

SHORT COMMUNICATION

Acute and chronic toxicity of four commonly used agricultural pesticides on the Asian common toad, *Bufo melanostictus* Schneider

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Abstract: Laboratory and field studies provide evidence that pesticides may play a role in population declines, range reductions and species extinctions of amphibians. The present study examined the acute and chronic toxicity of four commonly used agricultural pesticides, chlorpyrifos, dimethoate, glyphosate and propanil on the survival, growth and development of malformations in the Asian common toad, *Bufo melanostictus*, under laboratory conditions. The 48 hour LC₅₀ values of the chemicals were within the Pesticide Area Network (PAN) specified limits, except for propanil, which was less than the PAN specified value. Acute exposure to high concentrations of propanil may have a high direct toxic effect on the Asian common toad. The survival of the tadpoles with chronic exposure to ecologically relevant doses of the four pesticides was significantly reduced (survival in chlorpyrifos 39%, dimethoate 41%, glyphosate 36% and propanil 40% in the highest concentration) than in the control group (93%). Exposed tadpoles took more time to metamorphose but were larger in size than the control tadpoles. They also developed malformations at high frequencies (chlorpyrifos 30%, dimethoate 25%, glyphosate 35% and propanil 15% in the highest concentration). Malformations were mainly axial, including kyphosis (hunched back) and scoliosis (curvature) while skin ulcers and oedemas were also observed. Severe limb malformations such as extra or missing limbs as reported for other species of amphibians exposed to pesticides were not observed in the Asian common toad. None of the tadpoles in the control group had any malformations. Glyphosate exposed metamorphs recorded the highest mortality and malformations at high concentrations (1.0 ppm). However, a profound toxic effect was observed in chlorpyrifos exposed group even at low concentrations (0.1 ppm). The study shows that exposure to commonly used agrochemicals poses serious risk to amphibians in Sri Lanka and highlights the importance of investigating the level of agricultural pesticides in freshwater ecosystems and their effect on non-target organisms.

Keywords: *Bufo melanostictus*, chlorpyrifos, dimethoate, glyphosate, malformations, propanil.

INTRODUCTION

Amphibians are the most threatened of all vertebrate taxa in the world with more than 32% of the species threatened and more than 43% experiencing some form of population decline (Stuart *et al.*, 2004). A number of factors may affect amphibian populations, including habitat degradation, diseases and pathogens, invasive predators and competitors, depleted ozone and the presence of pollutants (Blaustein & Wake, 1995; Alford & Richards, 1999; Cowman & Mazanti, 2000; Sparling *et al.*, 2001; Blaustein *et al.*, 2003). Pesticides widely used in agriculture have received the attention of many ecologists to study their toxicity to non-target organisms. Pesticides can have many adverse effects on amphibians, which can be direct or indirect at lethal or sublethal level. The sublethal effects to pesticides on amphibians include abnormal growth, development and behaviour, which in turn may alter susceptibility to predation and competition, and decrease reproductive success (Bridges, 1999; Sparling, 2003; Boone & Semlitsch, 2002; Reylea, 2005). The effect of pesticides have been widely studied in ranids such as the northern leopard frog (*Rana pipiens*), the mink frog (*R. septentrionalis*), the green frog (*R. clamitans*), the bullfrogs (*R. catesbeiana*) and the foot hill yellow legged frog (*R. boylii*) (Berril *et al.*, 1994; Bridges & Semlitsch, 2000; Christin *et al.*, 2003; Hayes *et al.*, 2003; Sparling & Fellers, 2007; 2009). Hind limb deformities in wild caught green frogs, northern leopard frog, American toad (*Bufo americanus*) and bullfrogs

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have been observed in pesticide contaminated areas in the St. Lawrence River valley of Quebec, Canada (Ouellet *et al.*, 1997).

