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ORIGINAL ARTICLE

Mixture toxicity of microcystin-LR, paraoxon and bromadiolone in *Xenopus laevis* embryos

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Abstract

OBJECTIVES: Apart from infections and habitat loss, environmental pollution is another major factor of global decline of amphibians. Using the model of *Xenopus laevis* embryos, we test the hypothesis that combined exposure of amphibians to natural toxins and anthropogenic pollutants induces more pronounced adverse effects than single exposures.

METHODS: Experimental procedures adhered to Frog Embryo Teratogenesis Assay – Xenopus standards (FETAX). Exposure groups included controls, solvent (dimethyl sulfoxide) controls, and embryos exposed for 96 h to single, double and triple action of paraoxon (P), bromadiolone (B), and microcystin-LR (M), added to the FETAX medium at a dose of 300, 350, and 500 $\mu g.L^{-1}$, respectively. Studied responses of *X. laevis* embryos included mortality and malformations, head-to-tail length, total antioxidant capacity, lipid peroxidation, and caspase-3 activity.

RESULTS: The triple combination induced the highest mortality. Malformations in embryos significantly prevailed only in B-, and B+P-exposure groups. Apart from the single exposure to B, the tested substances and their combinations inhibited the embryonic growth. Triple exposure had the most pronounced effect both on the growth inhibition and total antioxidant capacity. Lipid peroxidation was increased after B+M exposure, while single and combined exposures to B and P had an opposite effect.

CONCLUSIONS: This study helps to understand adverse effects of environmental pollution by natural toxins and agrochemicals in amphibians. The results allow for risk assessment of environmental pollution and findings of low concentrations of contaminants in aquatic environments. Further research to address issues such as mixture toxicity to metamorphosing and adult amphibians is necessary.

Abbreviations

ANOVA	- analysis of variance
B	- bromadiolone
C	- control
DMSO	- dimethyl sulfoxide
FETAX	- Frog Embryo Teratogenesis Assay - Xenopus standards
FRAP	- ferric reducing antioxidant power assay
LSD test	- the least significant difference test
M	- microcystin-LR
P	- paraoxon
TBARS	- total thiobarbituric acid reactive species

INTRODUCTION

Amphibians have been documented to decline globally due to a variety of factors (Parmesan 2006; Hof *et al.* 2011). Infections such as chytridiomycosis or red leg disease are associated with substantial amphibian mortality and, among other stressors, environmental pollution is supposed to be a contributing factor (Bradford 1991; Hayes 2006; Peterson *et al.* 2013). Amphibians are closely bound to the aquatic environment, some of them for their whole life cycle. Moreover, they have a sensitive and permeable skin, which allows xenobiotics to enter their bodies. Regarding the thin protective mucous layer of eggs, embryos and adults, there is a high risk of severe adverse effects of environmental pollutants in amphibians (Glennemeier & Denver 2001).

Toxins of natural as well as anthropogenic origin are present together in the environment (Dvorakova *et al.* 2002; Strum *et al.* 2010; Sanchez-Barbudo *et al.* 2012; Chromcova *et al.* 2013). Environmental pollution is a key topic all over the world and there are Environmental Quality Standards for individual pollutants anchored in legislation of many countries. Toxicological experiments are used as a tool to evaluate these pollutant limits, but they are mostly focused on single substance evaluations and mortality endpoints (Buryskova *et al.* 2006; Damkova *et al.* 2009, 2011; Peckova *et al.* 2009; Adamovsky *et al.* 2013). There are only a few combined exposure experiments dealing with the possible modulation of effects by multiple toxins *in vivo* (Pikula *et al.* 2010; Ondracek *et al.* 2012; Osickova *et al.* 2012, 2014). However, toxins are mostly present in sublethal doses as a mixture under environmental conditions (Sih *et al.* 2004; Kortenkamp *et al.* 2007). Importantly, agrochemicals are considered one of three major factors effecting malformations in amphibians (Loeffler *et al.* 2001).

The aim of this study was to test the hypothesis that co-exposures to cyanotoxins and two pesticides (an acetylcholinesterase inhibitor and anticoagulant) in sublethal doses may cause different effects than single substance exposures. Therefore, we compared the action of microcystin-LR (M), bromadiolone (B) and paraoxon (P) (i.e., the active metabolite of the organophosphorus insecticide parathion) in single, double or triple exposures and evaluated their effects on growth inhibition, mortality, induction of malformations, apoptosis, and total antioxidant activity. Since Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) was designed as a screening test to identify potential human teratogens and to examine newly-produced chemical substances by measuring their effects on mortality, malformation and growth inhibition of *Xenopus laevis* embryos, it is an appropriate method for testing the effects of co-exposures to toxins on amphibian early life stages (ASTM 1998).

