INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print), 2222-3045 (Online)
http://www.innspub.net
Vol. 6, No. 3, p. 307-314, 2015

RESEARCH PAPER OPEN ACCESS

Toxicological impact of a mimetic estrogen specie: mancozeb on a tadpoles of the green frog (*Rana saharica)*

Benosmane Sana*, Djebar Mohammed Reda, Berrebah Houria, Alayat Amel, Benamara Marwa, Zouaghi Mohamed Fateh, Amamra Rima

Laboratory of Cellular Toxicology, Department of Biology, Faculty of sciences, University of Badji Mokhtar, Annaba, Algeria

Key words: Tadpole, physiology, biometry, respiration, reactive oxygen species.

Article published on March 27, 2015

Abstract

The presence in the environment of chemicals with hormone-mimetic properties generates concerns about their effects on aquatic organisms. Due to their estrogenic activity, they are part of the chemical compounds whose concentration level in the environment isn't monitored. The estrogenic substances can be of natural origin, but also of synthetic origin such as certain pesticides. In order to prevent the risks associated with better use and exposure to xeno-estrogens, we have investigated the effects of estrogen mimetic (MOS) fungal origin (Mancozeb) on the morphology of bio-indicators organisms of pollution: the tadpoles of the green frog (*Rana saharica*.) Treated with different concentrations 0.25, 0.5, 0.75 and 1 mg/l over a period of 5 weeks. This xeno-estrogen induced disturbances of growth and a condition index tadpoles showing a net delay of the breeding and a affection of sexual maturity. We also observe that the exposure of tadpoles populations to different concentrations of the fungicide disrupts their respiratory metabolism in dose-dependent manner. The inhibition of respiration resulted an increase in free radical production, which is confirmed by the test of quantization of reactives metabolites of oxygen (d-ROMs) which highlights a significant production of reactive oxygen species (ROS).

***Corresponding Author:** Benosmane Sana ✉ sanou.3.6@hotmail.fr

Introduction

Among the many chemicals released into the environment, drug residues occupy a prominent place. Among those, hormones or hormone analogs including xeno-estrogen compounds similar to estrogen. These chemical compounds, whose structure is identical to that of estrogen, are considered endocrine disruptors (Martin *et al.,* 2004). their mechanisms of action are manifold since they can potentially act on all the steps of endocrine regulation, since the synthesis of hormones to the response of target cells. To date, the best-described mechanisms of action are those meditated by nuclear steroid receptors, particularly estrogen receptors.

A disruption of the endocrine system can affect an organization for the development of the embryo growth and all critical life stages.

For ten years, it has been shown that some molecules, widespread in the environment, have estrogen-mimetic activity in fish and amphibians and are able to modulate transcription through different mechanisms of some estrogen-dependent genes (Flouriot *et al.,* 1997; Petit *et al.,* 1997; Le Guevel and Pakdel, 2001) and affect the reproduction of individuals (Brion *et al.,* 2004; Nash *et al.,* 2004).

Today, the issue of endocrine disrupters is largely focused on the problems of reproduction and genotoxicity, there are various studies in this areas such as those of Petridis *et al.* (2009) which measures the genotoxic potention of (xeno-) oestrogens in the bivalve *mollusc Scrobicularia plana* and those of Li *et al.* (2015) which work on the induction genes encoding chemical efflux proteins in gram-negative bacteria cause by estrogen Mimics, while we rarely found the effect of xeno-estrogens on oxidative stress of living things..

In order to contribute for prevent the most of the risks related to the use and exposure to xenoestrogens. it seemed to us interesting to do a study on the toxicological impact of an estrogen mimic fungal from family of dithiocarbamates: the Mancozeb, on a biological model considered an organic pollution indicator: tadpoles of the green frog (Rana saharica).

We have focused our study of the first level on the study of some physical parameters of an artificial environment created in the laboratory (pH and T °) coupled with other biometric parameters (weight, linear evolution and condition index) during a period of treatment. On another level we aim to evaluate the energy potential of tadpoles: O_2 consumption and production of reactive oxygen species (ROS) by an analysis system designed for the overall evaluation of oxidative stress, by means of a test of d-ROM.

Material and methods

Biological material

The biological material used in our study is the tadpole Green frog "*Rana saharica*." (Boulenger, 1913). The samples come from the El Kala region which is considered the least polluted site in the region (Brahamia and Semouk, 2010).