In Sri Lanka, 103 species of anurans and caecillians have been described so far (Manamendra-Arachchi & Pethiyagoda, 2006), which counts for 2% of the world's known anuran species (IUCN, 2006). An assessment completed in 2004 revealed that a total of 34 species of amphibians are known to have become extinct in the world and most of them belong to Sri Lankan amphibian fauna (Manamendra-Arachchi & Pethiyagoda, 2006; Meegaskumbura & Manamendra-Arachchi, 2005). The exact causes of this amphibian crisis or the factors threatening Sri Lankan amphibian populations are not yet identified. Occurrence of malformations in local amphibian populations has been observed in protected areas (Rajakaruna *et al.*, 2007) and subsequent laboratory studies have provided evidence that a larval stage of a digenetic trematode may be responsible for high mortality and malformations (Rajakaruna *et al.*, 2008; Jayawardena *et al.*, 2010). A recent laboratory study provides evidence that pesticides may play a role in amphibian declines, reporting high mortality and malformation in the common hourglass tree frog, *Polypedates cruciger* exposed to pesticides (Jayawardena *et al.*, 2010).

A large amount of agricultural pesticides are used in paddy, coconut and other crops in Sri Lanka and the run-off carries pesticides that might affect aquatic stages of amphibians directly (Pethiyagoda & Manamendra-Arachchi, 1998). The present study explores the acute and chronic effects of four commonly used agrochemicals on the Asian common toad, *Bufo melanostictus* under laboratory conditions. The Asian common toad has an island-wide distribution associated with human habitats and is known to have expanded its natural ranges and established a higher relative dominance following habitat disturbances (Manamendra-Arachchi & Pethiyagoda, 2006). It is known to be less sensitive to the perturbations in the environment and hence is an ideal amphibian species to study the effect of pesticides.

METHODS AND MATERIALS

Egg strands of newly spawned *B. melanostictus* were collected from the Peradeniya University Park and were brought to the laboratory. The eggs were placed in a glass trough containing dechlorinated tap water. Emergent tadpoles from four clutches were used in the study. Tadpoles were fed with ground commercial fish feed three times a day.

Commercial formulations of four commonly used

agricultural pesticides including two organophosphorous insecticides, chlorpyrifos and dimethoate and two herbicides, glyphosate and propanil were used. Active ingredient of these pesticides has been diluted in different solvents/surfactants. Commercially available chlorpyrifos (Lorbosan EC 40[®] or Pattas[®]) is dissolved in xylene (1, 2, 4-Trimethyl benzene) and is diluted in water (20-30 mL/10 L, depending on the target pest) before applying to the field (4 - 8 L/ ha). Dimethoate (Dimethoate EC 40[®]) is dissolved in water and, before application in the field, is dissolved in water once again (20 - 40 mL/10L, depending on the target pest) and applied at 4 - 8 L/ ha depending on the extent of the pest attack. Commercially available glyphosate, known as Roundup[®] or Glyphosate[®], is a combination of glyphosate and the surfactant, polyoxyethylene amine (POEA) and is applied after dissolving in water (i.e. for coconut plantations 1.44 kg/ha). Propanil (3,4 - dichloropropionanilide or 3,4 DPA[®]), dissolved in organic solvents such as cyclohexanone and petroleum solvents, is one of the most common herbicides used in paddy fields in the dry zone of Sri Lanka.

Samples of commercial formulations of the four test pesticides were obtained from the Pesticide Registrar's Office, Peradeniya. The pesticides used in this study are referred to based on the active ingredient (chlorpyrifos, dimethoate, glyphosate and propanil) throughout the manuscript.

Five days post-hatch tadpoles (Gosner stage 25-26; Gosner, 1960) were exposed to high concentration of the four pesticides for 48 h. After a preliminary range finding exposure, a dilution series of 2.5, 2.0, 1.5, 1.0 and 0.5 ppm was used for chlorpyrifos and propanil, and a higher series of 25.0, 18.75, 15.00, 11.25 and 9.50 ppm was used for dimethoate and glyphosate. The pesticides were diluted in dechlorinated tap water, which was also used as the control medium. Lethal concentrations, LC₅₀, LC₁₀ and LC₉₀ (concentration at which 50%, 10% and 90% of tadpoles die, respectively) of the four pesticides were determined. Tadpoles from three clutches were used in the acute exposure studies. Tadpoles were placed in glass tanks (measuring 15 x 15 x 25 cm) containing 2 L of the test solution and the mortality at 48 h after exposure was recorded.