MATERIALS AND METHODS

Experimental procedures adhered to FETAX standards (ASTM 1998) with some minor modifications to fit our specific conditions. Briefly, parent *Xenopus laevis* were induced to initiate the mating amplexus by injection of human chorionic gonadotropin (HCG; N.V. Organon, Oss, Holland) into the dorsal lymph sac at a dose of 300 IU and 150 IU for females and males, respectively, to obtain eggs for testing. Egg deposition ensued within 12 hours of hormone administration. Mid-blastula to early gastrula developing embryos were then selected for the test and randomly assigned to groups of 25 specimens per 60 mm glass Petri dishes containing 10 ml of the standard FETAX solution (625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \bullet 2H_2O$, and 75 mg $MgSO_4$ per litre of distilled water; pH adjusted to 7.6–7.9). Petri dishes with embryos were kept at 23±1 °C.

Microcystin-LR (Microcystin-LR 1 mg, Enzo Life Sciences, Lausen, Switzerland) was added to the test medium at a dose of 500 µg per litre of the FETAX solution. It had to be diluted in 250 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Therefore, a DMSO-solvent control group was included in the study. The concentration of 300 $\mu g.L^{-1}$ of paraoxon (Paraoxon-ethyl, Pestanal®, Fluka Analytical, Sigma-Aldrich) was used for single and combined paraoxon-treated groups. Bromadiolone (Bromadiolone, Pestanal®, Fluka Analytical, Sigma-Aldrich) at a concentration of 350 $\mu g.L^{-1}$ was used for groups B, B+M, B+P, and B+M+P. As a rule, exposure concentrations were derived to approximate a quarter of reported LC_{50} for aquatic animals, if available (Snawder & Chambers 1989; Dvorakova *et al.* 2002; Rao 2005).

Nine exposure groups in the present study included controls (C), solvent (DMSO) controls (C_{DMSO}), and embryos exposed to single and combined doses of paraoxon, bromadiolone, and microcystin, i.e., P, B, M, P+B, B+M, P+M, and P+B+M. The study was conducted as three independent experiments each including two replicate dishes per each substance and their combinations and four replicate dishes per both controls. The three independent experiments were conducted using embryos from different male/female pairs of *X. laevis*. Dead embryos were recorded and removed at 24-hour intervals while changing the exposure solutions. Embryos were also examined for behavioural abnor-

malities. Embryos from each replicate and control dish that survived 96 hours of test duration were divided into two groups in equal numbers, the first was fixed in 3% formalin and the second was deep-frozen immediately and stored (–80 °C) until biochemical analysis. A binocular dissection microscope was used to evaluate embryos for malformations and to measure head-to-tail length of embryos.

Total antioxidant capacity and lipid peroxidation parameters were measured in whole embryo samples as described previously (Benzie & Strain 1996; Paskova *et al.* 2008; Pohanka *et al.* 2009). The level of lipid peroxidation in embryos was assessed as total thiobarbituric acid reactive species (TBARS). The total antioxidant capacity was measured using the ferric reducing antioxidant power assay (FRAP). Caspase-3 activity was measured using the commercially available Caspase 3 Assay Kit (CASP-3-C, Sigma-Aldrich) as recommended by the producer. The caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline moiety. P-nitroaniline has a high absorbance at 405 nm.

Statistica for Windows® 10 (StatSoft, Inc., Tulsa, OK, USA) was used to compare effects in different experimental groups using procedures of one-way analysis of variance (ANOVA), post-hoc analysis of means by the LSD test, Levene's method to test for the homogeneity of variances, log-transformation of non-homogenous parameters prior to analysis and comparison with the non-parametric Kruskal-Wallis test. The levels of significance used were either $p<0.05$ or $p<0.01$.