The samples are kept in breeding in plastic boxes and monitor the temperature and pH according to standards (Chagra *et al.,* 2007).

Chimical materiel

In our study, we used "The Mancozeb" ethylene-bis-dithiocarbamate, metal fungicide of synthetic origin that is part of the dithiocarbamate group. A similar structure to natural hormones estradiol (Gomez, 2003; Paro *et al.,* 2012) and is used in the control of a wide range of pathogens including burns, mildew and scab on potatoes and other crops. Broad-spectrum, non-systemic, it acts as disturbance of lipid metabolism. (PPDB, 2014).

Processing mode

The experiments are conducted on tadpoles in the early stages of larval development (stage 26-32) and ending with the stages of pre metamorphosis (37-39) (Gosner, 1960), this imposes an exposure time limited

to 5 weeks.

We used a sample (T) and 04 concentrations of Mancozeb (C_1, C_2, C_3 and C_4), respectively equal to 0.25, 0.5, 0.75 and 1 mg /l.

These concentrations are determined from an LC50 of acute toxicity test trials in Fish and Aquatic Invertebrates 1mg /l <LC50 <10mg /l.

The samples consist of 10 tadpoles per aliquot for each beaker (Botosoa, 2010).

Parameters studied

The pH and temperature

The measurement of pH and temperature of our samples is performed every 24 hours using a pH meter (type HANNA HI2211 pH / ORP meter) and a temperature sensor.

Biometric parameters

The tadpoles are weighed weekly using a precision balance (type Sartorius TE 124S). Changes in the size is measured every week, by placing the tadpole on filter paper by lengthening beside a meter tape, the tadpole length is the distance between the ends.

Condition index

The change in the condition index versus time and the size of the tadpole allows to know the breeding period and to determine the approximate size at first maturity (Le cren, 1951). According Le cren (1951) the condition index is expressed by the following formula: $CI = (W/L^3) \times 100$

W: Total weight (g)

L: Length of the tadpole (cm)

Polarographic measurement

O_2 consumption is measured by a Hansatech type oxygen electrode to measure rates of O_2 in the order of nano-mole by the method of Chagra *et al.* (2007), about the same size tadpoles and even weight after 3 weeks of treatment with estrogen mimetic "Mancozeb".

Quantification of ROS (reactive oxygen species)

The rate of ROS is quantified in blood samples collected from tadpoles treated for 3 weeks with increasing concentrations of Mancozeb. It was measured using the test d-ROM. This test was carried out on an analysis system of free radical system (FRAS4) spectrophotometer which is a new analytical system consisting of a photometer with a dedicated integrated centrifuge, designed for the overall evaluation of oxidative stress, we used the d-ROM test, a small sample of capillary blood. The initials FRAS means Free Radical Analytical System. FRAS 4 is a highly innovative system that provides real-time information on the global oxidative balance of an individual (Celi *et al.,* 2010).

In the test d-ROM, hydroperoxides in a blood sample of 20 µL react with a chromogenic substrate at 37 °C to develop a derivative color whose intensity is directly proportional to the concentration of d-ROMs, according to the law of Beer-Lambert (Alberti *et al.*, 2000). The color intensity is quantified by FRAS4 and the results are expressed in units Caratelli (U.Carr.), where a U.Carr. is equivalent to 0.08 mg/ 100 ml of hydrogen peroxide (Trotti *et al.,* 2002).

Result

pH change and temperature

The pH is a very important parameter for amphibian physiology. Indeed, the acidic conditions may slow the development of embryos and tadpoles (Jung and Jagoe, 1995) and reduced survival (Dunson and connell, 1982). The pH varies between a minium of 7,0 and a maximum of 9,0.

The temperature should be checked regularly, this factor can be decisive for the evolution and growth of tadpoles. During treatment tadpoles, we have maintained a constant temperature favorable to their development between 16 and 20 °C.

Variation of biometric parameters tadpoles treated with different concentrations of Mancozeb

Figure 1 illustrates the effect of Mancozeb on

biometrics tadpoles during a period of 5 weeks. Our results show a highly significant delay (p = 0.000) in the evolution of the weight and size of tadpoles treated compared to controls.

