Turkish Journal of Biology

Volume 33 | Number 3 Article 1

1-1-2009

The toxicity and teratogenicity of gibberellic acid (GA3) based on the frog embryo teratogenesis assay-Xenopus (FETAX)

AYPER BOĞA

SEÇİL BİNOKAY

YAŞAR SERTDEMİR

TÜBİTAK

Turk J Biol
33 (2009) 181-188

doi:10.3906/biy-0807-30

The toxicity and teratogenicity of gibberellic acid (GA_3) based on the frog embryo teratogenesis assay-Xenopus (FETAX)

Ayper BOĞA[1], Seçil BİNOKAY[1], Yaşar SERTDEMİR[2]

[1]Department of Physiology, Faculty of Medicine, Çukurova University, Adana - TURKEY

[2]Bioistatistics, Faculty of Medicine, Çukurova University, Adana - TURKEY

Received: 23.07.2008

Abstract: This study investigated the developmental toxicity of a plant growth regulator (a type of pesticide) using the frog embryo teratogenesis assay-*Xenopus* (FETAX). *Xenopus laevis* embryos were exposed to 11 different concentrations of gibberellic acid (GA_3), from stage 8 to 11, for 96 h under static renewal test conditions. The median lethal concentration (LC_{50}), malformation (EC_{50}), non-observed adverse effect concentration (NOAEC), and lowest observed adverse effect concentration (LOAEC) were calculated.

The corresponding LC_{50} and EC_{50} values determined for GA3 exposure were 1117.5 mg/L and 658 mg/L, respectively. The TI (LC50/EC50) value calculated for GA_3 was 1.69. Different anomalies occurred in the embryos, depending on the GA_3 concentration. Based on these results, we conclude that GA_3 is toxic and teratogenic to *Xenopus laevis* embryos. Moreover, our results confirm that the FETAX assay can be a useful pretest for integrated biological hazard assessment of chemical agents used in agriculture.

Key words: Gibberellic acid, FETAX, Teratogenicity, Toxicity, *Xenopus laevis*

Gibberellik asit'in (GA_3) toksisite ve teratojenitesinin kurbağa embriyo teratogenezis testi (FETAX) kullanılarak değerlendirilmesi

Özet: Çalışmada, bitki büyüme düzenleyicisi gibberellik asitin (GA_3) (bir pestisit tipi) potansiyel gelişimsel toksisitesi FETAX testi kullanılarak değerlendirildi. *Xenopus laevis* embriyoları (evre 8-11); 96 saatlik statik yenileme test koşullarında, gibberellik asitin 11 konsantrasyonuna maruz bırakıldı. Muamelenin sonucunda, ortalama letal konsantrasyon (LC_{50}), ortalama malformasyon (EC50), etkinin hiç görülmediği konsantrasyon (NOAEC) ve etkinin ilk görüldüğü en düşük konsantrasyon (LOAEC) saptandı.

Gibberellik asite muamele sonucu *Xenopus* embriyolarında elde edilen LC50 ve EC50 değerleri sırayla 1117,5 mg/L and 658 mg/L, TI (LC_{50}/EC_{50}) değeri (GA_3)= 1,69 olarak bulundu. Gibberellik asit konsantrasyonunun artışına paralel olarak embriyolarda farklı tip anomaliler gözlendi. Bu sonuçlara göre, gibberellik asitin *Xenopus laevis* embriyoları için toksik ve teratojenik bir madde olduğunu söyleyebiliriz. Ayrıca, elde ettiğimiz sonuçlar FETAX'ın tarımda kullanılan kimyasalların biyolojik zarar değerlendirmesinde bir ön test olarak kullanılabileceğini teyit etmektedir.

Anahtar sözcükler: Gibberellik asit, FETAX, Teratojenite, Toksisite, *Xenopus laevis*

Introduction

The loss of global biodiversity and the emergence of infectious diseases are 2 of the most pressing environmental concerns, and the underlying causes are both complex and intertwined. Many amphibian species have disappeared or are in decline throughout the world. In addition, more than 60 different amphibian species with severe abnormalities have been observed in the United States of America and several other countries. Amphibians are considered by many biologists to be excellent bioindicators of the health of an environment (1).

