Using fish embryos in assessment the toxicity of brominated phenols

Tamer El-Sayed Ali

Oceanography Department, Faculty of Science, Alexandria University, Egypt.
Email: tameraly@yahoo.com

Abstract

Brominated phenols or polybrominated diphenyl ethers (PBDEs) are flame-retardant potential toxic chemicals that accumulate in human tissues. To monitor general toxicity of this class of metabolites, embryos of zebrafish (*Danio rerio*) were exposed to gradual concentrations of 2-hydroxy brominated diphenyl ether 123 (2OH-BDE123). Exposures were done by immersion of 1 hour post fertilization (hpf) zebrafish eggs to 72 hpf, nominal concentration range of 0.03 - 2.5 µM. Embryos/ larvae were assessed daily for death and structural defects. Results revealed that concentrations from 0.3 µM of such metabolites were toxic to the developing zebrafish causing serious morphological deformations. Both toxicity incidence and potency were correlated with the concentration applied.

Keywords: *Danio rerio* - Metabolites - Toxicity - embryo test

1. Introduction

Fish embryos represent an attractive model for environmental risk assessment of chemicals since they offer the possibility to perform small-scale, high-throughput analyses (Scholz *et al*., 2008). Embryos of zebrafish have often been used in toxicity studies of environmentally relevant substances (Kosmehl *et al*., 2006; Scholz *et al*., 2008, Kammann *et al*., 2009, El-Sayed Ali and Legler, 2011).

Zebrafish (*Danio rerio*) is an emerging toxicity screening model for both human health and ecology (Padilla *et al*., 2011). It is a suitable experimental organism in toxicological research due to its short spawning intervals, easy manipulation and adaptation to the laboratory setting, rapid development and generation time, transparent eggs which can be maintained in a relatively small space, (Nagel, 2002; Braunbeck *et al*., 2005) facilitating visual identification of morphogenetic movements and organogenesis with a standard dissecting microscope. Beyond their application for determining the acute toxicity, fish embryos are also excellent models for studies aimed at the understanding of toxic mechanisms and the indication of possible adverse and long-term effects. (Scholz *et al*., 2008). Several endpoints can be detected in parallel, thereby enhancing the toxicity spectrum covered by the assay (Kammann *et al*., 2009).

PBDEs are a class of chemicals that accumulate in human tissues and are potential toxicants. Concentrations of PBDEs in human tissues have increased recently due to the industrial revolution. Although many PBDEs have not been well studied, new concern has been raised on their hydroxylated congeners. In aquatic organisms, PBDEs can be converted to mainly hydroxylated (-OH) forms after biotransformation, and their hydroxylated metabolites are found to be diverse among living organisms (Hakk and Letcher, 2003). Hydroxylated polybrominated diphenyl ethers or brominated phenols (OH-PBDEs) have been reported to be found in aquatic and terrestrial wild life and human plasma. Many of these compounds are naturally occurring while others are thought to be either metabolic or environmental transformation products of the commercially produced PBDEs which are a class of brominated flame retardants (Legler and Brouwer, 2003). High concentrations of OH-BDEs have also been detected in algae and sponges as their natural anti-bacterial products to prevent infection (Sharma *et al*., 1970; Handayani *et al*., 1997; Malmvärn *et al*., 2005).

The present paper aimed to study the hydroxylated BDE 123 metabolite, an important metabolite within the pool of hydroxylated PBDEs retained in biological systems and have potential endocrine disrupting properties (Canton *et al*., 2005; Hamers *et al*., 2006) and to test the *in vivo* toxicity and teratogenic action of 2-OH-BDE123 on developing zebrafish (*Danio rerio*) embryos .

2. Materials and Methods

2.1. Metabolite solutions

Five stock solutions (0.3, 1, 3, 10 and 25 mM) of 2OH-BDE123 were dissolved in dimethyl sulfoxide (DMSO, 0.01%) immediately prior to use and then directly diluted 10000 times in Dutch standard water (nominal concentrations: 0.03, 0.1, 0.3, 1 and 2.5 µM). Solvent (DMSO, 0.01%) and positive (6-OH-BD47) controls were incorporated in the experiment.

