Original article DOI: 10.1515/aiht-2015-66-2642

Acute toxicity of maneb in the tadpoles of common and green toad

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[Received in March 2015; CrossChecked in March 2015; Accepted in September 2015]

Pesticides used in agriculture can have hazardous effects on aquatic organisms, and amphibians are even more threatened than other aquatic vertebrates. Maneb is widely used to control fungal diseases on crops, fruits, and vegetables. The aim of this study was to investigate the acute toxic effects of maneb on the common (*Bufo bufo*) and green toad (*Pseudepidalea viridis*) tadpoles. Tadpoles at the development stage 21 were exposed to maneb (0-5 mg L^{-1}) for 120 h. Maneb LC_{50} values at hour 120 were 1.966 mg L^{-1} for *B. bufo* and 0.332 mg L^{-1} for *P. viridis*. To the best of our knowledge, these are the first published LC_{50} findings for the two species. Visceral oedema and tail deformations were observed in both species. We also observed liver necrosis, pronephric tubule deformations, somite deteriorations, and visceral oedema at maneb concentrations ≥0.1 mg L^{-1} for *B. bufo* and ≥0.05 mg L^{-1} for *P. viridis*. Our results show that *B. bufo* tadpoles have a much higher resilience to maneb than *P. viridis* tadpoles. This resilience seems to be related to the larger size of the *B. bufo* tadpoles and their ability to metamorphose faster in adverse conditions. Future research should look into the mechanisms of toxic action of maneb in anurans.

KEY WORDS: Bufo bufo*; fungicide; histopathology; morphology;* Pseudepidalea viridis

Pesticides used in agriculture often cause damage and death in non-target organisms and their presence in the environment is gradually increasing as a result of unconscious and/or uncontrolled use (1). One of the most affected organisms among aquatic vertebrates are amphibians, as they use shallow, lentic, and seasonal puddles for breeding and spend their entire larval life in water (2, 3). The decline in the populations of some amphibian species, which is reported on the global scale, has urged toxicologists to intensify their studies in these species (4-6).

Maneb (manganese ethylene-bis-dithiocarbamate, CAS Number: 12427-38-2) is a dithiocarbamate pesticide that disturbs endocrine function (7). In agriculture it is often used as a fungicide to treat fruits, vegetables, wheat, and shelled crops such as walnut, hazelnut, and pistachio (8) and is quite common in Turkey (9).

The *Bufonidae* are one of the largest families of anurans and its genera *Bufo* and *Pseudepidalea* are widely distributed in Turkey. Widespread in Europe and northern Eurasia, the common toad (*Bufo bufo*) is also common in our country, especially in north-western Anatolia, Thrace, the Aegean region, and along the Black Sea and Mediterranean shores, whereas the green toad (*Pseudepidalea viridis*) is widespread in eastern Europe, Russia, and Thrace (10).

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To the best of our knowledge there are only two studies to have studied the toxic effects of maneb in amphibians (11, 12). Considering how common *B. bufo* and *P. viridis* are in Turkey, we decided to be the first determine the lethal concentrations of maneb in tadpoles of these two species and to see which morphological and histological anomalies were likely to occur at their most sensitive developmental stages (13, 14).

MATERIALS AND METHODS

Animals

The *B. bufo* and *P. viridis* tadpoles were obtained from adult specimens collected in Çanakkale, Turkey in February 2010. The adult specimens were carefully collected by hand from areas near seasonal puddles, generally after the sunset, and brought to the laboratory in wet cloth bags. In order for spawning to take place, the specimens in amplexus were placed in plastic containers (259×476×209 mm) with 5-cm-deep tap water and tree branches. The adult specimens were removed after the fertilisation and the fertilised *B. bufo* and *P. viridis* eggs were divided into four equal parts and transferred to 350×350×250 mm glass aquaria with 10-cm-deep tap water. Water temperature, dissolved oxygen, and pH were controlled using a Thermo Orion 3 Star portable ecological kit (Thermo Fisher Scientific, Waltham, MA, USA) and ranged from 15 to 17 °C, 8.2 to 8.5 mg L^{-1}, and

7.5 to 7.7, respectively. At the early stages of development, the tadpoles were fed vegetable feed for fish, and at later stages boiled lettuce and spinach. The light/dark cycle corresponded to natural seasonal conditions at this stage of tadpole development.

