organs. Such plasticity, in most cases, plays a positive role as an adaptation for new conditions. However, under the presence of toxins such as butachlor, a change in tail structure occurs in such a way that the normal tail deviates from its straight form, which is a negative consequence.

CONCLUSION

This study illustrates that environmental pollutants can have complex effects that are not only concentration-dependent, but vary between different species. In fact, since butachlor (a widely-used herbicide) has environmental toxicity for aquatic animals including amphibians, farmers and growers are advised to replace butachlor (used in the first week after transplanting to eliminate weeds in rice) with an alternative non-chemical/biological control if applicable. Herbicide mixtures with fewer side effects on non-target organisms are also suggested. Removing toad spawns from paddy fields and transferring them to a secured temporary pool before butachlor spraying is highly recommended.

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