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ORIGINAL ARTICLE

Genotoxicity of Three Avermectins on *Polypedates megacephalus* Tadpoles Using the Comet Assay

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Abstract Avermectins are a new class of macrocyclic lactones derived from mycelia of the soil actinomycete, and are used as effective agricultural pesticides and antiparasitic agents. However, run-off from crops treated with avermectins may contaminate various bodies of water, and accumulated to certain concentrations to impact the development of aquatic animals. Here, we tested the genotoxicity of three avermectins (abamectin, ABM; ivermectin, IVM; and emamectin benzoate, EMB) on *Polypedates megacephalus* tadpoles by the alkaline single-cell gel electrophoresis assay. Tadpoles were treated for 48 h in the laboratory with different concentrations of these three agents, 0.006, 0.012, 0.018, 0.024, 0.030 mg/L for ABM, 0.003, 0.006, 0.009, 0.012, 0.015 mg/L for IVM and 0.04, 0.06, 0.08, 0.10, 0.12 mg/L for EMB, and then measured their DNA damage by the Comet assay tail factor %. The concentrations of resulted in highly significant increases in DNA damage of the tadpoles were found above the concentration threshold of 0.012 mg/L ABM, 0.003 mg/L IVM and 0.06 mg/L EMB and linear correlations between the intensity of DNA damage and the concentrations of these three avermectins. Our results showed clearly that avermectins caused dose dependent DNA damage on amphibian tadpoles, and there might be a control on the misuse of avermectins.

Keywords *Polypedates megacephalus*, tadpole, avermectins, abamectin, ivermectin, emamectin benzoate, DNA damage, comet assay

1. Introduction

Amphibian decline in almost all over the world (Stuart *et al.*, 2004; Xie *et al.*, 2007), with diverse speculations regarding the causes (Ankley *et al*., 1998; Davidson, 2004; Wang and Jia, 2009). Chemical contamination in aquatic environment as a consequence of pesticide application continues to be postulated as a contributing factor for the decline (Berrill *et al*., 1997; Mann and Bidwell, 2001). Indeed, amphibians may be at greater risk of the toxic effects of pollutants than other aquatic vertebrates due to their special physiological and life history characteristics. Amphibian skins are highly absorptive, contaminants have the potential to easily permeate the epidermis (Tyler, 1994), and some amphibians often prefer to breed in shallow, lentic, or ephemeral water bodies, where contaminants may accumulate without dilution (Duellman and Trueb, 1994). Avermectins and their derivatives are very effective agricultural pesticides and antiparasitic agents, and nowadays are used widely in veterinary, and agricultural fields. About 2500 tons of avermectins is produced annually in China, with production expected to increase in the future (Sun and Meng, 2009). Avermectins are a new class of macrocyclic lactones derived from mycelia of the soil actinomycete, *Streptomyces avermitilis*, with four closely related major components, A1a, A2a, B1a and B2a, and four minor components, A1b, A2b, B1b and B2b, which are lower homologs of the corresponding major components (Danishefsky *et al.*, 1989). These compounds were reported to be possessing insecticidal, acaricidal and nematicidal properties and the mechanism of toxicity is fundamentally different from those associated with current natural and synthetic pesticides (Putter *et al*., 1981). Among these components, the B1 fractions (ABM, abamectin, a blend of B1a and B1b avermectins) display the most effective antiparasitic activities (Egerton *et al.*, 1979) and was selected for

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development to control phytophagous mites and insect pests on a variety of agricultural and horticultural crops worldwide (Reddy, 2013). Ivermectin (IVM, 22, 23-dihydroavermectins B1) is semisynthetic derivatives of avermectins B1 with the same effective antiparasitic activity and registered and widely used in veterinary medicine against scab mites (Currie and McCarthy, 2010). Emamectin benzoate (EMB), 4'-deoxy-4'-epi-methyl amino benzoate salt of avermectins B1, is structurally similar to natural fermentation products. It is a mixture of two avermectin homologues: a major constituent (≥ 90%) MAB_1a and a minor constituent (≤ 10%) MAB_1b. It has unprecedented potency against a broad spectrum of lepidopteron pests and is used for controlling lepidopteron pests in agricultural fields (Singh *et al.*, 2013).