Five days post-hatch tadpoles (Gosner stage 25-26; Gosner 1960) were exposed to ecologically relevant concentrations of the four pesticides in similar glass tanks containing 2 L of the test solution. The tadpoles were exposed to a concentration series of the pesticide similar to the concentrations found in the field. Concentration series of chlorpyrifos was prepared as

0.05, 0.10, 0.25 and 0.5 ppm based on previous reports (Moore *et al.*, 1998; Mazanti *et al.*, 2003). Chlorpyrifos concentrations in small water bodies adjacent to agricultural lands have been estimated to be in the range of 0.1 ppm up to 1.0 ppm (Moore *et al.*, 1998; Mazanti *et al.*, 2003). Concentration series for dimethoate, glyphosate and propanil were 0.25, 0.50, 0.75 and 1.00 ppm. Information from the Pesticide Registrar Office and a recent study on glyphosate concentrations in the field reported as 10 ppm in well water (Navaratne *et al.*, 2009), were considered in preparing the ecologically relevant concentration series of the pesticides. Tests were initiated with 20 tadpoles per tank, exposing them to test concentrations of each pesticide and a control containing dechlorinated tap water. Dechlorinated tap water was used to dilute the test chemicals. The tadpoles were fed with commercial fish feed. Considering the rate of degradation of the chemicals in water and minimizing the stress due to extensive handling of tadpoles, the medium in the tanks was renewed weekly. Survival of the tadpoles was recorded daily until they underwent metamorphosis. Snout-vent length (SVL) of the tadpoles was measured to the nearest 0.01 cm using a digital vernier caliper and the body weight to the nearest 0.001 g was measured at metamorphosis using an analytical balance. The time required for forelimb emergence of half the number of tadpoles in each treatment (TE_{50}) was also recorded. Exposure was repeated using tadpoles from three different egg clutches. All the tanks were kept at room temperature with daytime temperature varying between 27 - 31 °C under a natural photoperiod of approximately 12:12 h.

Malformations were recorded at 10 d and 30 d post-hatch (Gosner stages 27 and 31) and at metamorphosis. The percentage malformation was calculated as the number of malformed individuals at a given age divided by the initial number of tadpoles. The dead malformed individuals were also included in calculations.

At metamorphosis, all the malformed individuals were anesthetized (using tricaine methane sulfonate MS-222) and preserved in 5% formalin. Laboratory rearing of the tadpoles and anesthetizing and killing of the malformed amphibians were carried out according to the protocols approved by the Canadian Council on Animal Care (1993). Malformations were categorized using the Field Guide to Malformations of Frogs and Toads (Meteyer, 2000).

The LC values of the pesticides were calculated using the EPA Probit Analysis Programme Version 1.5 (EPA, 2006) with the pooled data from three egg clutches. Results of the pesticide exposure at ecologically relevant

doses were analysed using MINITAB 14.0 for Windows. An *F*-test showed that at the 0.05 significance level, the variance of the data from three clutches could not be ruled out to be different and hence the data were pooled and analysed. The individual effect of concentration on the survival of the tadpoles was analysed using a chi-square test. A correlation analysis was performed to test the relationship of survival, growth parameters (body weight, SVL and TE_{50} values) and percentage malformations with the concentrations of each chemical. The effect of each pesticide on growth parameters was analysed using one-way ANOVA. The differences in growth parameters and malformations among the four pesticides were compared using an analysis of co-variance (ANCOVA).

RESULTS

The 48 hour LC_{50} values of *B. melanostictus* for the four pesticides, chlorpyrifos, dimethoate, glyphosate and propanil are given in Table 1. LC_{90} and LC_{10} values were also reported with 95% confidence interval (CI) limits. The highest LC_{50} value was reported for glyphosate (45.94 ppm) and the lowest value for propanil (1.46 ppm; Table 1).

Tadpoles exposed to the four pesticides at ecologically relevant concentrations showed a decrease in survival, differences in growth pattern compared to the control and they also developed malformations.