RESULTS

As shown in Table 1, there is a significant increase in mortality of *X. laevis* embryos exposed to bromadiolone, bromadiolone combined with paraoxon, and paraoxon combined with microcystin LR. The triple combination of all tested substances induced the highest mortality compared with controls. The total prevalence of malformations in *X. laevis* embryos was significantly induced only in the bromadiolone-, and bromadiolone + paraoxon-exposure groups ($p<0.05$). Within 24 h of exposure frog embryos in all paraoxon-exposed groups manifested circling and rolling movements. Apart from the single exposure to bromadiolone, the tested substances and their double and triple combinations significantly inhibited the growth of *X. laevis* embryos compared to control (Figure 1). The growth inhibition effect of the triple combination of tested substances differed significantly ($p<0.05$) from all other exposure groups except for bromadiolone+paraoxon. A considerable increase of ferric reducing antioxidant power was observed in all exposure variants except for the groups exposed to bromadiolone, paraoxon, and paraoxon combined with microcystin LR (Figure 2). Triple exposure had the most pronounced effect both on the growth inhibition and the ferric reducing antioxidant power. Compared with both controls and the triple combination group lipid peroxidation was lower in single and combined exposures to bromadiolone and paraoxon and paraoxon+microcystin LR, exposure to bromadiolone combined with microcystin LR lead

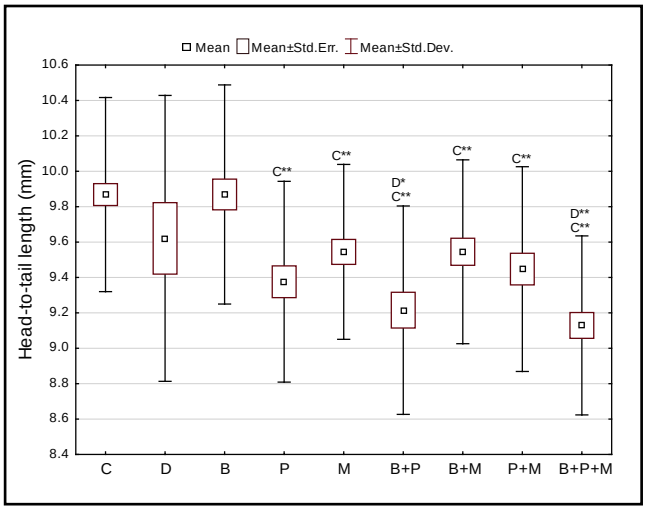

Fig. 1. Growth inhibition of *Xenopus laevis* embryos exposed to single and combined doses of bromadiolone (B), paraoxon (P), and microcystin LR (M), * = $p<0.05$, ** = $p<0.01$ when compared against control group (C) and DMSO solvent control group (D).

Tab. 1. Mortality and malformations (%) in control (C) and DMSO solvent control (C_{DMSO}) groups, and *Xenopus laevis* embryos exposed to single and combined doses of bromadiolone (B), paraoxon (P), and microcystin-LR (M).

	C	C_{DMSO}	B	P	M	B+P	B+M	P+M	B+P+M
Mortality	4.00	1.33	10.66 C*D**	4.00	5.33	8.00 C*D*	1.33	14.66 C**D**	16.00 C**D**
Malformations									
Edema	0	0	13.33	4.00	4.00	5.33	5.33	6.66	4.00
Tailless embryos	4.00	4.00	0	0	0	10.67	1.33	2.67	1.33
Edema & tailless	0	1.33	2.67	4.00	4.00	0	0	0	1.33
Total	4.00	5.33	16.00 C*D*	8.00	8.00	16.00 C*D*	6.66	9.33	6.66

* = $p<0.05$, ** = $p<0.01$ when compared against control group (C) and DMSO solvent control group (D).

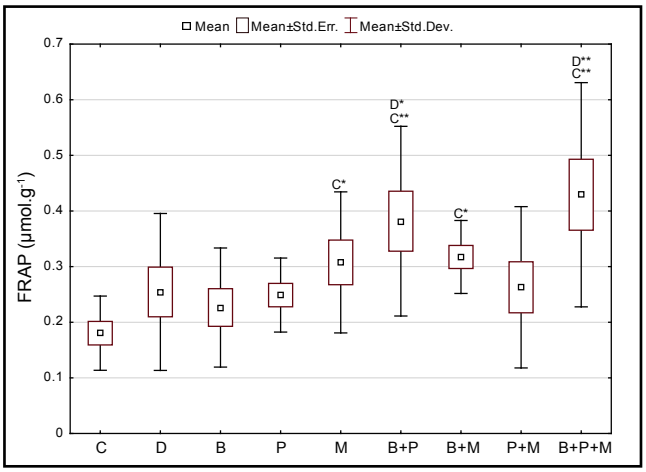

Fig. 2. Ferric reducing antioxidant power (FRAP assay) in *Xenopus laevis* embryos exposed to single and combined doses of bromadiolone (B), paraoxon (P), and microcystin LR (M), * = $p<0.05$, ** = $p<0.01$ when compared against control group (C) and DMSO solvent control group (D).