Many chemicals are currently used in agriculture and plant growth regulators (PGRs) are among those widely employed (2). These chemicals, including gibberellins (GAs), exhibit great structural diversity. GAs belong to a large family of tetracyclic diterpenoid compounds, some of which function as endogenous plant growth regulators (3). Gibberellin was discovered in 1926 by a Japanese scientist, Eiichi Kurosawa, who was studying *bakanae*, the "foolish seedling" disease in rice. It was first isolated in 1935 by Teijiro Yabuta from fungal strains (*Gibberella fujikuroi*) provided by Kurosawa. Yabuta named the isolate gibberellin (4).

Gibberellins (GAs) are plant hormones that regulate growth and influence various developmental processes, including stem elongation, germination, dormancy, flowering, sex expression, enzyme induction, and leaf and fruit senescence (5). Gibberellic acid (GA_3) was the first gibberellin to be structurally characterized. Currently, there are 136 GAs that have been identified from plants, fungi, and bacteria (6).

The major ecological effects of GAs were not adequately addressed in early studies (7). Data obtained from such studies indicated that GAs were not likely to cause adverse effects in non-target avian, aquatic, and insect species In addition, these studies claimed that there were no potential risks to agricultural workers associated with eye, dermal, or inhalation exposure. These studies suggested that due to the lack of acute GAs toxicity they should placed in toxicity categories III (slightly toxic) and IV (non-toxic), and did not necessitate any additional evaluation regarding worker exposure (7).

Nonetheless, many recent studies report contradicting results. The Pesticide Action Network North America (PANNA) Pesticides database suggests that GA_3's acute toxicity, carcinogenicity, ground water contaminating characteristic, and developmental or reproductive toxicity should be reevaluated (8).

Due to their toxicity, pesticides are potentially hazardous to all organisms and the environment. According to the study, the 4 routes of exposure are dermal (skin), inhalation (lungs), oral (mouth), and ocular (eye) (9).

Although plant growth regulators are used to promote germination, flowering, proliferation, and fertilization in a wide variety of crops, little is known about the teratogenic effects of high doses of GA_3 in mammals; in fact, only 2 studies on teratogenicity in rats have been published (10,11). In addition, Uçkan et al. (2008) reported that GA_3 exposure adversely affected the developmental duration of egg to adult, adult longevity, fecundity, and sex ratio in the larval endoparasitoid, *Apanteles galleriae* (12).

Many types of agents in an aquatic environment bind to sediments, an important route of exposure in frog species (13). Additionally, humans may be exposed to GA_3 residues through their diet. Because of the hazards of GA_3 to aquatic organisms, evaluation of GA_3 toxicity is important for reducing the potential hazardous effects on aquatic organisms (14).

While several environmental agents are well known to cause developmental toxicity in humans (e.g. lead, ionizing radiation), many others are suspected as potential agents of developmental toxicity on the basis of data emerging from experimental animal studies (e.g. some pesticides) (15). Researchers suggest that the use of pesticides is correlated with anuran hind limb deformities in the St. Lawrence River Valley in Quebec (16).

Numerous laboratory studies have shown that many different contaminants can kill or deform amphibians. Deformed frogs have been observed in or near sources of human drinking water, and many malformed amphibians are observed in agricultural areas in which insecticides and fertilizers are used extensively (17).

According to Fort et al. (2004), only recently has evidence of the adverse effects of exposure to a variety of different physical and chemical stressors been provided; therefore, it is important to understand the effect on amphibians, and especially their embryos, of exposure to different kinds of environmental pollutants (18).

From the outset, pesticides have been suspected as a probable cause of amphibian declines. Due to their permeable skin, amphibians are especially vulnerable to foreign chemicals in the environment. In addition, most amphibians undergo a delicate, hormone-driven transformation from the larval to adult stage—a period of particular susceptibility to endocrine-disrupting chemicals (19).