The percentage survival of the exposed metamorphs was reduced compared to that of the control group (93%; Table 2). At the highest concentration of the pesticide (chlorpyrifos 0.5 ppm and 1.0 ppm for others) glyphosate recorded the lowest survival rate (36%) followed by chlorpyrifos (39%) with propanil and dimethoate showing 40% and 41% survival, respectively (Table 2). The decrease in survival in exposed groups was significantly high in all four pesticides at all four concentrations compared to the control group (Chi square test, $p < 0.05$; Table 2). Survival decreased with

Table 1: The 48 hour lethal concentration (at 95% CI) of the four pesticides at five days post-hatch tadpoles of *B. melanostictus*

Pesticide	LC_{10} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)
Chlorpyrifos	1.41	1.47	2.11
Dimethoate	6.04	8.89	13.09
Glyphosate	27.91	45.94	75.64
Propanil	0.95	1.46	2.25

ppm = parts per million

Table 2: Percentage survival of *B. melanostictus* tadpoles and metamorphs exposed to four pesticides at different concentrations

Pesticide	Exposure level (ppm)	Percentage survival (%)		
		10 days post-hatch tadpoles (p)	30 days post-hatch tadpoles (p)	Metamorphs (p)
Chlorpyrifos	0.05	70 (0.000)	65 (0.000)	59 (0.000)
	0.10	59 (0.000)	54 (0.000)	43 (0.000)
	0.25	51 (0.002)	46 (0.001)	40 (0.001)
	0.50	50 (0.008)	44 (0.004)	39 (0.008)
Dimethoate	0.25	65 (0.000)	60 (0.000)	56 (0.000)
	0.50	61 (0.002)	55 (0.001)	51 (0.001)
	0.75	59 (0.002)	56 (0.001)	48 (0.001)
	1.00	50 (0.004)	44 (0.002)	41 (0.003)
Glyphosate	0.25	56 (0.001)	51 (0.000)	50 (0.000)
	0.50	61 (0.001)	56 (0.000)	51 (0.000)
	0.75	55 (0.002)	46 (0.001)	44 (0.000)
	1.00	54 (0.001)	44 (0.000)	36 (0.000)
Propanil	0.25	64 (0.002)	56 (0.000)	50 (0.001)
	0.50	51 (0.001)	44 (0.000)	39 (0.001)
	0.75	56 (0.000)	43 (0.000)	39 (0.001)
	1.00	58 (0.004)	49 (0.001)	40 (0.001)
Control	0	100	99	93

ppm = parts per million

Table 3: Comparisons of mean snout-vent length (SVL) and mean body weight of *B. melanostictus* metamorphs exposed to ecologically relevant concentrations of the four pesticides with those of the control group using one-way ANOVA

Pesticide	Exposure level (ppm)	Mean SVL ± SD (cm)	F	Mean weight ± SD (g)	
				F	
Chlorpyrifos	0.05	0.76 ± 0.05	192.24	0.069 ± 0.02	63.22
	0.10	0.81 ± 0.05	240.98	0.071 ± 0.02	132.58
	0.25	0.92 ± 0.08	213.65	0.086 ± 0.02	125.74
	0.50	1.09 ± 0.01	387.16	0.108 ± 0.02	166.50
Dimethoate	0.25	0.71 ± 0.00	67.16	0.061 ± 0.01	15.26
	0.50	0.78 ± 0.07	78.82	0.067 ± 0.01	28.11
	0.75	0.81 ± 0.07	95.66	0.079 ± 0.01	37.30
	1.00	0.81 ± 0.13	177.46	0.083 ± 0.01	56.85
Glyphosate	0.25	0.77 ± 0.04	79.67	0.060 ± 0.01	27.94
	0.50	0.76 ± 0.02	137.82	0.068 ± 0.02	19.45
	0.75	0.93 ± 0.04	112.39	0.081 ± 0.00	30.70
	1.00	1.12 ± 0.08	180.63	0.083 ± 0.01	47.79
Propanil	0.25	0.78 ± 0.01	91.51	0.060 ± 0.00	31.31
	0.50	0.78 ± 0.05	125.79	0.068 ± 0.01	42.50
	0.75	0.82 ± 0.05	146.99	0.072 ± 0.01	59.53
	1.00	0.98 ± 0.03	199.38	0.073 ± 0.01	39.56
Control	0	0.72 ± 0.04	-	0.054 ± 0.00	-

df = 1; p < 0.001 for all the comparisons; SD = standard deviation

increasing concentration of pesticides (Table 2), but this relationship was significant only in the chlorpyrifos treated group (Pearson Correlation, $r = -0.879$, $p = 0.05$).