More and more frequent application and broad array of uses, the potential negatively impact of these three agents needs to be carefully considered. After the investigation of acute toxicities, the genotoxicity of these avermectins to *Polypedates megacephalus* tadpoles were evaluated in this study using the Alkaline Single-Cell Gel Electrophoresis Assay (SCGE) or Comet assay, an effective and sensitive assay for testing DNA damage caused by mutagens (Tice, 1995).

2. Materials and Methods

2.1 Chemicals Normal-melting-point agarose (NMA), Low-melting-point agarose (LMA), Triton X-100, and Tris (Tris hydroxymethyl) aminonethane hydrochloride were obtained from BBI (Ontario, Canada). Dimethylsulfoxide (DMSO) and ethidium bromide (EtBr) were purchased from Amersco (UKAS), while methylmethane sulfonate (MMS) and Trypan-blue dye was obtained from Sigma (St. Louis, MO). Other general reagents and chemicals used for the comet assay were purchased from Sangon Biotech (Shanghai) Co., Ltd. ABM and IVM were provided by Chengdu Aikeda Chemical Product Co., Ltd. (Chengdu, China), and EMB was provided by Yinnong Biochemical Industry Co., Ltd. (Huizhou, China).

2.2 Animals *Polypedates megacephalus* (Anura: Rhacophoridae) is a medium-sized treefrog, widely distributed in southeastern China. It was chosen as the test animal for this study due to its presence in many disturbed agricultural areas, and its reproductive period is relatively long (Cai, 1979). The chance for the tadpoles to contact these agents is very high. The tadpoles were collected from farm fields in Geling Town, about 50 km southwest from Fuzhou, Fujian Province, China, and reared to Gosner-stage 37–38 tadpoles in the laboratory (Gosner, 1960).

2.3 Treatment All tadpoles were held in glass tanks in dechlorinated water and fed with eel fodder and yolk. After 5–7 days of acclimation, healthy tadpoles with the same stage were selected for the genotoxic tests.

The maximum test concentrations used in the assay were based on approximate 60% of the 48 h LC_{50} concentrations for ABM (0.030 mg/L), IVM (0.015 mg/L) and EMB (0.120 mg/L), and then a series of dilutions were made from these concentrations (Table 1). These 48 h LC_{50} concentrations were derived from a report on acute toxicity in *P. megacephalus* tadpoles that will be published separately (in preparation). 60% of the 48 h LC_{50} concentrations for these agents were chosen as the maximum test concentrations since more than the concentrations could induce a part of tadpole death and disturbed experimentation. A 48-h exposure to all concentrations resulted in 100% tadpole survival.

A total of 408 tadpoles were divided into three parts (i.e., replicated 3 times) with each part consisting of 136 individuals. There were 8 tadpoles per group including negative, positive controls and various treated groups were conducted in the dark in 2 L beakers containing 1500 mL of dechlorinated water, 1500 mL of 3.125 mg/L MMS, or 1500 mL of the various concentrations of these avermectins.

2.4 Alkaline Comet Assay The procedure described by Ralph *et al*. (1996) and Geng *et al.* (2010) was employed, with some modifications. All animals were processed individually. The animals were truncated tails and placed immediately into 1 mL of cold phosphate buffered saline (PBS, calcium- and magnesium- free) for 5 min. Each roughened microscope slide was coated with 200 μL of 0.7 % NMA at 37°C, and then covered with a coverslip and transferred to a humidified box at 4°C for 25 min to allow the solidification of agarose. The erythrocytes (30 μL) were then mixed with 0.7 % LMA (100 μL) and this suspension was pipetted onto fully frosted slides and covered with coverslips. The slides were stored in the dark at 4°C for 30 min to allow complete polymerization of the agarose. The coverslips then were removed and the slides were immersed into freshly made lysing solution (pH = 10) and incubated at 4°C in the dark for 2 h. After lysis, the slides were drained and placed in an alkaline electrophoresis buffer for 30 min. For the electrophoresis, the power supply was set at 20 V and the current adjusted to 200 mA by slowly changing the buffer level in the tray. Slides were electrophoresed in the dark at 4°C for 30 min. After electrophoresis, the slides were placed in a staining

Table 1 Detection of DNA damage with DNA damage frequency and cell viability in erythrocytes of *Polypedates megacephalus* tadpoles (Gosner-stage 37-38) after a 48 h exposure to different concentrations of abamectin, ivermectin and emamectin-benzoate.