Effect of different concentrations of Mancozeb on changes in condition index (CI)

Figure 3 shows the variations in the condition index tadpoles treated with different concentrations of Mancozeb. We note that a condition index tends to increase a highly significant (p = 0.000) in tadpoles treated especially for concentrations C_2, C_3 and C_4 compared to control dice the 2nd week.

Effect of different concentrations of Mancozeb on the respiratory metabolism

Figure 04 illustrates the effects of Mancozeb on the respiratory metabolism of tadpoles at different processing times. The level of oxygen consumption continues to decline in a highly significant (p= 0.000) and dose-dependent, this decrease is of the order of 70% to 80% compared to controls.

Table 1. Quantitative variations reactive oxygen metabolites observed in the blood of tadpoles treated with various concentrations mancozeb.

d-ROMs (U.Carré)	T	0,25 mg/l	0,5 mg/l	0,75 mg/l	1 mg/l
	266	269	298	314	464

Evolution of the rate of ROS

Table 1 shows the evolution of ROS levels in the blood of witnesses tadpoles treated with increasing concentrations of Mancozeb. Our results show that as the lowest concentration of Mancozeb, the rate of ROS tends to increase and this increase is dose-dependent and highly significant from the concentration: 0,75 mg /l of blood.

Discussion

Monitoring of physical parameters of the environment of tadpoles during treatment.

This study was conducted to investigate the consequences of estrogen mimetic pollution from agricultural origin in one species of amphibians, tadpoles of the green frog (*Rana saharica).* These bio-indicator species are very sensitive to the slightest changes in their environment and any changes in their environment could affect their embryonic development and their physiology (Joly, 2000).

Indeed, the acidification of lakes and temperature variations are a threat to the survival of amphibians (Joly, 2000; Chagra *et al.,* 2007) Thus the works of Denver (1997), of Lillywhite *et al.* (1999) and of Schonweger *et al.* (2000) show that a temperature varying between 1 and 10 °C is harmful to the development of the tadpole and temperatures ranging from 10 and 25 °C are the most favorable while that Mikkelsen and Jenssen (2006) state that the ideal temperature for the development of amphibians should be between 16 and 20 °C. In our work, we have taken account of these data and the temperatures were monitored and maintained at values favorable to the development of tadpoles ie around 15 and 16 °C.

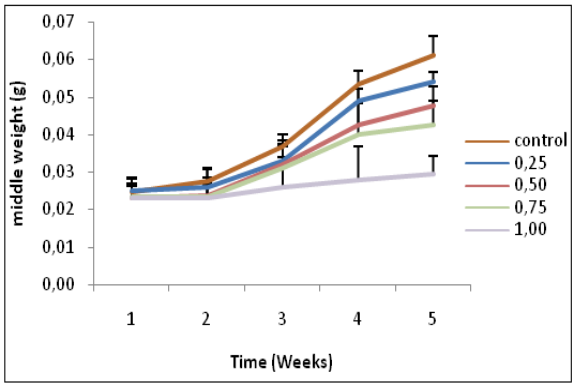

Fig. 1. Evolution of the average weight of tadpoles treated with different concentrations of Mancozeb.

Impact of the estrogen mimetic (Mancozeb) on the growth and condition index of tadpoles.

Many studies have shown that a wide range of contaminants and pollutants are responsible for the achievement of the amphibian population in their life

and their diversity: pesticides, fungicides, herbicides and fertilizers, and many other molecules (Sparling *et al.*, 2000).

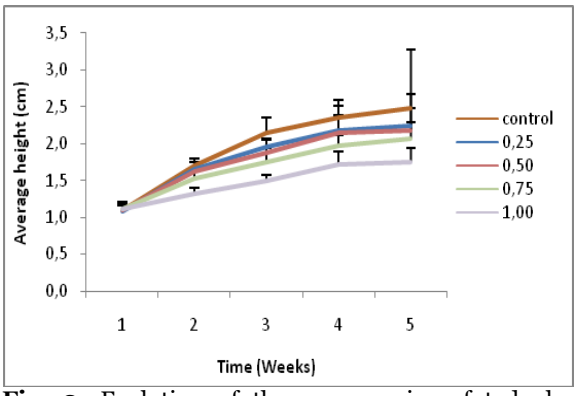

Fig. 2. Evolution of the average size of tadpoles treated with different concentrations of Mancozeb.