In the other three pesticides survival decreased with the concentration but the relationship was not statistically significant (Pearson Correlation, $p > 0.05$).

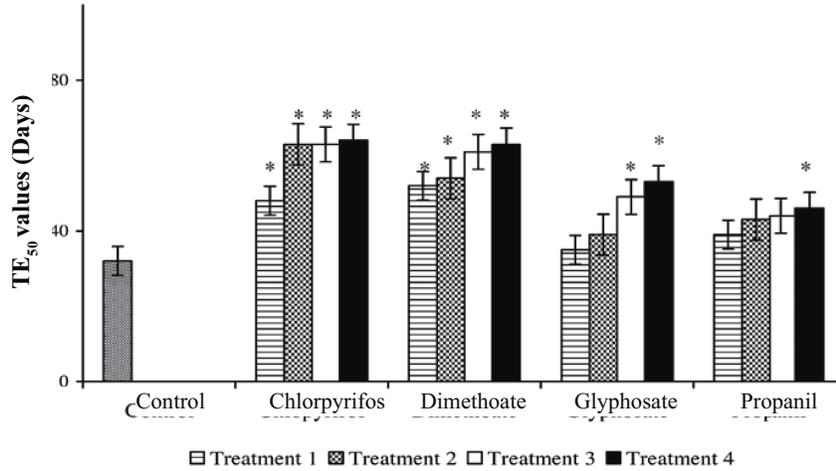


Figure 1: Average TE₅₀ (time required for forelimb emergence in half the number of tadpoles) values of tadpoles of *B. melanostictus* exposed to chlorpyrifos, dimethoate, glyphosate and propanil at different concentrations (exposure levels are given as Treatment 1 = 0.05 ppm, 2 = 0.10 ppm, 3 = 0.25 ppm and 4 = 0.5 ppm for chlorpyrifos, and Treatment 1 = 0.25 ppm, 2 = 0.50 ppm, 3 = 0.75 ppm and 4 = 1.0 ppm for other chemicals). *Represents the TE₅₀ values significant at $p < 0.05$ (ANOVA) compared to the control

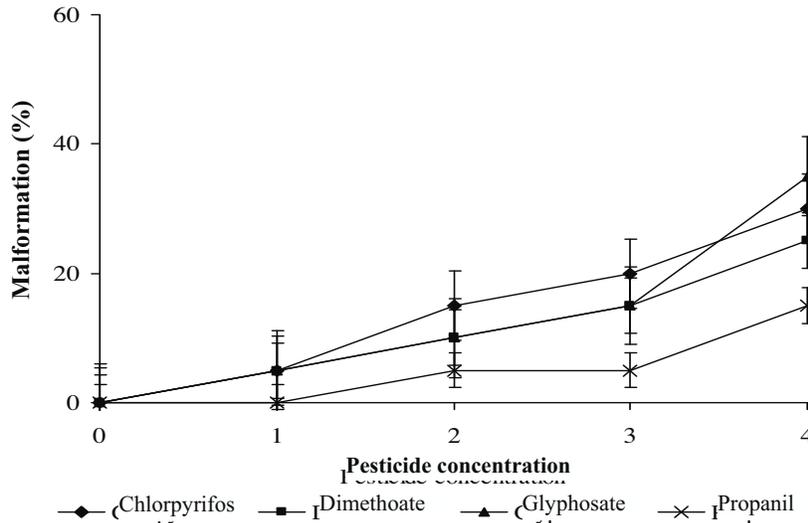


Figure 2: Percentage incidence of malformation observed in 10 days post-hatch tadpoles of *B. melanostictus* exposed to different concentrations of the four pesticides. 0 = control, 1 - 4 denotes the increasing order of the pesticide dose (1 = 0.05 ppm, 2 = 0.1 ppm, 3 = 0.25 ppm and 4 = 0.5 ppm for chlorpyrifos, and 1 = 0.25 ppm, 2 = 0.5 ppm, 3 = 0.75 ppm and 4 = 1.0 ppm for other pesticides).