Experimental design

The tadpoles were divided in six groups of about 20 per species (in triplicate), of which one was control (unexposed) and the remaining five were exposed to 98 % maneb (Sigma-Aldrich, Saint Louis, MO, USA) in one of the following concentrations: 0.01, 0.05, 0.1, 1, and 5 mg L^{-1}. Exposure started at their developmental stage 21, when the external gills are fully developed, and lasted 120 h.

The stock solution was prepared afresh at the start of exposure and applied to the groups by thinning it out with soft water to the concentrations mentioned above. Our maneb concentrations for the acute toxicity study are based on the reported maximum solubility in water at 25 °C, which is 6 mg L^{-1} (15). Dead tadpoles were recorded and removed together with faeces every 24 hours.

Morphological study

The tadpoles were measured for wet weight (g), total body length (mm), tail length (mm), and width (mm) at the beginning and the end of the experiment. Wet weight was measured on a scale sensitive to 0.0001 g. The tadpoles were placed onto a slide of determined weight and excess water was dried with paper. To measure total body length, tail length, and width we used a digital compass sensitive to 0.01 mm. These measurements were made on 10 tadpoles from each group.

For detailed morphological exam we used an Olympus SZ binocular microscope (Olympus, New York, NY, USA) with bottom illumination. We looked for anomalies such as visceral oedema, tail deformations, and spinal curvature, and took microscopic photographs (at 8x and 40x magnification).

Histopathological study

At the end of the experiment (hour 120), all surviving tadpoles were evaluated for histopathological changes by fixing them in Bouin's solution for 8 h and then preserving them in 70 % ethyl alcohol (Sigma-Aldrich) until the preparation day. On the preparation day, the tadpoles were passed through 80 %, 90 %, 96 %, and absolute ethyl alcohol series and later through xylene (Sigma-Aldrich) and paraffin series to be embedded in paraffin blocks. We made 8-µm-thick cross sections and stained them with haematoxylin & eosin (H&E; Sigma-Aldrich) for histopathological examination with an Olympus CX21 light microscope. We also took photographs with a camera adapted to the Olympus BX51 light microscope and analysed them with Olympus Analysis LS software.

Statistical analysis

For statistical analysis we used the SPSS 17.0 program (IBM, New York, NY, USA). Lethal concentrations (LC_{10}, LC_{50}, and LC_{90}) were calculated with Finney's probit analysis. With this method we created a concentration-survival curve showing the percentages of surviving tadpoles in relation to maneb concentrations. Tadpole survival in each group was also estimated using the Kaplan-Meier estimate, and the differences between the groups were assessed with the log-rank (Mantel-Cox) test for pairwise comparisons.

Morphological measurement data were tested for normality with the Kolmogorov-Smirnov test, and differences in measurements (wet weight, total body length, tail length, and width) between the groups with one-way analysis of variance (ANOVA). Differences <0.05 were considered statistically significant.

RESULTS

Lethal concentrations and survival

Table 1 shows the $LC_{10,}$ $LC_{50,}$ and LC_{90} values of maneb in *B. bufo* and *P. viridis* tadpoles after 120 h of exposure. To the best of our knowledge, these are the first LC findings in these two species.

Table 1 *The 120-hour lethal concentrations (LC_{10}, LC_{50}, and LC_{90}) of maneb in* B. bufo *and* P. viridis *tadpoles*

Species	Lethal concentrations (mg L^{-1})		
	LC_{10} (95 % CI)	**LC_{50} (95 % CI)**	**LC_{90} (95 % CI)**
B. bufo	0.700 (0.071-1.341)	1.966 (0.818-3.313)	5.518 (3.280-23.457)
P. viridis	0.066 (0.011-0.015)	0.332 (0.145-0.626)	1.664 (0.857-5.446)

Figure 1 shows the survival rates in *B. bufo* and *P. viridis* tadpoles from the beginning (0 h) to the end (120 h) of the experiment.

Morphological findings

Table 2 shows the number of morphological anomalies in the ten analysed *B. bufo* and ten *P. viridis* tadpoles.