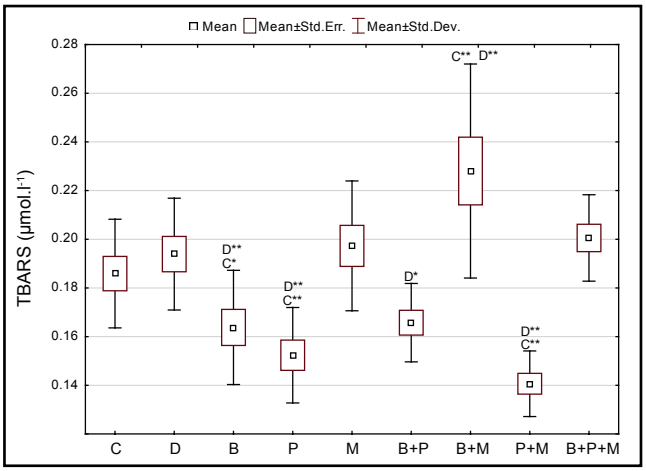

Fig. 3. Lipid peroxidation (TBARS assay) in *Xenopus laevis* embryos exposed to single and combined doses of bromadiolone (B), paraoxon (P), and microcystin LR (M), * = $p<0.05$, ** = $p<0.01$ when compared against control group (C) and DMSO solvent control group (D).

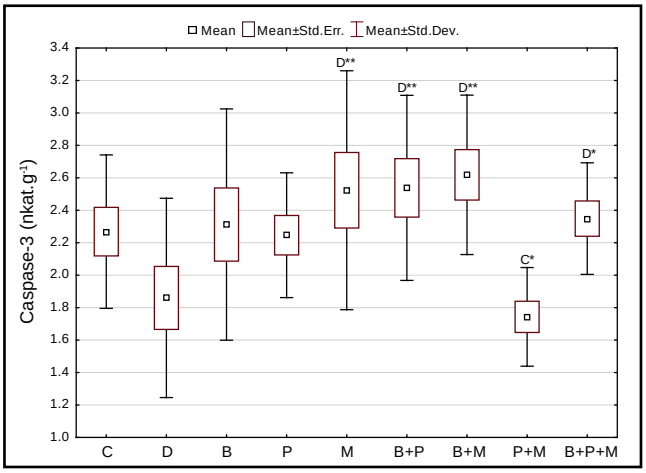

Fig. 4. Caspase-3 activity in *Xenopus laevis* embryos exposed to single and combined doses of bromadiolone (B), paraoxon (P), and microcystin LR (M), * = $p<0.05$, ** = $p<0.01$ when compared against control group (C) and DMSO solvent control group (D).

to increased lipid peroxidation (Figure 3). The only observed decrease in caspase-3 activity against controls concerned the group exposed to paraoxon combined with microcystin LR (Figure 4). Some other differences were noted when comparing exposure groups against the DMSO solvent control group.

DISCUSSION

The present study employing a standard amphibian model to evaluate risks in the aquatic environment confirmed that combined exposure to multiple toxins enhances some of their adverse effects. It has already been shown that such studies may provide environmentally relevant data (Pikula *et al.* 2010; Bandouchova *et al.* 2011) and that two stressors in combination can be far deadlier than one (Sih *et al.* 2004). FETAX is a whole-embryo test for the characterization of developmental toxicity potential of chemicals and complex mixtures. As FETAX screens for malformations during embryonic organogenesis which is highly conserved in vertebrates, potential mammalian and human developmental toxicants and teratogens may be predicted with accuracy greater than 85% (ASTM 1998). While mortality, malformations, and growth inhibition are the primary endpoints, data obtained over a concentration range allow calculation of medium lethal and/or malformation inducing concentrations (ASTM 1998). Since this study focused at testing for differences among single, double or triple exposures to microcystin-LR, bromadiolone and paraoxon, ranges of several concentrations that would considerably increase the total number of embryos and replicate dishes were not examined in this experiment. Values for the 50% lethal concentration, the 50% effective concentration for malformations and the teratogenic index are therefore lacking in the present results.

Bromadiolone induced the greatest rates of mortality and malformations in single exposures among the three tested substances. It has probably also driven the toxic effect in combined exposures. Paraoxon was responsible for abnormal movements in embryos exposed to this acetylcholinesterase inhibitor. While degradation of paraoxon in river water results in a decrease to zero concentration within 13 days (Zhao & Hwang 2009), concentrations of organophosphorus pesticides of environmental interest amount to 40 µg.L^{-1} (Castillo *et al.* 1997). Interestingly, microcystin-LR has shown lower mortality and malformation rate than reported previously (Dvorakova *et al.* 2002; Buryskova *et al.* 2006). Likewise, exposure to cyanobacterial biomass containing microcystin-LR via diet has not dramatically affected the development of *X. laevis* tadpoles (Zikova *et al.* 2013). On the other hand, in contrast with a previous report (Dvorakova *et al.* 2002) a significant growth inhibition of *X. laevis* embryos was observed in the group treated with microcystin-LR in the present study. Microcystin-LR was diluted in DMSO. The lower

mortality and malformation rates in the B+M exposure group compared with single exposure to bromadiolone may be due to pleiotropic and protective effects of DMSO against the mixture-induced toxicity.