Amphibians may be also harmed by the so-called inert ingredients added to improve the performance of a pesticide. Surfactants, for example, are detergent-like additives used to facilitate the dispersion and absorption of pesticides. Recent studies have demonstrated that the surfactants in a sample of 300 common pesticides increased the toxicity of the pesticides to tadpoles more than 10-fold that of the toxicity of the underlying active ingredient, glucose-phosphate. (20).

The frog embryo teratogenesis assay-*Xenopus* (FETAX) bioassay has been used to screen several environmental pollutants, but embryotoxic effects on amphibians in response to plant growth regulators has not been reported to date. As such, the present study used FETAX and focused on determining the characteristic amphibian abnormalities induced by different kinds of pesticides commonly used for agricultural production in Turkey.

As an alternative bioassay FETAX provides a rapid, simple, and cost-effective method for evaluating the mechanisms of developmental toxicity, on a preliminary basis. FETAX is a 96-h whole-embryo static renewal assay that uses *Xenopus laevis* embryos (1,21). It is essentially an organogenesis test—and organogenesis is highly conserved across amphibians and mammals. The first 96-h of embryonic development in *Xenopus* parallels many major processes observed in human organogenesis. Thus, FETAX should be useful in predicting potential human developmental toxicants and teratogens (1,22,23).

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) reported the validity of FETAX as a screening assay for detecting potential human teratogens, and for use in ecotoxicological assessment of water/soil/sediment samples (15).

The United State Environmental Protection Agency (USEPA) has employed the FETAX protocol to examine the developmental toxicity of an herbicide, an organochlorine insecticide, and a fertilizer component in different species of frog (14).

In all of these studies *Xenopus laevis* embryos were also tested and were often (but not always) observed to be more sensitive to the detrimental effects of the chemicals tested (13). FETAX has been used for single chemicals and chemical mixtures (24), as well as to estimate the ecotoxicological efficiency of a water processing plant (19).

The aim of the present study was to investigate the teratogenic and toxic effects of GA_3 (widely used in agricultural and horticultural production) on *Xenopus laevis* embryos using the FETAX system, and the implications for human health.

Materials and methods

The toxicity test employed strictly followed the procedures of the American Society for Testing Materials (ASTM) Standard Guide (24).

Test substances

The pesticide, GA_3 (Gibgro-T), was purchased from Agtrol Chemical Products (Houston, TX, USA). Follicle stimulation hormone (FSH) and human chorionic gonadotropin (hCG, pregnyl, 5000 IU) were respectively obtained from Serono and Organon (İstanbul, Turkey).

Test organism and bioassay

The African clawed frog (*Xenopus laevis)* is the species commonly proposed as a model for frog research (25). Adult Xenopus breeding and embryo collection were performed as described in ASTM-E1439-98 ASTM (24). Adult *Xenopus laevis*, bred at the Physiology Department, University of Çukurova (Adana, Turkey), were housed in aquaria (95 × 60 × 44 cm) at 23 °C (±1 °C) under a 12:12 h light:dark cycle and were fed 4-5 pieces of chicken liver (3g) twice weekly.

In vitro fertilization

In vitro fertilization was performed according to Lindi et al. (26). *Xenopus* females, 5-6 per experiment, were injected with 700-1000 IU of hCG 16 h before females laid their eggs in petri dishes. Eggs were immediately inseminated with sperm suspension obtained by mincing the testes in 1-2 mL of cold DBT (De Boers Tris, in mM: NaCl 119, KCl 2.5, $CaCl_2$ 1.8, and Tris/HCl 15, at pH 7.5). After 2 min, 10 mL of FETAX (containing in mg/L: NaCl 625, $NaHCO_3$ 96, KCl 30, $CaCl_2$ 15, $CaSO_4$. $2H_2O$ 60, and $MgSO_4$ 70, at pH 7.8-8.0) solution was added to each petri dish. Successful insemination was detected after a few minutes; only the eggs oriented with the dark side (animal pole) up were considered. All irregularly segmented eggs were eliminated. Normal developing embryos were selected up to the late blastula stage (stage 8-11) and used for the FETAX assay.