B. bufo

At the end of the experiment, no morphological anomaly was found in control tadpoles (Figure 2a), 95 % of which survived, completed their operculum development, and reached stages 25 to 27, at which the hind limb starts to

Table 2 *Morphological anomalies in* B. bufo *and* P. viridis *tadpoles (N=10 each)*

Morphological anomalies	Maneb (mg L^{-1})											
	Control		0.01		0.05		0.1		1		5	
	B. bufo	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*
Visceral oedema	0	0	0	1	1	3	3	3	5	6	8	9
Tail deformation	0	0	0	1	1	2	4	3	5	7	7	8

develop. Maneb concentrations of 0.01 and 0.05 mg L^{-1} caused no significant anomaly except for slight tail deformations. In addition, no significant developmental delay was detected in these groups. In the tadpoles exposed to maneb concentrations of 0.1 and 1 mg L^{-1} the most significant anomaly was visceral oedema. Tadpoles exposed to maneb at 0.1 mg L^{-1} were between developmental stages 23 and 26 (Figure 2b), whereas tadpoles exposed to 1 mg L^{-1} were between stages 22 and 24 (Figure 2c).

The concentration-dependent effects were also visible in tail deformations (Figure 3), which were the most prominent in tadpoles exposed to maneb at 1 mg L^{-1}.

Figure 4 shows average width, tail length, and total length at the end of the experiment and Figure 5 differences in weight gain (0-120 h) between the groups. With only two surviving tadpoles, the group exposed to 5 mg L^{-1} was not included in morphological evaluation.

P. viridis

Similar to *B. bufo*, no morphological anomaly was found in the *P. viridis* control tadpoles, which reached stages 26 to 28, and developed their hind limbs (Figure 6a). Tadpoles exposed to 0.01 mg L^{-1} maneb also showed no anomaly and reached stages 26 to 28. Maneb started to suppress development at 0.05 mg L^{-1}; this group reached developmental stages 23 to 26. In addition, it exhibited a few tail deformations. Groups exposed to maneb concentrations 0.1 and 1 mg L^{-1} had not completed their operculum development and reached stages 23 to 25 and 23 to 24, respectively (Figure 6b, 6c). They also exhibited severe visceral oedema. All tadpoles exposed to maneb at 5 mg L^{-1} died by the end of the experiment and were not included in the morphological study.

Figure 7 shows average width, tail length, and total length at the end of the experiment and Figure 8 differences in weight gain (0-120 h) between the groups.

Histopathological findings

Table 3 shows the number histopathological changes observed in ten *B. bufo* and ten *P. viridis* tadpoles.

B. bufo

Severe visceral oedema started to appear with maneb concentrations of 0.1, 1, and 5 mg L^{-1} (Figure 9).

Liver necrosis was observed at 0.1, 1, and 5 mg L^{-1} and was concentration-dependent (Figure 10).

Deformation of pronephric tubule epithelial cells was observed at maneb concentrations of 1 and 5 mg L^{-1} (Figure 11).

P. viridis

This species was more sensitive to maneb than *B. bufo*. Mild visceral oedema was already observed at 0.01 and