Test agents	Concentrations (mg/L)	No. of tadpoles	Cell viability (%)	DNA damage frequency (%)	Comet assay tail factor (%)
Negative control	Dechlorinated water	8	99.2	14.38 ± 3.51	4.80 ± 0.53
Positive control	MMS (3.125 mg/L)	8	90.3	85.23 ± 4.73**	30.05 ± 1.75**
Abamectin	0.006	8	96.5	69.38 ± 2.39**	7.99 ± 0.51*
	0.012	8	90.9	78.06 ± 3.43**	11.03 ± 0.59**
	0.018	8	91.6	83.25 ± 3.81**	20.80 ± 1.28**
	0.024	8	87.6	92.11 ± 4.06**	21.21 ± 1.17**
	0.030	8	85.9	96.67 ± 1.30**	24.49 ± 0.56**
Ivermectin	0.003	8	98.0	54.88 ± 3.18**	9.28 ± 0.80**
	0.006	8	97.3	80.38 ± 3.50**	16.43 ± 0.55**
	0.009	8	89.4	82.25 ± 2.96**	26.25 ± 0.27**
	0.012	8	92.1	87.63 ± 2.45**	29.87 ± 2.79**
	0.015	8	90.9	90.63 ± 1.85**	33.81 ± 2.77**
Emamectin benzoate	0.04	8	97.2	43.88 ± 5.64**	6.46 ± 1.06
	0.06	8	93.8	89.50 ± 3.59**	15.32 ± 1.78**
	0.08	8	94.1	95.63 ± 2.77**	16.87 ± 1.26**
	0.10	8	86.4	97.44 ± 1.50**	17.57 ± 0.65**
	0.12	8	85.5	99.00 ± 0.53**	31.29 ± 3.00**

Note: All comparisons are relative to the negative control. $^{*}P < 0.05$, $^{**}P < 0.01$

tray and covered with a pH 7.5 Tris-HCl neutralizing buffer in the dark for 15 min. This last step was repeated 3 times. The slides were drained, overlayered with 20 μg/mL EtBr, covered with coverslips, and examined at 400× using a fluorescence microscope. All slides were coded and examined blindly. Routinely, 100–120 cells were examined per animal.

2.5 Statistical Analysis The standard of classify comets as 0–4 class (Collins *et al*., 1995) was used according to degree of DNA damage using software CASP in the study (Figure 1). Comet assay tail factor % was used as DNA damage degree according to Valic *et al.* (2004). Comet assay tail factor % = ∑i×Fi (The "i" was coefficients of the various classify comets, with 2.5,12.5, 30, 67.5 and 97.5, respectively; and the "Fi" was the percent of various class damages). Prior to any statistical tests all variables were tested for normality using the Kolmogorov- Smirnov test and for homogeneity of variances using Bartlett's test. The results of the different treatment groups relative to the negative control groups were compared using non-parametric comparisons (Kruskal-Wallis test). Alpha levels of 0.05 and 0.01 were used to determine significance in all statistical analysis. Linear regression analyses were carried out to establish correlations between dose and DNA damage (Comet assay tail factor %). All data processing was made using statistical software SPSS 19.0.