FETAX procedure

Two range-determining and 3 definitive concentration-response experiments were conducted. During the assays, embryos were cultured in a tissue culture incubator at 23 ± 1.0 °C. For each assay, 20-25 embryos were randomly placed in covered 100-mm glass petri dishes in 50 mL of different concentrations of GA_3 solution—from 1-1300 mg/L. All concentrations were tested with 4 petri dishes, using a total of 80-100 embryos. For the control groups, only FETAX solution was used. For the experimental groups, stock GA_3 solution was prepared daily in the FETAX solution.

All solutions were replaced every 24 h during the 4-day test with daily prepared GA_3 stock solution. Dead embryos were removed and fresh solution was added. After 96 h of exposure (stage 46 embryos) (27,28), living larvae were fixed in 3% (w/v) formalin (pH 7.0) and the number of malformed embryos (29) was determined using a dissection microscope.

Analytical methods

Probit analysis was used to determine the 96-h median lethal (LC_{50}) and teratogenic (EC_{50}) concentrations. A teratogenic index (TI) was calculated based on the 96-h LC_{50} value to 96-h EC_{50} value ratio, as a means of assessing the teratogenic potential of GA_3. The teratogenic risk potential of GA_3 was analyzed according to ASTM standards (24). Unfortunately, the minimum concentration to inhibit growth (MCIG/LC_{50}) values were not analyzed due to a lack of head-tail length measurements.

Utilizing statistical software (SPSS v.10.0) the non-observed adverse effect concentration (NOAEC) and lowest observed adverse effect concentration (LOAEC) values were determined using Dunnett's multiple comparison procedure (26).

Results and discussion

In the present study GA_3 was investigated because data on its teratogenic effects on higher animals is very limited. Additionally, GA_3 is found in a wide variety of biologically active compounds. We observed that exposure to GA_3 induced teratogenicity in *Xenopus* embryos. This is the first study to examine the effect of GA_3 using FETAX; therefore, we could not compare our results with previous results. On the other hand, John (1979) suggested that indole acetic acid (IAA) (a type of PGR) causes teratogenic effects in rats, such as cleft palate, exencephaly, ablepharia, dilated cerebral ventricles, and crooked tail (10). Frukawa and Abe (2004) studied the in vivo teratogenicity of IAA in rats and showed that IAA induced microcephaly in rat fetuses (11).

During the experimental period the mean mortality rate in the control group in FETAX solution was 0.83% (range: 0%-2.5%). Control results met the criteria established by the ASTM standard for test acceptance (24). Table 1 shows the mean mortality rate and malformation rate in the control and GA_3-exposed groups during the 96-h test, from stage 8 to 11 of the embryonic developmental period, based on FETAX results. As can be seen in Table 1, the highest mortality rate (100%) in the GA_3-exposed groups occurred at the highest GA_3 concentration. The LC_{50}, EC_{50}, TI, NOAEC, and LOAEC values observed in this study are presented in Table 2**.** GA_3 was teratogenic to *Xenopus laevis* based on ASTM criteria (24) when TI was calculated (LC_{50}/EC_{50}). The LC_{50} and EC_{50} values for GA_3 were 1117.5 mg/L (range: 1036-1220 mg/L) and 658.617 mg/L (range: 610-711 mg/L), respectively (Figure).

Compared to NOAEC (0.1 ppm) and LOAEC (0.3 ppm) values, LC_{50} and the EC_{50} values were much higher, yet are compatible with the EPA's tolerance values for GA_3 residues (0.15 ppm).

Table 1. Mortality and malformation rates in the control and treatment groups, based on FETAX assay.

Treatment	Dose range (mg/L)	n	Mean mortality (%)	Mean malformation (%)
Control	-	360	0.87 (0-2.5)	2.5 (1.67-3.33)
GA_3	0.1-1300	1200	19.7 (0-100)*	18.96 (1.33-65)*

n: Total number of embryos exposed to test solutions during the 96-h exposure period.
*Statistically different than the control embryos ($P < 0.05$).

Table 2. GA_3 teratogenic index (TI), NOAEC, and LOAEC values.