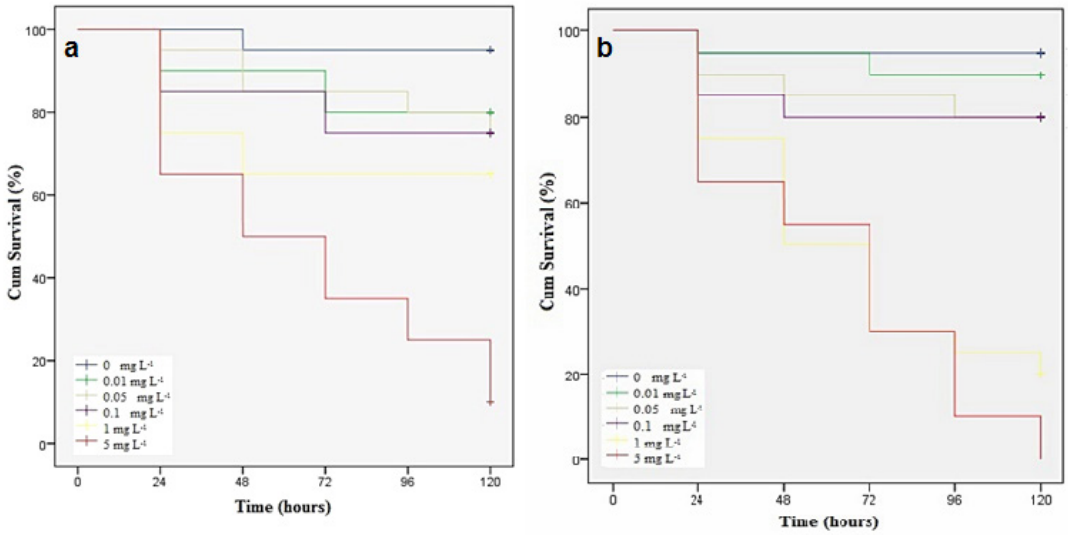

Figure 1 *Kaplan-Meier 120-hour survival curves at different maneb concentrations in: a)* B. bufo *and b)* P. viridis *tadpoles*

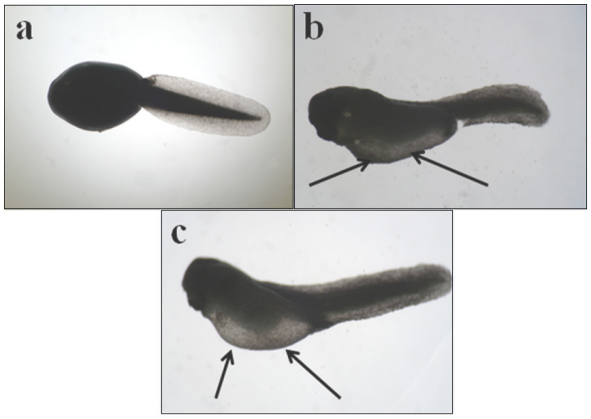

Figure 2 *Visceral oedema (e) in* B. bufo *tadpoles exposed to maneb: a) control (stage 27); b) 0.1 mg L^{-1} (stage 23); c) 1 mg L^{-1} maneb (stage 22)*

0.05 mg L^{-1} and became severe at 0.1 and 1 mg L^{-1} (Figure 12).

Advanced liver necrosis was observed at 1 mg L^{-1} (Figure 13).

Whereas tubular deformation started to appear at 0.01 mg L^{-1}, and it was obviously observed at 0.1 and 1 mg L^{-1} of maneb (Figure 14).

DISCUSSION

To the best of our knowledge this acute toxicity study is the first to report lethal maneb concentrations in *B. bufo* and *P. viridis* tadpoles. Maneb was highly toxic to either of the species even at lower concentrations.

Like all adverse effects observed in our study, tail deformations were concentration-dependent. Deformed tails severely limit the survival of a larva. A similar finding to ours was reported by Bancroft et al. (11) in *Xenopus laevis* larvae exposed to maneb.

Furthermore, maneb slowed down the development of either species in a concentration-dependent manner. Others

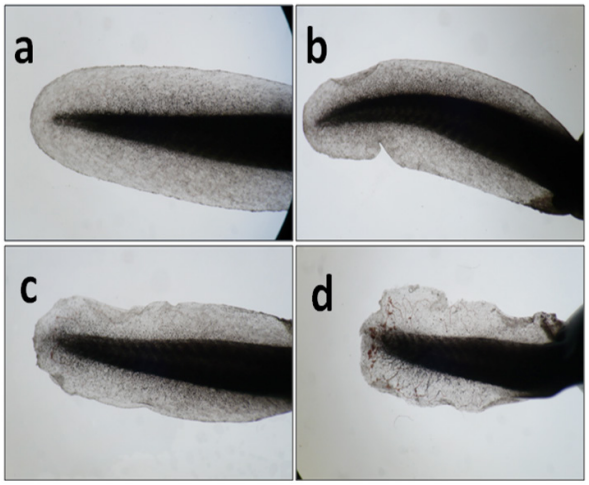

Figure 3 *Tail deformations in* B. bufo *tadpoles exposed to maneb: a) control (stage 27); b) 0.05 mg L^{-1} (stage 27); c) 0.1 mg L^{-1} (stage 23); d) 1 mg L^{-1} (stage 22)*