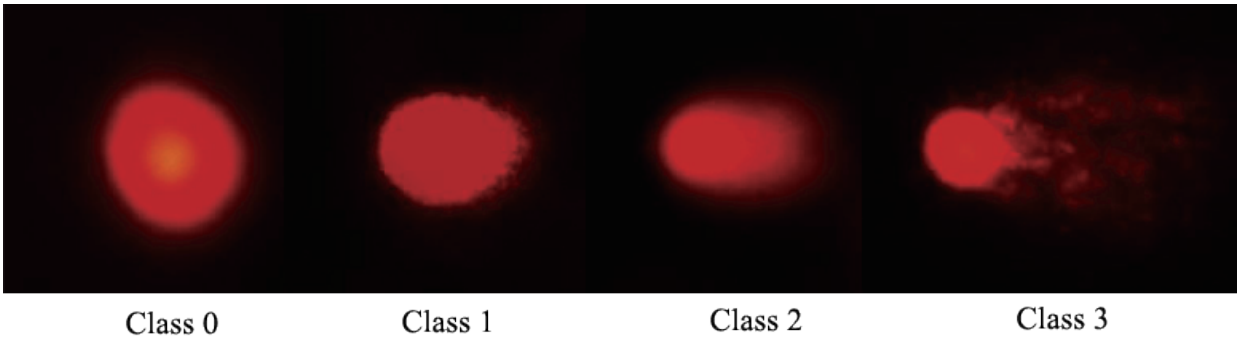

Figure 1 Classification of comets as 0–3 class in erythrocytes of *Polypedates megacephalus* tadpoles.

3. Results

No death and morbidity of the tadpoles were observed after the treatment. DNA damage degrees (Comet assay tail factor %) with DNA damage frequency and cell viability in each treatment group were summarized in table 1.

As shown in table 1, *Polypedates megacephalus* tadpoles exposed to the lower concentrations of EMB (0.040 mg/L) did not show a significant increase in the mean Comet assay tail factor % compared to those of the negative control ($P > 0.05$). However, the tadpoles exposed to the lower concentrations of ABM (0.006 mg/L) showed a significant increase in DNA damage ($P < 0.05$), and the tadpoles exposed to other concentrations of the three avermectins showed a highly significant increase in DNA damage ($P < 0.01$). Similarly, the tadpoles exposed to MMS (3.125 mg/L) showed a strong significant increase in DNA damage ($P < 0.01$).

The three avermectins increased the DNA damage observed in the tadpoles in a dose-responsive manner. There were strong linear correlations between the DNA damages and the concentrations of the three test substances (Figure 2). The cellular distributions of DNA damages in tadpoles are shown in Figure 3. Of the tadpoles treated with increasing concentrations of the three test substances, higher proportions of cells had greater amount of DNA damage than those of the negative control.

4. Discussion

Cell viability was found to be more than 85 %, measure up the most current internationally accepted standards for conducting the comet assay (Tice *et al*., 2000), using the Trypan-blue dye exclusion technique. DNA damage frequency and DNA damage degree were evaluated in the testes of three avermectins-exposed *Polypedates megacephalus* tadpoles. The results indicate that the comet assay can detect DNA damage induced by exposing *P. megacephalus* tadpoles to avermectins.

Although numerous studies report on the toxicities of avermectins (Madsen *et al.*, 1990; Herd, 1995; Davies *et al*., 1998; Katharios *et al*., 2002; Jensen *et al.*, 2003; Jencic *et al.*, 2006; Sanderson *et al.*, 2007; Yu *et al.*, 2007; Fanigliulo and Sacchetti, 2008; Jiang *et al.*, 2008; Römbke *et al.*, 2009; Egeler *et al.* 2010; Römbke *et al.*, 2010; Tang *et al.*, 2011; Prichard *et al.*, 2012; Bansod *et al.*, 2013), little information is available on their genotoxicities. The 96-h LC_{50} values of ABM to *Brachydanio rerio*, *Oncorhynchus mykiss* and *Pelophylax nigromaculatus* were 55.1 μg/L (Tisler and Erzen, 2006), 3.2 μg/L (Jencic *et al.*, 2006) and 43.2 μg/L (Wang and Zhao, 2013), respectively. The 96-h LC_{50} values of IVM to *Salmo gairdneri* and *Xenopus laevis* larvae were 3.3 μg/L (Bloom and Matheson, 1993) and 5.5 μg/L (Martini *et al*, 2012), respectively. The 96 h-LC_{50} values of EMB to *Brachydanio rerio* and *Rana zhenhaiensis* tadpoles were 0.113 mg/L (Wei *et al.*, 2008) and 0.129 mg/L (Chen *et al.*, 2011) , respectively. The adverse effect