LC_{50} (95%CI)	EC_{50} (95%CI)	TI[a] (LC_{50}/EC_{50})	NOAEC	LOAEC
1117.5	658.617	1.69 [d]	0.1	0.3

LC_{50}, EC_{50}, and TI are expressed as mg/L.
[a]Teratogenic index (TI) determined by LC_{50}/EC_{50}.
NOAEC and LOAEC are expressed as mg/L.
[d]Substance is classified as teratogenic by ASTM (2004), based on LC_{50}/EC_{50}.

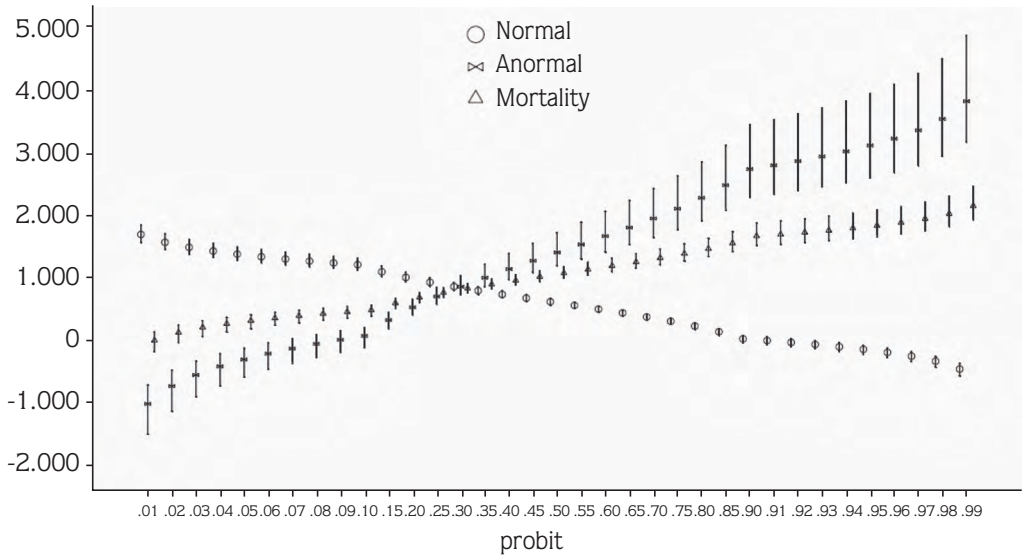

Figure. Estimated 95% confidence intervals for dilution by probit values adjusted for heterogeneity

In the present study we observed different types of malformations in *Xenopus laevis* embryos that were caused by GA_3. FETAX test results concerning the types and rates of malformation in *Xenopus laevis* embryos are shown in Table 3. The incidence of gut malrotation was 3.6% (n = 60) when exposed to GA_3 at concentrations ranging from 0.1 to 1100 to mg/L, and the hemorrhage incidence rate was 0.1% (n = 2) at 0.1 mg/L and 5 mg/L. Eye malformations included microphthalmia (10%, n = 167) when exposed to GA_3 at 1.0-1300 mg/L, microphthalmia (0.2%, n = 3) at 100 mg/L, 800 mg/L, and 400 mg/L, misplaced lens (0.5%, n = 9) at 0.2-5 mg/L, and cyclopia (0.1%, n = 2) at 1 mg/L and 60 mg/L. Body malformations included

Table 3. Different types of malformation caused by different concentrations of GA_3, based on FETAX.

Pesticide	Number of malformations	Concentration (mg/L)	Common Malformation Types (%)
GA_3	175	1-1300	Microcephaly (10.5)
	167	1-1300	Microphthalmia (10)
	151	0.5-1300	Kinked tail (9.6)
	96	0.1-1300	Edema (5.7)
	69	0.1-1300	Dermal blister or bullae (4.1)
	60	0.1-1100	Gut malrotation(3.6)
	33	400-1000	Micromelia (2.3)
	9	0.2-5	Misplaced lens (0.5)
	5	0.6,0.7-400	Anencephaly (0.3)
	3	100,400,800	Macropthalmia (0.2)
	2	0.1,5.0	Hemorrhage(0.1)
	2	1,60	Cyclops(0.1)
	2	5	Somite abnormalities (0.1)
	1	1000	Macroencephaly (0.06)