2.2. Fish maintenance

Formatted: After: 0.63 cm, Pattern: Clear (White)

Zebrafish were raised and kept under standard laboratory conditions at about 28°C and a photoperiod of 14:10 h. light: dark (Brand *et al*., 2002). Fish were fed with dry fish feed, Tetra-Pro Flakes (Tetra GmbH, Germany) in the morning and hatched brine shrimp (Artemia cysts from INVE, Grantsvillle, UT, USA) in the afternoon. The fish were acclimated in glass aquaria containing copper free water. Typically, the eggs were spawned synchronously at dawn of the next morning. One hour later, eggs quality has been checked under the microscope (Leica MZ 75), being sure to select the healthy, fertilized eggs for the experiment. Fish breeding and embryo manipulation were conducted according to Westerfield *et al*. (1997).

2.3. Embryo test

Fertilized eggs (1 hour post fertilization, hpf) were selected and placed in 24-well cell culture sterilized plates (one embryo/well). Embryos were exposed to the previously mentioned concentrations of AP at the 4:8 – cell stage (1:1.25 hour post fertilization, hpf). 24 embryos/concentration were used and incubated at 28°C. Embryos/larvae were screened daily - till 72 hpf - and scored for survival, alterations in morphology, developmental abnormalities and endpoints of toxicity (Nagel, 2002). Toxic/lethal end points (coagulation, missing heart beat, missing somites, missing tail detachment, missing spontaneous movement) and non-lethal malformations (pericardial or yolk sac oedema, bent notochord, fin malformation, no pigmentation, incomplete head and eye development) were reported separately.

2.4. Morphological observation of embryos

Developed embryos/larvae were examined and photographed daily by a stereo microscope. Paint shop Pro. 8 image analysis software was utilized to control a Roper digital camera on the microscope. Images were depicted at all treatment levels to complete the picture of the morphological abnormalities in different organs.

2.5. Calculation of LC_{50} and EC_{50}

The LC_{50} and EC_{50} were calculated at 120 h post fertilization from concentration-% lethality and concentration-% effect curves, respectively for all end points separately as well as for the sum of lethal affected embryos. Logistic curves with binomially distributed errors were used to describe the relationships. From these, LC_{50} and EC_{50} values and their 95% confidence intervals were calculated using Graph Pad Prism 5.01.

3. Results

Embryo-toxicity assay in the developing zebrafish was optimized. The dose-related effects of the metabolite were determined following 72 h exposure starting at 1 hour post fertilization (hpf). The results showed a very high toxic potential for this compound related to the levels (Figures 1-3).

The groups treated with 0.03 and 0.1 µM 2OH-BDE123, no effects were reported during all the test period, presenting embryos similar to those of the DMSO control group. 20% of the embryos subjected to 0.3 µM of 2OH-BDE123 were coagulated or non-lethally mal-formed at 48 hpf and the 100% mortality was shown at 72 hpf. Concentrations 1 and 2.5 µM were lethal within the first few hours of exposure, all embryos stopped their development in the epiboly stage. The developmental effects of 2OH-BDE123 were dose dependent with an EC_{50} value of 0.19 µM for all endpoints and LC_{50} of 0.41 µM. Embryos exposed to 6-OH-BD47, showing slow developed embryos with neither heart beat nor detached tail from the beginning and being in this form during all the test period without further growth, it's used only for checking the validity of the test condition during the study period.

24 hpf

Embryos subjected to the concentrations of 0.03, 0.1 and 0.3 µM were not altered morphologically, compared with those of the DMSO control group during the first 24 h of development, showing well developed healthy embryos with somites, yolk sac, tail, head, eyes, prominently sculptured brain and few pigment cells are present along the axis dorsal to the yolk extension and on the dorsal part of the yolk ball (Figure 1, A), similar to the control ones. While, embryos exposed to 1 and 2.5 µM showed coagulation resulted from development stop in epiboly stage (Figure 1, B).

48 hpf

Embryos exposed to concentrations of 0.03 and 0.1 µM showing embryos with well-developed notochord, otolith, caudal fin, head, eyes and pigment extends the whole length of the body (Figure 2, A), similar to the control group embryos. The 0.3 µM treated-group showing slight delay in the growth with oedema and a slightly unstraight notochord with mal-formed tail (curved, short, no tail fin) in 12% of the embryos (Figure 2, B). However, blood circulates through a closed set of channels and clear heart beats were measured and ranged between 119-120 beats/ min., as all other groups. Also, 8% of the embryos subjected to the 0.3 µM were coagulated (Figure 2C), the rest (80%) of this group was healthy as those of the control group.