Figure 3: Composite of malformations observed in *B. melanostictus* exposed to pesticides: A) curvature in the vertebral column (scoliosis), B) hunched back (kyphosis), C) oedema in early stage tadpoles, D) ulcer on the underside of the body, and E) kyphosis in late stage tadpoles

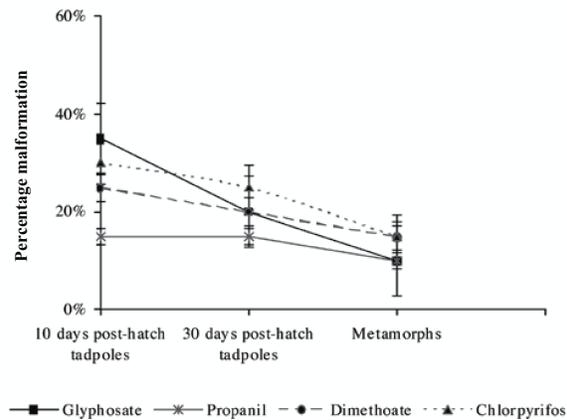


Figure 4: Percentage incidence of malformations at 10 days and 30 days post-hatch tadpoles and metamorphs of *B. melanostictus* exposed to the highest concentration of the pesticide (0.5 ppm for chlorpyrifos and 1.0 ppm for dimethoate, glyphosate and propanil). The percentage malformation was calculated as the number of abnormal individuals (including the dead abnormal ones) at a given age divided by the initial number of tadpoles.

Individuals exposed to the pesticides took more time to metamorphose while showing increases in the growth parameters (SVL, body weight and TE_{50}) compared to the control group (Table 3; Figure 1). Exposed metamorphs were larger than the control tadpoles and showed a significantly high enhancement in growth in all treatments (one way ANOVA, $p < 0.05$; Table 3). The SVL almost doubled in the tadpoles exposed to 1 ppm compared to the control tadpoles (one way ANOVA, $F_{1, 145} = 180.63$, $p < 0.001$). Moreover, growth of the metamorphosed individuals was also affected significantly by the pesticide type (ANCOVA, $p < 0.05$) with chlorpyrifos having the most profound effect followed by glyphosate. The increase in growth was positively correlated with increasing concentration of the pesticides, and the correlation between pesticide concentration and both SVL and weight measurements were significant for all the pesticides (Pearson Correlation, $p < 0.05$).

The average TE_{50} value for *B. melanostictus* was 32 days (range 28- 34 days) in the control, while in exposed groups it exceeded 70 days (range 58-73 days) with chlorpyrifos recording the highest value of 73 days (Figure 1). This lengthening of development period was significantly high for chlorpyrifos and dimethoate exposed groups for all concentrations tested (one way ANOVA, $p < 0.05$; Figure 1). In the glyphosate treated group significant lengthening of the development period was observed with 0.75 ppm (one way ANOVA, $F_{1, 6} = 148.86$, $p < 0.001$) and 1.00 ppm (one way ANOVA, $F_{1, 6} = 201.57$, $p < 0.001$) compared to the control. However, in propanil treated tadpoles a significant difference was observed only in 1.00 ppm treatment (one way ANOVA, $F_{1, 6} = 18.16$, $p < 0.01$) compared to the control.

Varying percentages of malformations were observed in tadpoles and metamorphs in all the treated groups whereas no malformations were observed in the control group (Figure 2). The highest percentage malformation was recorded in the tadpoles exposed to 1 ppm glyphosate (35%; Figure 2). The percentage malformation positively correlated with the concentration of the pesticides and a significant relationship was observed in chlorpyrifos and dimethoate exposed groups (Pearson Correlation, chlorpyrifos; $r = 0.992$, $p = 0.008$, dimethoate; $r = 0.983$, $p = 0.017$, respectively).