Production of bromadiolone and its use as a rodenticide may result in its release and toxicity in the environment (Beklova *et al.* 2007; Krizkova *et al.* 2007). Bromadiolone has shown toxicity for aquatic organisms. For example, the LC_{50} (96 h) for various fish species ranges from 1.4 to more than 3 $mg.L^{-1}$ (Rao 2005). Bromadiolone was shown to be stable in wastewater for at least 3 days and environmental water samples may contain approximately 0.56 $\mu g.L^{-1}$ of second generation anticoagulant rodenticides (Chen *et al.* 2014). Bromadiolone is more effective than the first-generation anticoagulant warfarin. While warfarin is commonly used to prevent thrombosis and reduce the risk of embolism in humans, its administration during pregnancy may induce the so-called warfarin embryopathy associated with nasal cartilage hypoplasia as well as abnormalities of the axial and appendicular skeleton (Hall *et al.* 1980). Embryotoxicity induced by bromadiolone in the present study is, therefore, in line with the pharmacological action of anticoagulants involving inhibition of vitamin K-dependent synthesis of proteins such as osteocalcin (Menon *et al.* 1987), which is associated with hydroxyapatite crystals in the extracellular skeleton matrix, and matrix γ-carboxyglutamic protein that predominates in embryonic bone and cartilage extracellular matrix.

Caspase 3, a cysteine protease, is one of the critical enzymes that mediate cell death and are important to the process of apoptosis (Porter & Janicke 1999). However, the results of the present study are rather non-persuasive regarding differences in this parameter. Several exposure groups induced its activity in comparison to solvent control, but the increase was not statistically significant compared to blank control. Apart from their primary modes of action, toxic substances can elicit clinical responses that may include oxidative stress as an unspecific biochemical process involved in the adverse reaction to many stressors (Vitula *et al.* 2011). It has already been shown that antioxidant/oxidative stress parameters were more sensitive, yet unspecific, indicators of exposure to toxins when compared with standard plasma biochemistry. Multivariate principal component analysis confirmed marked joint effects in the overall pattern of antioxidant/oxidative stress responses of the triple exposure group of birds treated with the same toxins as in the present study (Ondracek *et al.* 2012). Similar findings concerning the efficiency of activation of the antioxidant system were reported earlier in birds (Paskova *et al.* 2008, 2011). Two parameters not associated with the primary mode of action of the toxins were used in the present study to evaluate modulations of the antioxidant system and oxidative stress related cellular damage in *X. laevis* embryos. The ferric reducing antioxidant power is a clinical marker of the total antioxidant capacity that depends on non-enzymatic antioxidants including ascorbic and uric acids, bilirubin, vitamin E, α-tocopherol, and albumin (Benzie & Strain 1996; Pohanka *et al.* 2009). Lipid peroxidation indicates oxidative damage to membrane lipids (Halliwell & Gutteridge 2007; Paskova *et al.* 2008; Paskova *et al.* 2011). The most pronounced effect of triple exposure of *X. laevis* embryos was found with the ferric reducing antioxidant power. Stimulation of the total antioxidant capacity may, therefore, be responsible for the non-significant effects of lipid peroxidation observed in the triple exposure group. Significantly increased TBARS levels as a parameter of damage to membrane lipids were shown only in the group exposed to bromadiolone+microcystin LR, but not in the single exposures to these substances, when compared with both controls. More pronounced responses of oxidative stress parameters (FRAP, TBARS, glutathione reductase) were observed in tissues of Japanese quails exposed to single and combined doses of paraoxon, bromadiolone, and microcystins in cyanobacterial biomass in comparison against healthy controls (Ondracek *et al.* 2012).

To conclude, the present study contributes to the understanding of adverse effects of combined exposure to natural toxins and agrochemicals in amphibian embryos. The results may be used for risk assessment of environmental pollution and evaluation of findings of low concentrations of contaminants in aquatic environments. However, further research into this and other issues such as mixture toxicity to metamorphosing and adult amphibians is necessary.

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