72 hpf

Chorion of the embryos was ruptured and the larvae were hatched with quite elongated pectoral fin buds and vigorous heart beats were observed in the DMSO

Formatted: After: 0.63 cm

control group and those treated with 0.03 and 0.1 µM of 2OH-BDE123 (Figure 3A). Also, it was shown that the yolk sac started to be shrunk making the pericardial cavity more conspicuous (Figure 3A). For the rest of the embryos (80% of the total recorded at the beginning, 20% abnormal embryos, were dead after 48 h examination) treated with 0.3 µM, 32% of the embryos were coagulated, 12% were unhatched (Figure 3C), the rest were hatched with slight growth retardation, delayed development of caudal fin and curved notochord were shown (Figure 3B). Also reduction in heart beats number (80 beats/min.) was detected in the hatched larvae.

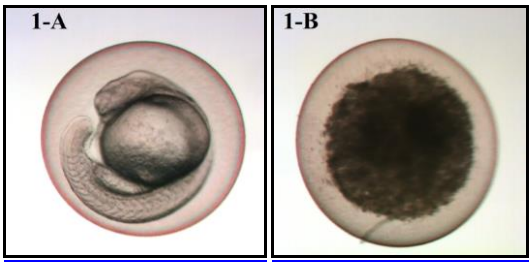

Figure 1. Morphological alterations in zebrafish embryos exposed to different concentrations of 2OH-BDE123 and were photographed live in lateral orientation through a stereomicroscope at 24 h post fertilization (hpf). Embryos exposed to concentrations of 0.03, 0.1 and 0.3 µM, showing well developed embryo with yolk sac, tail, head, eyes and pigmentation similar to the control group embryos (1-A). Embryos exposed to 1 and 2.5 µM showing development stop in epiboly stage (coagulate, 1-B), (×4).

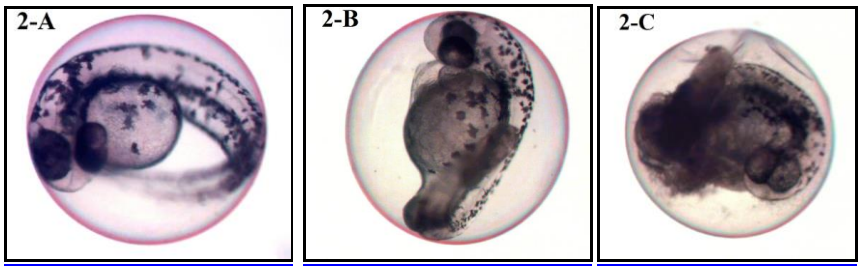

Figure 2. Morphological alterations in zebrafish embryos exposed to different concentrations of 2OH-BDE123 and were photographed live through a stereomicroscope at 48 h post fertilization (hpf). Embryos exposed to concentrations of 0.03 and 0.1 µM, showing embryos with well developed notochord with muscles, otolith, caudal fin, head, eyes and pigmentation similar to the control group embryos (2-A). 12% of the 0.3 µM treated group, showing mal-formed embryos with yolk sac oedema and mal-formed short tail with no tail fin (2-B) and 8% of the embryos started to coagulate (2-C), (×4).

Formatted: After: 0.63 cm, Pattern: Clear (White)

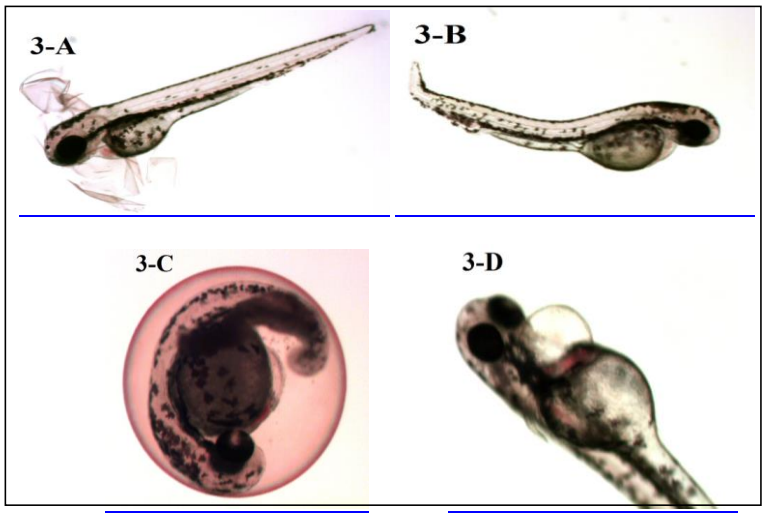

Figure 3. Morphological changes in zebrafish embryos exposed to different concentrations of 2OH-BDE123 and were photographed live in lateral orientation through a stereomicroscope at 72 h post fertilization (hpf). Embryos exposed to concentrations of 0.03, 0.1 µM showing well developed hatched larvae similar to the control group larvae (3-A, ×2). The majority of embryos exposed to 0.3 µM showing hatched larvae with a curved notochord and severe oedema (3-B & D, ×2), 12% of the embryos of this group presented delayed growth or no more growth than recorded at 48 hpf, i.e. unhatched larvae (3-C, ×4).