Malformations were mainly seen in the spine such as hunched back (kyphosis) and curvature (scoliosis) while oedemas and skin ulcers were also observed (Figure 3). Percentage malformations increased with increasing concentration of the pesticide. During metamorphosis some of the malformations disappeared, especially those in the tail region with the resorption of tail. Hence, the percentage malformation decreased with age (Figure 4).

DISCUSSION

This study shows that the tested concentrations of commonly used pesticides such as chlorpyrifos, dimethoate, glyphosate and propanil have a significant effect on the survival and growth of the common toad. Exposure to pesticides also induced malformations in tadpoles and metamorphs. Based on LC₅₀ values assigned to most sensitive (crabs, mayfly larva, etc.) and least sensitive (trout, amphibians, small mammals, etc.) animal groups, chlorpyrifos and dimethoate are classified as moderately toxic insecticides (0.10 - 10 ppm) while glyphosate and propanil are classified as slightly toxic herbicides (10–100 ppm; Kegley, 2007). The 48 hour LC₅₀ values for *B. melanostictus* were within PAN (Pesticide Area Network) specified limits for all the chemicals except for propanil. The LC₅₀ values of propanil for *B. melanostictus* (1.46 ppm) was lower than PAN reported values (10–100 ppm; Moore *et al.*, 1998; Kegley, 2007), which shows that this species is more sensitive to acute exposure to high concentrations of propanil than the most sensitive animals specified by PAN. Similarly, the LC₅₀ value of propanil for the common hourglass tree frog (Jayawardena *et al.*, 2010), bog frog and clawed toad (*Xenopus laevis*) are also below the PAN specified range (Kegley, 2007) indicating that the effect of propanil is more profound in these four species of anurans, and probably in all amphibians in general.

Exposure to pesticides at the ecologically relevant doses had a significant effect on the survival in a dose dependent manner. The highest mortality (64% at 1 ppm) was observed in glyphosate exposed tadpoles followed by chlorpyrifos (61% at 0.5 ppm). The increase in mortality was significantly high in all the exposed concentrations compared to the control group. However, chlorpyrifos exposed tadpoles had a consistently high mortality throughout the concentration series. Different pesticides have different targets. Some pesticides are endocrine disruptors interfering with hormonal processes. Pesticides, which are enzyme inhibitors, such as acetylcholine esterase (ChE) inhibitors, interfere with the proper functioning of the nervous system (Hayes *et al.*, 2006). Organophosphorous insecticides such as chlorpyrifos and dimethoate are well known ChE inhibitors, which bind with ChE in animals and disrupt their neural functioning (Hayes *et al.*, 2006). Disrupted ChE activity is known to cause increased mortality, reduced activity, increased vulnerability to predators, and reduced growth of tadpoles (Cowman and Mazanti, 2000). Chlorpyrifos particularly disrupts both cholinergic and adrenergic functions in the nervous system (Suwalsky *et al.*, 2003). This might cause a substantial effect on survival even at very low concentrations. Moreover, Suwalsky *et al.* (2003)

demonstrated that high solubility of chlorpyrifos in lipid medium enhances its interference on the neuroepithelial junction. It affects ion transport across the cell membrane and induces changes in the lipid-protein interface of the cell membrane resulting in heavy mortality (Suwalsky *et al.*, 2003).

Glyphosate and propanil are herbicides targeted to disrupt the photosynthetic pathway and therefore considered to have little effect on animals. However, this study shows that these two herbicides significantly affected the survival, growth and the development of malformations in *B. melanostictus*. Acute exposure to high concentrations of propanil and chronic exposure to ecologically relevant doses of glyphosate had a high direct lethal effect on this species. In the commercial formulation of glyphosate the added surfactant (POEA) is known to be more toxic than the active constituent, glyphosate (Wan *et al.*, 1989). Even though the current study did not isolate the impacts of the active ingredient glyphosate and the surfactant, laboratory studies have shown that glyphosate alone has a low toxicity while the surfactant can be highly toxic to a variety of taxa including amphibians (Mann and Bidwell, 1999; Lajmanovich *et al.*, 2003; Howe *et al.*, 2004).