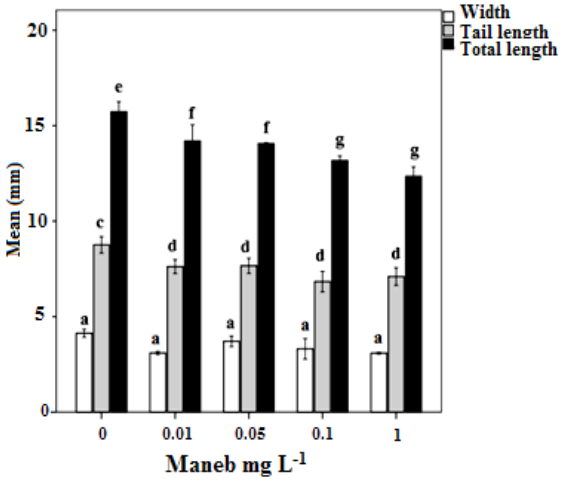

Figure 4 *Width, tail length, and total length of* B. bufo *tadpoles exposed to maneb. Bars represent mean values (±SEM). Different letters denote significant differences between bars (ANOVA,* p<*0.05)*

have also reported that exposure to maneb suppresses growth and development (16, 17).

The organs where histopathological changes are first observed in organisms exposed to a pollutant are the liver and the kidneys. These changes directly affect the life of an organism. Our necrosis and pronephric tubule deformation findings show that maneb affects these organs severely. Similar histopathological findings were reported in albino mice exposed to maneb and zineb (18).

However, judging by the survival rates and adverse morphological, histopathological, and developmental effects, *B. bufo* is more resilient to maneb than *P. viridis*. This difference can partly be related to the fact that the *B. bufo* tadpoles were significantly bigger than the *P. viridis* tadpoles. According to Geng et al. (19), the larger the body size, the stronger the resilience to pesticide effects. Resilience is also related to growth rate and the time needed

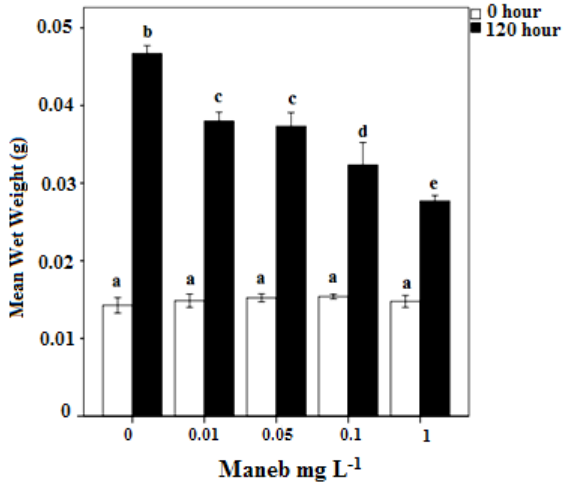

Figure 5 *Mean (±SEM) wet weight in* B. bufo *tadpoles at the beginning and the end of the experiment. Different letters denote significant differences between bars (ANOVA,* p<*0.05)*

Table 3 *Histopathological findings in* B. bufo *and* P. viridis *tadpoles (N=10 each)*

Histopathological findings	Maneb (mg L^{-1})											
	Control		0.01		0.05		0.1		1		5	
	B. bufo	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*	*B. bufo*	*P. viridis*
Epithelial cell deformation	0	0	0	1	0	3	2	4	4	8	7	8
Liver necrosis	0	0	0	1	0	1	3	3	6	6	8	9
Visceral oedema	0	0	0	1	0	4	3	4	6	6	8	8
Tubular deformation	0	0	0	1	0	3	1	5	3	8	7	9