4. Discussion

Considering the limited information of the effect of phenolic metabolites (Canton *et al*., 2008), this study investigates the effects of environmentally relevant concentrations of 2OH-BDE123 (0.03 : 2.5 µM) on the embryos morphology. The toxicity level varied according to the concentration, i.e, for the highest nominal concentrations of 1 and 2.5 µM, the experiment was terminated at 24h examination, whereas for 0.3 µM the lethality and non-lethality endpoints were shown at 72 hpf, explaining the acute immediate toxicity of the first concentration and the non-lethal action (endpoints are inhibition of the embryonic development and oedemas) of the second one during the first 72 hpf. This study demonstrates that the developmental effect of phenolic metabolite (2 OH-BDE123) is dose dependent with a LC_{50} value of 0.41 µM and EC_{50} for lethal endpoints of 0.19 µM,. Very Recently, El-Sayed Ali (2010) demonstrated that metabolites of the same family (6 OH-BDE90) caused lethal as well as non-lethal malformation during zebrafish embryo development, presenting similar acute toxicity and non lethal endpoints at near similar levels.

The rate of uptake, metabolism and excretion are important parameters determining the bioavailable and internal, effective concentrations of an environmental chemical in an organism. Metabolization of phenols in the fish causing numerous direct and indirect effects ranging from changes in gene expression (Arukwe *et al*., 2002; Larkin *et al*., 2003) through induction of estrogen responsive genes (Arukwe *et al*., 2001) and protein (Andreassen *et al*., 2005) and effects on brain muscarinic receptors (Jones *et al*., 1998) to increase apoptosis (Weber *et al*., 2002), expression of acute phase protein (Baldwinm *et al*., 2005) and changes in phase II detoxication (Hughes and Gallagher, 2004).

A number of studies have provided evidence that embryos exhibit a strong bioconcentration potential (Gorge and Nagel 1990; Wiegand *et al*. 1999; Schreiber *et al*., 2009). The degree of toxicity of these compounds varies according to the dose and exposure period (El-Sayed Ali, 2010). Additionally, the nature of its effects on the zebrafish embryo differs according to the embryonic/larval phase. Similarly, the effect of their metabolites could be interpreted.

This work explores the potential of the effects of the phenolic PBDE in living organisms. Such information would be valuable for better estimating the risk of such class of chemicals and for following up their mode of

Formatted: After: 0.63 cm

actions. These, studies based on the metabolites are an important part of risk assessment of such xenoestrogens on different organisms.

Acknowledgments

The author would like to express thanks to Professor Juliette Legler, Institute of Environmental Studies, Vrije University, Amsterdam, The Netherlands for giving the opportunity to realize this work by using the institute facilities.

References

Andreassen, T.K.; Skjoedt, K., Korsgaard, B.: 2005, Upregulation of estrogen receptor alpha and vitellogenin in eelpout (*Zoarces viviparus*) by waterborne exposure to 4-tert-octylphenol and 17beta-estradiol. *Comparative Biochemistry and Physiology*, C 140: 340-346.

Arukwe, A.; Kullman, S.W. and Hinton, D.E.: 2001, Differential biomarker gene and protein expressions in nonylphenol and estradiol-17beta treated juvenile rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology*, C 129: 1-10.

Arukwe, A.; Kullman, S.W.; Berg, K.; Goksoyr, A. and Hinton, D.E.: 2002, Molecular cloning of rainbow trout (*Oncorhynchus mykiss*) eggshell zona radiata protein complementary DNA: mRNA expression in 17beta-estradioland nonylphenol-treated fish. *Comparative Biochemistry and Physiology*, B 132: 315-326.