kinked tail (9.6%, n = 151) when exposed to GA_3 at 0.5-1300 mg/L, somite abnormalities (0.1%, n = 2) at 5 mg/L, and micromelia (2.3%, n = 33) at 400-1000 mg/L. Malformations of the head included microcephaly (10.5%, n = 175) when exposed to GA_3 at 1-1300 mg/L, anencephaly (0.3%, n = 5) at 400 mg/L and 0.6-0.7 mg/L, and macroencephaly (0.06%, n = 1) at 1000 mg/L. Dermal blisters or bullae were observed 4.1% (n = 69) at all concentrations and were evident all over the body surface, especially along the dorsal fin. Edema, seen in 5.7% of the embryos (n = 96) at all concentrations, was evident over the entire embryo, especially the gut.

In addition to the effects mentioned above, general embryonic health may also be adversely affected by the accumulation of GA_3 in the living embryos. These effects may be produced directly by the GA_3 agent or as a result of its biodegradation via different enzymes in aquatic organisms. Such biotransformation may enhance of reduce the toxicity/teratogenicity of GA_3.

The (USEPA) completed an assessment of the potential human health and environmental risk associated with the pesticidal use of GA_3 and its related isomers. Based on this assessment, the US EPA suggested that GA_3 was a non-toxic compound (14). It is currently unknown if the toxicity to *Xenopus laevis* embryos observed in the present study was a direct effect, or the result of biodegradation or of unknown factors produced during the exposure (9).

It is well recognized that different mechanisms of embryonic development can be affected by different concentrations of GA_3. The dose-dependent increases in the incidence of edema, tail flexure, microphthalmia, microcephaly, edema, and bullae observed in this FETAX assay study could not be compared with similar studies using amphibians and other vertebrates due to lack of available data.

Conclusion

Our results show that GA_3 is toxic and/or teratogenic to *Xenopus* embryos. The teratogenic index (TI) value indicates that the tested chemical should be considered a developmental toxicant, based on TI results greater than 1.5, according to ASTM (26) and NICEATM (15). The potential risk of GA_3 causing malformations in humans and its teratogenicity are not known for certain. As accessing the risk of exposure to GA_3 on the basis of this in vitro study is not sufficient, more data is needed on discharges and concentrations of this pesticide in the environment in order to perform a more reliable risk assessment.

Acknowledgements

This work was supported by the Scientific Research Office of Çukurova University (I.U BAP) (project no: TF2004BAP5).

The authors acknowledge Professor Veyis TANSI from the University of Çukurova, Faculty of Agriculture for reviewing the manuscript.

Corresponding author:
Ayper BOĞA
Department of Physiology,
Faculty of Medicine,
Çukurova University,
Adana - TURKEY
E-mail: aypbog@cu.edu.tr