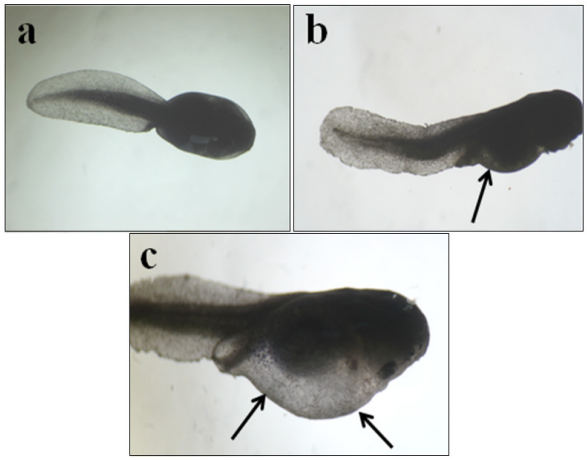

Figure 6 *Visceral oedema (arrow) in* P. viridis *tadpoles exposed to maneb: a) control (stage 26); b) 0.1 mg L^{-1} (stage 23); c) 1 mg L^{-1} (stage 23)*

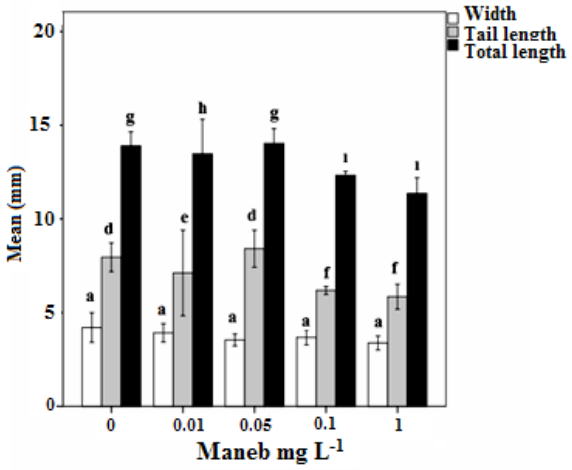

Figure 7 *Width, tail length and total length of* P. viridis *tadpoles exposed to maneb. Bars represent mean values (±SEM). Different letters denote significant differences between bars (ANOVA, p<0.05)*

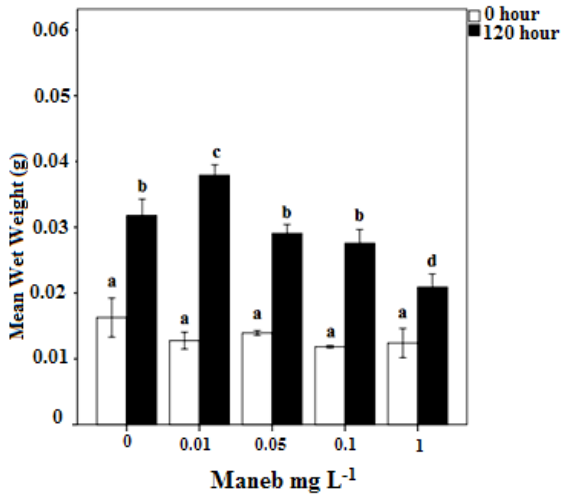

Figure 8 *Mean (±SEM) wet weight in* P. viridis *tadpoles at the beginning and the end of the experiment. Different letters denote significant differences between bars (ANOVA, p<0.05)*

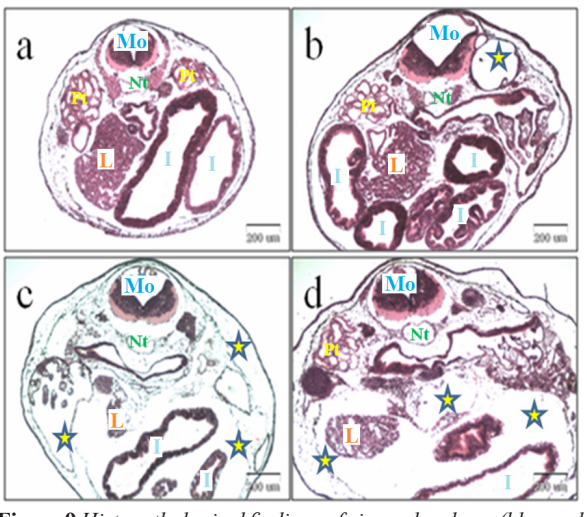

Figure 9 *Histopathological findings of visceral oedema (blue and yellow star) in* B. bufo *tadpoles exposed to maneb: a) control; b) 0.1 mg L^{-1}, c) 1 mg L^{-1}; d) 5 mg L^{-1}. (Mo: medulla oblongata; Pt: pronephric tubule; Nt: notochorda; L: liver; I: intestine)*