Baldwinm, W.S.; Roling, J.A.; Peterson, S. and Chapman, L.M.: 2005, 'Effects of nonylphenol on hepatic testosterone metabolism and the expression of acute phase proteins in winter flounder (*Pleuronectes americanus*): comparison to the effects of Saint John's Wort', *Comparative Biochemistry and Physiology*, C 140: 87–96.

Brand, M.; Granato, M. and Nüsslein-Volhard, C.: 2002, "Keeping and raising zebrafish" in: C. Nüsslein- Volhard and R. Dahm (eds.), Zebrafish: A Practical Approach. IRL Press, Oxford, pp. 261.

Braunbeck, T.; Boettcher, M.; Hollert, H.; Kosmehl, T., Lammer, E.; Leist, E.; Rudolf, M. and Seitz, N.: 2005, Towards an alternative for the acute fish LC(50) test in chemical assessment: the fish embryo toxicity test goes multi-species-an update', *ALTEX*, 22:87-102.

Canton, R. F.; Sanderson, J. T.; Letcher, R. J.; Bergman, A. and van den Berg, M.: 2005, Inhibition and induction of aromatase (CYP19) activity by brominated flame retardants in H295R human adrenocortical carcinoma cells. *Toxicological Sciences*, 88 (2): 447-455.

Canton，R.F.; Scholten, D.E.; Marsh, G.; de Jong, P.C. and van den Berg, M.: 2008, Inhibition of human placental aromatase activity by hydroxylated polybrominated diphenyl ethers (OH-PBDEs). *Toxicology and Applied Pharmacology*, 227 (1): 68-75.

El-Sayed Ali, T.: 2010, 'eratogenic effect of phenolic metabolites on zebrafish (*Danio rerio*) embryos. *Egyptian Journal of Aquatic Research*, 36(4): 661-667.

El-Sayed Ali, T. and Legler, J.: 2011, Developmental toxicity of nonylphenol in zebrafish (*Danio rerio*) embryos. *Indian Journal of Marine Sciences*, 40(4): 509-515.

Gorge, G. and Nagel, R.: 1990, Kinetics and metabolism of 14c-lindane and 14c-atrazine in early life stages of zebrafish (*Brachydanio-Rerio*). *Chemosphere*, 21: 1125-1137

Hakk, H. and Letcher, R.J.: 2003, Metabolism in the toxicokinetics and fate of brominated flame retardants-a review. *Environment International*, 29: 801-828.

Hamers, T.; Kamstra, J.H.; Sonneveld, E.; Murk, A.J.; Kester, M.H.; Andersson, P.L.; Legler, J. and Brouwer, A.: 2006, In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. *Toxicological Sciences*, 92(1): 157-173.

Handayani, D.; Edrada, R.A.; Proksch, P.; Wray, V.; Witte, L. and van Soest, R.W.; Kunzmann, A.: 1997, Soedarsono Four new bioactive polybrominated diphenyl ethers of the sponge *Dysidea herbacea* from West Sumatra, Indonesia. *Journal of Natural Products*, 60(12): 1313-1316.

Hughes, E.M. and Gallagher, E.P.: 2004, Effects of 17-beta estradiol and 4-nonylphenol on phase II electrophilic detoxification pathways in largemouth bass (*Micropterus salmoides*) liver. *Comparative Biochemistry and Physiology*, C 137: 237-247.

Jones, S.B.; King, L.B.; Sappington, L.C.; Dwyer, F.J.; Ellersieck, M. and Buckler, D.R.: 1998, Effects of carbaryl, permethrin, 4-nonylphenol, and copper on muscarinic cholinergic receptors in brain of surrogate and listed fish species, *Comparative Biochemistry and Physiology*, C 120: 405-414.

Kammann, U.; Vobach, M.; Wosniok, W.; Schäffer, A. and Telscher, A.: 2009, Acute toxicity of 353-nonylphenol and its metabolites for zebrafish embryos. *Environmental Science and Pollution Research*, 16: 227-231.

Kosmehl, T.; Hallare, A.V.; Reifferscheid, G.; Manz, W.; Braunbeck, T. and Hollert, H.: 2006, A novel contact assay for testing genotoxicity of chemicals and whole sediments in zebrafish embryos. *Environmental Toxicology and Chemistry*, 25: 2097-2106.

Larkin, P.; Knoebl, I. and Denslow, N.D.: 2003, Differential gene expression analysis in fish exposed to endocrine disrupting compounds.

Comparative Biochemistry and Physiology, B 136: 149-161.

Legler, J.; Brouwer, A.: 2003, Are brominated flame retardants endocrine disruptors?. *Environment Int*ernational, 29: 879-885.