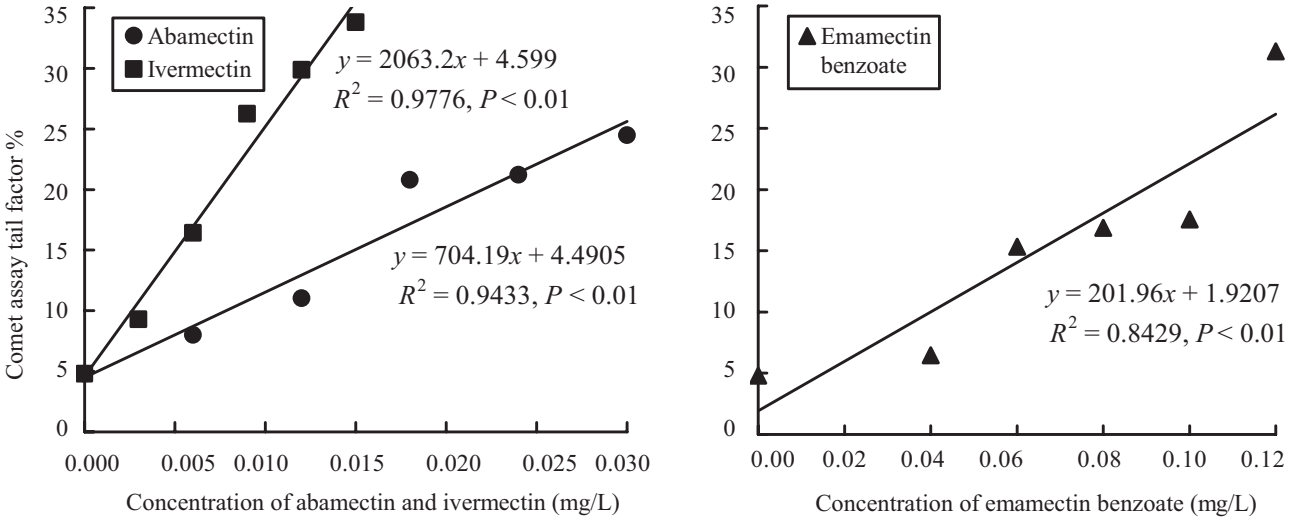

Figure 2 Linear correlations between the DNA damages (Comet assay tail factor %) of tadpoles and the concentrations of abamectin, ivermectin and emamectin benzoate.