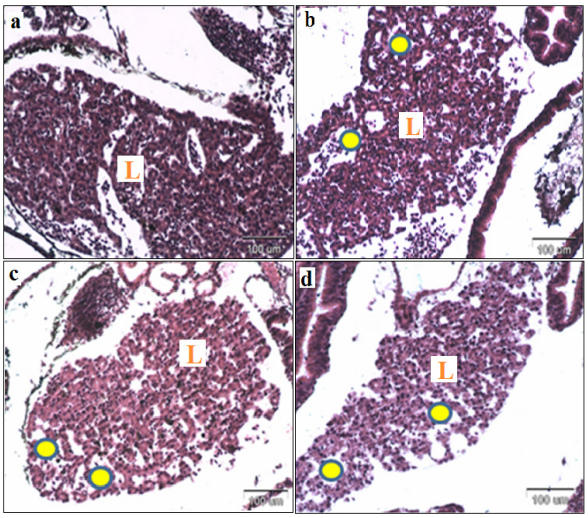

Figure 10 *Liver necrosis (blue and yellow ellipse) in* B. bufo *tadpoles exposed to maneb: a) control; b) 0.1 mg L^{-1}; c) 1 mg L^{-1}; d) 5 mg L^{-1}. (L: liver)*

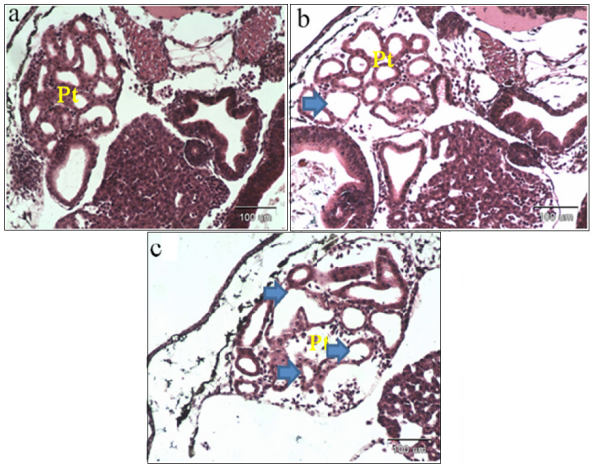

Figure 11 *Pronephric tubule epithelial cell deformations (blue arrow) in* B. bufo *tadpoles exposed to maneb: a) control group; b) 1 mg L^{-1}; c) 5 mg L^{-1}. (Pt: pronephric tubule)*

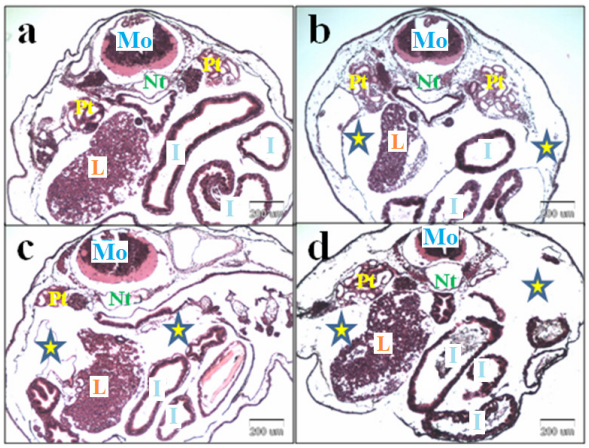

Figure 12 *Histopathological findings of visceral oedema (blue and yellow star) in* P. viridis *tadpoles exposed to maneb: a) control; b) 0.05 mg L^{-1}; c) 0.1 mg L^{-1}; d) 1 mg L^{-1}. (Mo: medulla oblongata; Pt: pronephric tubule; Nt: notochorda; L: liver; I: intestine)*