All these xenobiotics are toxic directly through a breach in the immediate environment of the animals or indirectly by reducing their growth through a breach of their endocrine or inducing immuno-suppression (Sparling *et al.*, 2000). Moreover, the nature and intensity of the toxic effects of a fungicide on a body depends on its concentration in the target organ. This concentration is related to the administered dose, its distribution and its metabolism (Muckter, 2003). According to the works of Yoon *et al.* (2003), treatment of Xenopus embryos by increasing concentrations of a fungicide (benomyl) causes not only an increase in the number of defects from the lowest concentrations tested (5, 10, 15 µM), but also the death of all the embryos with the highest concentration (20 µM).

Our results show that in the presence of Mancozeb, tadpole growth is inhibited, the toxicity of this xenobiotic in tadpoles is manifested primarily through a delay in weight gain and size at all concentrations tested (0.25, 0.50, 0,75et 1 mg /l). However, and at the highest concentration, we recorded a mortality rate of about 70% and beyond 6 weeks of treatment.

According Castelle *et al.* (1994), fungicides pose a real threat to most species of amphibians. Bridges and Semlitsch (2000), Kiesecker *et al.* (2001) and Yu *et al.* (2013) reported a malfunction in sexual development and a much higher frequency of defects during development in amphibians subjected to treatment with fungicides. Malformations may include additional members, abnormal digestive systems and reduced the size and weight of control tadpoles compared to treated as was observed in our study.

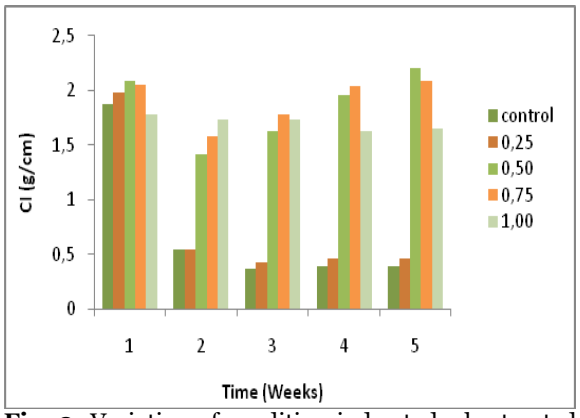

Fig. 3. Variation of condition index tadpoles treated with different concentrations of Mancozeb.

Simmaco *et al.* (1998) found that the chemical stress can induce a decrease in concentrations of thyroid hormones in the blood of tadpoles exposed to fungicides. This may have implications for the metamorphosis of these animals and Blaustein and Johnson (2003) confirm that the biotic stress is the main cause of the appearance of malformations in amphibians and indirectly contributes to the simple deletion of their immune defense.

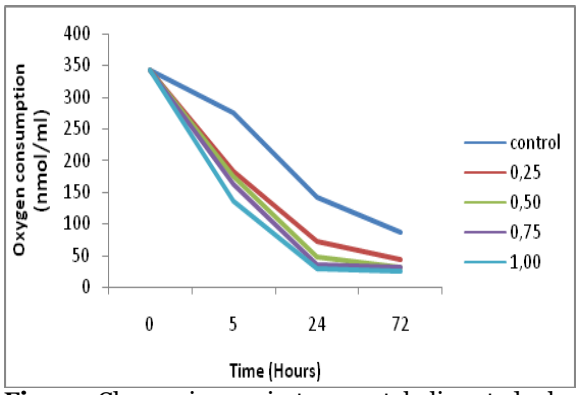

Fig. 4. Change in respiratory metabolism tadpoles treated with different concentrations of Mancozeb.

Among the first effects of MOS we have highlighted the decline of both the weight and the size of the

treated tadpoles, confirmed by the value of the condition index showing good growth retardation may affect sexual maturation as suggest Laskowski and Hopkin (1996), which confirmed that the inhibition of growth induced by pollutants can result metabolic disorders, behavioral or a disturbances of neuroendocrine control. At the individual level, the delay of the period of sexual maturity is one of the most significant consequences of the inhibition of the growth of treated animals.

Evaluation of energetic parameters of tadpoles in the presence of a Mancozeb.