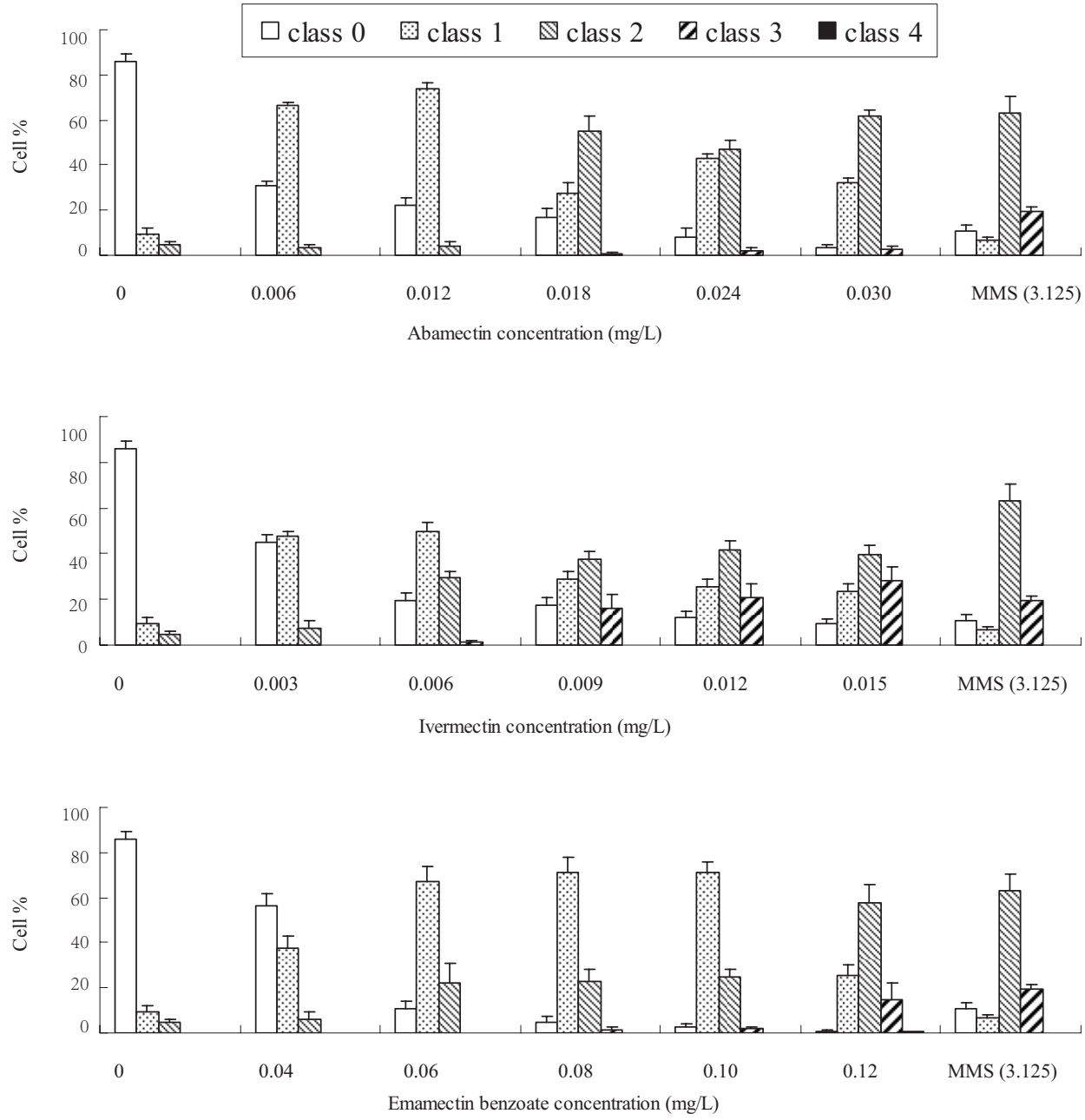

Figure 3 Distribution of DNA damage (based on damage class of DNA patterns pooled across 8 tadpoles in each dose group) observed at the cellular level in *Polypedates megacephalus* tadpoles after exposure for a 48 h period to selected concentrations of abamectin, ivermectin and emamectin benzoate.

of ABM was found on male rat fertility (Elbetieha and Da'as, 2003) and it might have reproductive toxicity (Bing, *et al.*, 2008). Wang and Zhao (2013) found that ABM can induce micronucleus and nuclear anomalies in erythocytes of *Pelophylax nigomaculatus*. Zhang *et al.* (2014) recently reported an in situ assay for quantifying genotoxicity of IVM to the tadpoles at Gosner stage 30-33 in laboratory conditions using alkaline SCGE, and EMB was also found to produce genotoxicity on *Rana zhenhaiensis* tadpoles (Fang *et al.*, 2010).

According to these results above and our finding that avermectins can cause DNA damage in tadpoles at the concentrations below the recommended applied levels (Xu *et al.*, 2010), we consider it possible that avermectins are carcinogenic, and confirm it has the negative impact on the development of tadpoles. Amphibian tadpoles were found to be susceptible to genetic damage caused by short-term exposure to low concentrations of chemicals

(Ralph *et al*., 1996; Clements *et al*., 1997). Our study also shows amphibian tadpoles may be considered as a sensitive biomonitor for detecting the genotoxic potential of avermectins.

In conclusion, because of their genotoxic effects at relatively low concentrations, dose- dependent responses, frequent application and broad array of uses, avermectins likely pose a threat to organisms inhabiting in small water bodies.

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