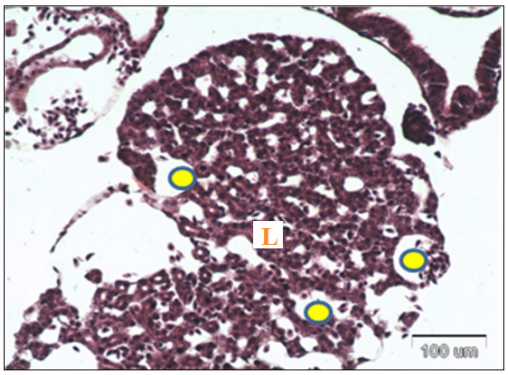

Figure 13 *Liver necrosis (blue and yellow ellipse) in* P. viridis *tadpoles exposed to 1 mg L^{-1} maneb. (L: liver)*

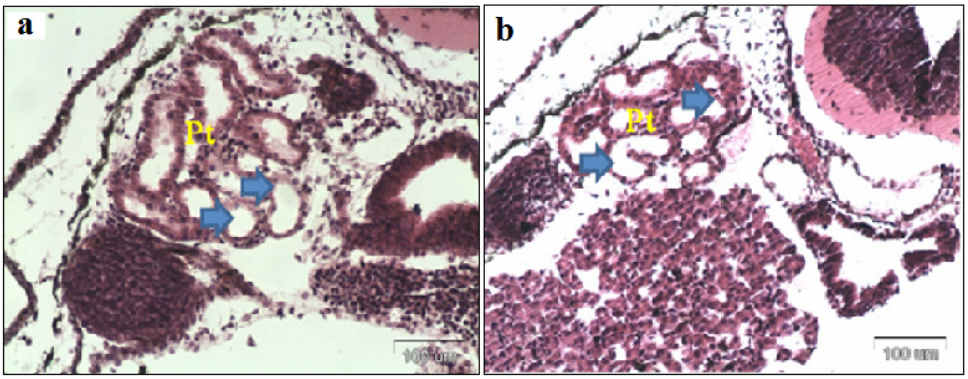

Figure 14 *Pronephric tubule epithelial cell deformations(blue arrow) in* P. viridis *tadpoles exposed to maneb: a) 0.1 mg L^{-1} and b) 1 mg L^{-1}. (Pt: pronephric tubule)*

for a larva to metamorphose. Nunes et al. (20) reported earlier that *B. bufo* tadpoles were able to shorten the time to metamorphosis to cope better with adverse conditions.

While our study may have established the toxic effects of maneb in the two species, it has not addressed the biological mechanisms of its action, and we hope that future studies with different concentrations and frog and toad species will be able to answer this question.

Acknowledgements

This study was part of a PhD thesis and was approved by the Çanakkale Onsekiz Mart University Scientific Research Projects Commission (BAP 2010/02).

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Akutna toksičnost maneba u punoglavaca smeđe i zelene krastače

Pesticidi u poljoprivrednoj uporabi mogu biti opasni za vodene organizme, a najugroženiji među vodenim kralježnjacima su vodozemci. Maneb se rabi za suzbijanje gljivičnih bolesti usjeva, voća i povrća. Cilj ovog istraživanja bio je utvrditi akutnu toksičnost maneba u punoglavaca smeđe (*Bufo bufo*) i zelene krastače (*Pseudepidalea viridis*). U tu smo svrhu punoglavce u 21. stadiju razvoja izložili manebu (0-5 mg L^{-1}). Po svršetku izloženosti (120 h), LC_{50} maneba iznosio je 1,966 mg L^{-1} u *B. bufo* te 0,332 mg L^{-1} u *P. viridis*. Koliko znamo, ove vrijednosti dosada nisu objavljene za ove dvije vrste. Osim letalne koncentracije, pri koncentracijama maneba ≥0.1 mg L^{-1} za *B. bufo* odnosno ≥0.05 mg L^{-1} za *P. viridis* utvrdili smo i nekrozu jetara, deformacije predbubrežnoga kanalića, propadanje somita te visceralni edem. Naši rezultati pokazuju da su punoglavci smeđe krastače značajno otporniji na maneb od punoglavaca zelene krastače.

KLJUČNE RIJEČI**:** Bufo bufo*; fungicid; histopatologija; morfologija;* Pseudepidalea viridis