This toxic effect is accompanied by disruption of respiratory metabolism. In fact we are interested in this type of metabolism because of its importance, as the level of the mitochondria, molecular oxygen undergoes successive cuts to form a water molecule. These reductions are the source of ROS formation which 75% are from endogenous source mainly the mitochondrial respiratory chain and the rest of exogenous source from free radicals in the redox cycle with the O_2. In our work, we have demonstrated an increase in O_2 consumption in treated tadpoles. This result is in agreement with those of Chagra *et al.* (2007) wich have also shown that a perturbation of the respiratory metabolism in tadpoles of (*Rana saharica*) treated with a fungicide artea 330 EC or Bouaricha *et al.* (2012) on a freshwater organism used as an alternative biological model *Paramcium sp.* treated by an insecticide based emamectin benzoate.

From these physiological and metabolic effects, we are interested to the involvement of ROS as a probable cause of this toxicity and for this we followed the rate of hydrogen peroxide in the blood of tadpoles. Thus, on the highest concentration of estrogen mimetic, we have demonstrated a high rate of H_2O_2 in the blood, confirming the involvement of oxidative stress in the induction of metabolic disturbances as well as physiological previously observed.

Conclusion

The estrogen mimetic Mancozeb we tested, is toxic and this toxicity was manifested by disturbances of some biometric parameters, physiological and metabolic of tadpoles. On the other hand, we have demonstrated induction of ROS through increased H_2O_2 levels at the highest concentrations of xenobiotic. The induction of oxidative stress encourages us to explore other avenues including carcinogenesis and DNA damage in tadpoles of the green frog (*Rana saharica).*

References

Alberti A, Bolognini L, Macciantelli D, Caratelli M. 2000. The radical cation of N,N-diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. Research on Chemical Intermediates **26,** 253–267.

Blaustein A, Johnson P. 2003. The complexity of deformed amphibians. Frontiers in ecology and the environment **1,** 87-94.

Botosoa JA. 2010. Purification and partial chemical and biological characterization of the active extracts from leaves *Pechia madagascariensis* (APOCYNACEAE). PhD thesis, University of Antananarivo, Madagascar.

Bouaricha H, Berrebbah H, Grara N, Djebar MR. 2012. Response of *paramecium sp*. with respect to an insecticide (Proclaim): growth, content of MDA, AChE activity and respiratory metabolism. Journal of Applied Sciences Research **8,** 4172-4180.

Boulenger GA. 1913. On a Collection of Batrachians and Reptiles made by Dr. H.G.F. Spurrell, F.Z.S., in: the Choco, Colombia. In: Proceeding of the Zoological Society of London **83,** 1019-1038.

Brahamia K, Semouk A. 2010. Tourist activity in a fragile snack space of El Kala National Park. El-Tawasso **26.**

Bridges CM, Semlitsch RD. 2000. Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. Conservation Biology **14,** 1490-1499.

Brion F, Tyler CR, Palazzi X, Garric J, Laillet B, Porcher JM, Flammarion P. 2004. Impacts of 17beta-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). Aquatic Toxicology **68,** 193-217.

Castelle AJ, Johnson AW, Conolly C. 1994. Wetlands and Stream Buffer Size Requirements. A Review. Journal of Environmental Quality **23,** 878-882.

Celi P, Sullivan M, Evans D. 2010. The stability of the reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests on stored horse blood. The Veterinary Journal **183,** 217–218.

Chagra A, Rouabhi R, Berrebbah H, Djebar MR. 2007. The toxicity study of a systemic fungicide: artea 330 EC on the physiology and the respiratory metabolism of the tadpole (*Rana saharica*). Communications of Agricultural and Applied Biological Sciences **72,** 191-5.

Denver RJ. 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. American Zoologist **37,** 172–184.

Dunson WA, Connell J. 1982. Specific-inhibition of hatching in amphibian embryos by low pH. Journal of Herpetology **16,** 314-316.

Flouriot G, Pakdel F, Ducouret B, Ledrean Y, Valotaire T. 1997. Differential regulation of two genes implicated in fish reproduction: vitellogenin and estrogen receptor genes. Molecular Reproduction and Development **48,** 317-23.

Gomez E. 2003. Emerging contaminants Liaison with estrogen receptor: examples for different classes of substances. HSM Hydrosciences, Montpellier 1, France.