Delayed metamorphosis was observed in all the tadpoles exposed to pesticides with a significant elongation of the growth period (tadpole stage). Delayed metamorphosis due to exposure to chemical contaminants has been reported in tadpoles of several species of amphibians. For example, the tadpoles of *Hyla arborea* upon exposure to dimethoate (Mizgirev *et al.*, 1984) and *Hyla chrysoscelis* exposed to chlorpyrifos and atrazine (Briston, 1998) have taken a longer period to metamorphose. Exposure to chlorpyrifos, dimethoate, glyphosate and propanil significantly lengthen the time to metamorphose in the tadpoles of *P. cruciger* (Jayawardena *et al.*, 2010). Delayed metamorphosis has a crucial impact on amphibian survival, because they often breed in temporary water bodies, particularly in agricultural and human altered habitats where the water bodies can dry up before the tadpoles complete metamorphosis. Longer the development period, higher the chances of exposure to predators, parasites and other etiologies in the environment leading to increased mortality. Therefore, it is likely that exposed individuals are at a disadvantage under natural conditions and may face threats and indirect mortality as a consequence of delayed metamorphosis. All the exposed tadpoles in previous studies that showed lengthening of development period are smaller in size at the time of metamorphosis (Mizgirev *et al.*, 1984; Briston, 1998; Jayawardena *et al.*, 2010). It has also been reported that the tadpoles of

R. sylvatica exposed to glyphosate (Vision®) in a conifer plantation showed a reduction in length and weight, compared to unexposed frogs (Glaser, 1998). In contrast, the metamorphs of *B. melanostictus* were larger than those in the control. Even though exposed tadpoles had a longer tadpole period, larger size at metamorphosis could be an advantage for the tadpoles of *B. melanostictus* as bigger size affects the individual's reproduction (Hayes et al., 2006), survival (Shenoy et al., 2009), immunocompetence (Christin et al., 2003; Carey et al., 2009) and ability to escape from predators and defending territories (Bridges, 1999).

The types of malformations observed were mainly axial. Studies on exposure to agricultural contaminants (Ouellet et al., 1997) reported severe limb deformities such as ectrodactyly (missing digit), polydactyly (extra digit), apody (missing foot), ectromely (missing limb) and polymely (extra limb) in amphibians. In the present study *B. melanostictus* exposed to pesticides did not produce severe limb malformations. However, malformations in the spine, such as kyphosis (hunched back) and scoliosis (curvature) were common. Moreover, skin ulcers and oedemas were also observed. Oedemas caused shifting of the centre of gravity resulting in twisting of the body axis, which in turn affected their swimming behaviour. Affected tadpoles were seen swimming upside down, not being able to balance the body while swimming. These oedemas upon rupturing were often fatal. Most of the malformations in the tail region disappeared as the tail was absorbed and these tadpoles metamorphosed into normal adults. Tadpoles of *P. cruciger* exposed to the same chemicals (Jayawardena et al., 2010) and *R. sylvatica* exposed to glyphosate (Vision®; Glaser, 1998) have shown a similar array of axial malformations where newly metamorphosed frogs had dorsally curved, laterally curved or undulated (like a wave) axial skeleton together with oedemas and abnormal tails. Even though the malformed tadpoles survive to metamorphose under laboratory conditions, they are vulnerable in the wild and are linked with indirect mortality due to increased predation (Sessions & Ruth, 1990). Hence, the mortality of exposed individuals could be much higher if the results are extrapolated into a field context.

The four pesticides tested in the present study are extensively used to control weeds and pests attacking rice, tea, coconut and other crops in Sri Lanka. Recently, an analysis of water samples from a well with possible exposure to agricultural run-off showed that water contained up to 10 ppm of glyphosate (Roundup®; Navaratne et al., 2009). Water from these wells are used by man for drinking, cooking, bathing and washing clothes, while such habitats provide excellent

breeding sites for many frog and toad species. Rice fields are also preferred habitats of amphibians and 18 species have been found breeding in rice fields throughout Sri Lanka (Bambaradeniya, 2000). Unlike other freshwater ecosystems where pesticide run-off is prevalent, rice fields are subjected to direct application of agrochemicals and hence the levels of these chemicals are expected to be much higher in rice fields. The results of this study underscore the importance of investigation of the levels of these agricultural pesticides in freshwater ecosystems.

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