References

1. Fort DJ, Stover EL, Farmer DR et al. Assessing the Predictive Validity of Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Teratogenesis Carcinogen Mutagen 20: 87-98, 2000.
2. Celik I, Turker M, Tuluce Y. Absisic acid and gibberellic acid cause increased lipid peroxidation and fluctuated antioxidant defense systems of various tissues in rats. J Hazard Mater 148: 623-629, 2007.
3. Kaneko M, Itoh H, Inukari Y, et al. Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants. Plant J 35: 104-153, 2003.
4. Gibberellins. www.en.wikipedia.org/wiki/Gibberellins, 2004. .
5. Celik I, Tuluce Y, Işık I. Evaluation of toxicity of absisic acid and gibberellic acid in rats: 50 days drinking water study. J Enzyme Inhib Med Chem 22(2): 219-226, 2007.
6. Gibberellin history: Function of gibberellin. www.plant-hormones.info/gibberellins.htm.
7. Gibberellins. http://www.epa.gov/pesticides/
8. Pesticide Action Network North America (PANNA). Pesticides info DATABASE. California Data. Pesticide Use Reports. http://www. panna.org/
9. The Pennsylvania State University (PENN STATE). Pesticide Safety Fact Sheet, Toxicity of Pesticides. www. pested.psu.edu/resources/facts/
10. John JA, Blogg CD, Murray FJ et al. Teratogenic effects of the plant hormone indole-3-acetic acid in mice and rats. Teratology 19(3): 321-4, 1979.
11. Frukawa S, Masayoshi A, Usuda K et al. Indole-3-acetic acid induces microencephaly in rat fetuses. Toxicologic Pathology 32:6 (659-667), 2004.
12. Uçkan F, Tüven A, Er A et al. Effects of gibberellic acid on biological parameters of the larval endoparasitoid ***Apanteles galleriae*** (Hymenoptera: Braconidae). Ann. Entomol. Soc. Am 101(3): 593-597, 2008.
13. Burkhart JC, Ankley G, Bell H et al. Strategies for assessing the implications of malformed frogs for environmental health. Environ Health Perspect Volume 108, Number 1, 2000.
14. United State Environmental Protection Agency (USEPA). Pesticide Registration (PR) Notice. 95-3. Reduction of Worker Protection Standard (WPS). www.epa.gov/PR_Notices.
15. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). FETAX background review document. Executive Summary, ES1-ES16, Appendix 13, FETAX TEST Method Protocol, 2004.
16. Quellet P. Characteristics and vertical Distribution of Atlantic cod (*Gadus morhua*) Eggs in the Northern Gulf of St. Lawrence and the possible effect of cold water temperature on recruitment. Can. J. Fish. Aquat. Sci 54: 211-223, 1997 .
17. Solcomhouse. Frogs. An Early Indicator of Global Disaster. http://www.solcomhouse.com/frogs.htm.
18. Fort DJ, Guiney PD, Weeks JA et al. Effect of methoxychlor on various stages of *Xenopus laevis*. Toxicol Sci 8(2): 454-466, 2004.
19. Gutleb AC, Appelman J, Bronkhorst MC. Delayed effects of pre and early life time exposure to polychlorinated biphenyls on tadpoles of two amphibian species (*Xenopus laevis* and *Rana temporaria*). Environ Toxicol Pharmacol 8(1): 1-14, 1999.
20. AmphibiaWeb. Worldwide Amphibian Declines: How big is the problem, what are the causes and what can be done? http://amphibiaweb.org/declines/declines. html
21. Mann RM, Bidwell R. Application of the FETAX protocol to assess the developmental toxicity of nonyphenol ethoxylate to *Xenopus laevis* and two Australian Frogs. Aquat Toxicol 51(1): 19-29, 2000.
22. Boğa A, Binokay S, Kendirci A, et al. Morphological changes in *Xenopus laevis* embryos with lithium and evaluation of the FETAX test. Turk J Biol 24:177-188, 2000.
23. Vismara C, Battista V, Valiati G et al. Paraquat induced embryotoxicity on *Xenopus laevis* development. Aquat Toxicol 49(3): 171-179, 2000.
24. American Society for Testing Materials (ASTM). Standart guide for conducting the frog embryo-teratogenesis assay - *Xenopus*, FETAX, E1439-98. In: ASTM Standarts on biological effects and environmental fate. Vol. 11. 05. Philadelphia, PA, 447-457, 2004.

25. Boğa A, Binokay S, Kendirci A, et al. Experimental embryology in *Xenopus laevis*. Turk J Biol 21:141-148, 1997.

26. Lindi C, Gigliola M, Rossi F et al. Effect of ethanol exposure on Xenopus embryo lipid composition. Alcohol Alcohol 36(5): 388-392, 2001.

27. Nieuwkoop PD, Faber J. Normal Table of *Xenopus laevis* (DAUDIN). Garland Publishing, INC. Newyork & London, 1994.

28. Özgünen T, Boğa A. *Xenopus laevis* Normal Tablosu [Normal Table of *Xenopus laevis*], Mesleki Çeviri Kitap, Çukurova Üniv., Adana; 2006 (Accepted).

29. Boğa A, Özgünen T. *Xenopus laevis* Anormal Atlası [Abnormal Atlas of *Xenopus laevis*]. Mesleki Kitap , Çukurova Üniv., Adana; 2006 (Accepted).