Gosner KL. 1960. A Simplified Table for Staging Anuran Embryos and Larvae. Herpetologica **16,** 183-190.

Joly P. 2000. Biological invasions: state of the art and perspectives. Journal of Ecology (Earth and Life) **7,** 21-35.

Jung RE, Jagoe CH. 1995. Effects of low pH and aluminum on body size, swimming performance and suceptibility to predation of green tree frog (*Hyla cinerea*) todpoles. Canadian Journal of zoology **73,** 2171- 2183.

Kiesecker JM, Blaustein AR, Belden LK. 2001. Complex causes of amphibian population declines. Nature **410,** 681-684.

Laskowski R, Hopkin SP. 1996. Effect of Zn, Pb and Cd on fitness in snails *(Helix aspersa).* Ecotoxicology Environmental Safety **34,** 59-69.

Le cren ED. 1951. The length-weight relationship and seasonal cycle in gonad weight I and condition in the perch (*Perca fluviatilis*). Journal of Animal Ecology **20,** 201-19.

Le guevel R, Pakdel F. 2001. Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods. Human Reproduction **16,** 1030-6.

Li X, Teske S, Conroy-Ben O. 2015. Estrogen mimics induce genes encoding chemical efflux proteins in gram-negative bacteria**.** Chemosphere **128,** 327-331.

Lillywhite HB, Zippel KC, Farrell AP. 1999. Resting and maximal heart rates in ectothermic

vertebrates. Comparative Biochemistry and Physiology **124,** 369-382.

Martin PM, Berthois Y, Sasco A. 2004. Xenoestrogens, environmental estrogens, endocrine disruptors. 26th days of the SFSPM, Nancy.

Mikkelsen M, Jenssen BM. 2006. Polychlorinated biphenyls, sex steroid hormones and liver reinoids in adult male European common frogs (*Rana temporaria*). Chemosphere **63,** 707-715.

Mückter H. 2003. What is toxicology and how does toxicity occur?. Best Practice and Research Clinical Anaesthesiology **17,** 5–27.

Nash J, Kime DE, Van der ven L, Wester P, Brion F, Maack G, Stahlschmidt-Allner P, Tyler CR. 2004. Environmentally relevant concentrations of the pharmaceutical, ethinyloestradiol, impact fish populations. Environmental Health Perspectives **112,** 1725-33.

Paro R, Tiboni GM, Buccione R, Rossi G, Cellini V, Canipari R, Cecconi S. 2012. The fungicide Mancozeb induces toxic effects on mammalian granulosa cells. Toxicology and Applied Pharmacology **260,** 155–161.

Petit F, Le Goff P, Cravedi JP, Valotaire Y, Pakdel F. 1997. Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. Journal of Molecular Endocrinology **19,** 321-35.

Petridis P, Awadhesh N, William J, Langston. 2009. Measurements of the genotoxic potential of (xeno-) oestrogens in the bivalve *mollusc Scrobicularia plana*, using the Comet assay**.** Chemosphere **128,** 327-331.

PPDB. 2014. Pesticide Mancozeb. Properties DataBase, University Hertfordshire.

Schonweger G, Schwerte T, Pelster B. 2000. Temperature-dependent development of cardiac activity in unrestrained larvae of the minnow Phoxinus-Phoxinus. American Journal of Physiology Regulatory Integrative and Comparatie Physiology **279,** 1634-40.

Simmaco M, Mignogna G, Barra D. 1998. Antimicrobial peptides from amphibian skin: What do they tell us?. Biopolymers (Peptide Science) **47,** 435-450.

Sparling DW, Linder G, Bishop CA. 2000. Ecotoxicology of Amphibians and Reptiles. In: SETAC Press, Pensacola, FL, *325*.

Trotti R, Carratelli M, Barbieri M. 2002. Performance and clinical application of a new, fast method for the detection of hydroperoxides in serum. Panminerva Medica **44,** 37–40.

Yu S, Wages MR, Cobb GP, Maul JD. 2013. Effects of chlorothalonil on development and growth of amphibian embryos and larvae. Environmental Pollution **181,** 329-34.

Yoon CK, Jin JH, Park JH, Youn HJ, Cheong SW. 2003. The fungicide benomyl inhibits differentiation of neural tissue in the *Xenopus* embryo and animal cap explants. Environmental Toxicology **18,** 327–337.