Malmvärn, A.; Marsh, G.; Kautsky, L.; Athanasiadou, M.; Bergman, A. and Asplund, L.: 2005, Hydroxylated and methoxylated brominated diphenyl ethers in the red algae *Ceramium tenuicorneand* and blue mussels from the Baltic Sea. *Environmental Science and Technol*ogy, 39 (9): 2990-2997.

Nagel, R.: 2002, DarT: the embryo test with the zebrafish *Danio rerio*-a general model in ecotoxicology and toxicology. *Altex-Altern Tierexp*, 19(Suppl 1): 38-48.

Padilla, S.; Corum, D.; Padnos, B.; Hunter, D.L.; Beam, A.; Houck, K.A.; Sipes, N.; Kleinstreuer, N.; Knudsen, T.; Dix, D.J. and Reif, D.M.: 2011, Zebrafish developmental screening of the ToxCast™ Phase I chemical library. *Reproductive Toxicology*, on line.

Scholz, S.; Fischer, S.; Gündel, U.; Küster, E.; Luckenbach, T. and Voelker, D.: 2008, The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. *Environmental Science and Pollution Research*, 15: 394-404.

Schreiber, R.; Altenburger, R.; Paschke, A.; Schüürmann, G. and Küster, E.: 2009, A novel *in vitro* system for the determination of bioconcentration factors and the internal dose in zebrafish (*Danio rerio*) eggs. *Chemosphere*, 77: 928-933.

Sharma, G.M.; Vig, B.; Burkholder, P.R.: 1970, Studies on the antimicrobial substances of sponges: Structure of a bromine-containing compound from marine sponge. *Journal of Organic Chemistry*, 35 (8): 2823-2826.

Weber, L.P.; Kiparissis, Y.; Hwang, G.S.; Niimi, A.J. and Jantz, D.M.: 2002, Increased cellular apoptosis after chronic aqueous exposure to nonylphenol and quercetin in adult medaka (*Oryzias latipes*). *Comparative Biochemistry and Physiology*, 131: 51-59.

Westerfield, M.; Doerry, E.; Kirkpatrick, A.E.; Driever, W. and Douglas, S.A.: 1997, An on-line database for zebrafish development and genetics research. *Seminars in Cell and Developmental Biol*ogy, 8: 477-488.

Wiegand, C.; Pflugmacher, S.; Oberemm, A.; Meems, N.; Beattie, K.; Steinberg, C. and Codd, G.: 1999, Uptake and effects of microcystin-LR on detoxication enzymes of early life stages of the zebra fish (*Danio rerio*). *Environmental Toxicology*, 14: 89-95.

Formatted: After: 0.63 cm, Pattern: Clear (White)

Formatted: Left: 2.2 cm, Right: 2.3 cm, Top: 2.6 cm, Bottom: 2.6 cm, Section start: Continuous, Width: 21 cm, Height: 29.7 cm, Header distance from edge: 2 cm, Footer distance from edge: 2 cm, Different first page header

Formatted: After: 0.63 cm

أستخدام أجنة أسماك الزيبرا لمعرفة التاثيرات السمية لمركب البرومو فينول

تامر السيد على

المعهد القومي لعلوم البحار والمصايد

يعتبر البرومو فينول مركب عضوى أيضي وسطي يدخل فى العديد من الصناعات , لذا فهو يتواجد في البيئات المائية من خلال الصرف الصناعى و يعتبر أحد الملوثات الخطرة على البيئة. لقد ركزت هذة الدراسة علي دراسة التاثيرات السمية لمركب البرومو فينول 2OH-BDE123 علي نمو أجنة أسماك الزيبرا، حيث تم تعريض بيض هذا النوع من الأسماك بعد ساعه واحده من تلقيحه الى تركيزات مختلفة ومتدرجة للمركب لمدة ٧٢ ساعة لمعرفة مدى تأثيره على نمو و معدلات الحياه لأجنه هذ النوع من الأسماك. ولقد أوضحت الدراسة التأثير السمى للمركب فى الأجنة تتناسب طردياً يتدرج مع درجة تركيزات الجرعة المستخدمة. كما أوضحت هذة الدراسة أيضا أن التعرض المبكر لهذا النوع من المركبات له تاثير مباشر علي نفوق هذه الأجنة و قد يسبب تشوهات عند تعرضها لجرعات اكبر من ٠.٣ µ مول .