Determination of 17β-Estradiol Concentration in Aquatic Environment of Peninsular Malaysia using the ELISA Technique

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Abstract: Effluents from municipal and industrial wastewater treatment plants, agricultural run-off and domestic drainage add numerous pollutants to the aquatic system. Among them is 17β-estradiol (E2), which is known as one of the strongest estrogenic chemical in the environment and commonly found in wastewater. This study was conducted to assess present concentration of E2 in aquatic environment of Peninsular Malaysia. Water samples were collected from rivers, estuaries and lakes at the northern, central and southern regions of Peninsular Malaysia. E2 concentration was determined using ELISA technique. Results revealed that the center region has the highest E2 average concentration (149.19 ng/L), followed by the northern region (95.04 ng/L) and southern region (15.66 ng/L). These results exhibit size of human population and activities may directly related with E2 elevation in aquatic environment as reflected by high human population in the central region. The lakes have the lowest concentration (11.83 ng/L), thus suggesting E2 discharges flow directly into river system. Comparison with other countries demonstrates E2 in aquatic environment in Peninsular Malaysia is higher several folds. If no immediate actions are implemented to control E2 level in the environment, there could be some adverse effects, especially to male aquatic animals, such as ova-testis, feminization, fertility reduction and villetogenin gene expression. Application of suitable bio-indicator is recommended in order to monitor estrogenic activities in aquatic environment.

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1. Introduction

Industrial, household, domestic and agricultural activities are among many other activities in this world that involved with the usage of chemicals. Most of these chemicals will end up in the water bodies and become contaminant as it show adverse effects to the organism that live in the water bodies and also human health. There is a wide range of chemical compound that can be found in the water bodies such as heavy metals, organochlorine pesticides, phthalates, alkylphenols, natural hormones and pharmaceuticals. Among these compounds, natural and synthetic estrogens are shown to be the most potent in in-vitro (Gutendorf and Westendorf, 2001) and in vivo studies (Laws et al., 2000). Estrogenic pharmaceuticals are prescribed principally for birth control and estrogen replacement therapy like mestranol or the estradiol valerate are widely used. These compounds, in addition to natural hormones, 17β-estradiol (E2), estrone (E1) and estriol (E3), are excreted by women and consequently found in wastewater (Lee and Peart, 1998; Aris et al., 2014). E2 is known as one of the strongest estrogenic chemical in the environment (Imai et al., 2005). A high concentration of E2 has shown to bring adverse effect to organism from molecular to a community level (Ismail et al., 2007a). There are concerns among scientists about the route of bioaccumulation of pollutants (including environmental hormones) in aquatic food web (Zulkifli et al., 2010, 2014). In order to understand this process, several biological indicators can be used to monitor the health of an environment or ecosystem through qualitative analysis such as behavior, morphology, population, biochemical process and others (Ismail et al., 1991, 1995, 2004; Ismail and Ramli, 1997; Mohamat-Yusuff et al., 2010, 2011, 2014; Sow et al., 2012, 2013; Khodadoust et al., 2012, 2013). The use of bio-indicator offers time integrated measures of those portions of the ambient pollutant load that are of direct ecotoxicological relevance (Rainbow, 1995). The purpose of this study is to assess present concentration of 17β-estradiol in aquatic environment of Peninsular Malaysia using the ELISA technique.

2. Material and Methods

Water surface sampling was conducted in the selected aquatic ecosystems (rivers, estuaries and lakes) from the north to the south of Peninsular Malaysia. Surface water samples were collected in triplicates from 99 stations. All water samples were placed in sterilized glass tubes and stored in a refrigerator (<8ºC) before further analysis. The 17β-estradiol (E2) analysis was conducted by using ELISA method for measuring E2 (Otsuka Lifescience Initiative, 2003; Ismail et al., 2007b) where it involved the use of 17β-estradiol EIA kit manufactured by the Yanaihara Institute Inc., Shizuoka, Japan. In brief, the first reaction was conducted by adding 50mL of Biotin-E2 Conjugate, 100ml of 17β-Estradiol standards or samples and 50 ml of anti-serum were added into the microplate wells, sealed and incubated for 18-20 hours at 4-8ºC. The second reaction was conducted by adding 100 ml of SAHRP, sealed and swirled for 2 hours at 20-30ºC. After that, 100ml of OPD solution was added and leaved in the dark for 20 minutes at 20-30ºC for reaction. The reaction was stopped by adding 100ml of stop solution (1M H_2SO_4). The absorbance was then measured at 490nm and 650nm. E2 concentrations in water samples were calculated using the SOFTmax Pro software produced by Molecular Devices Corporation, USA.

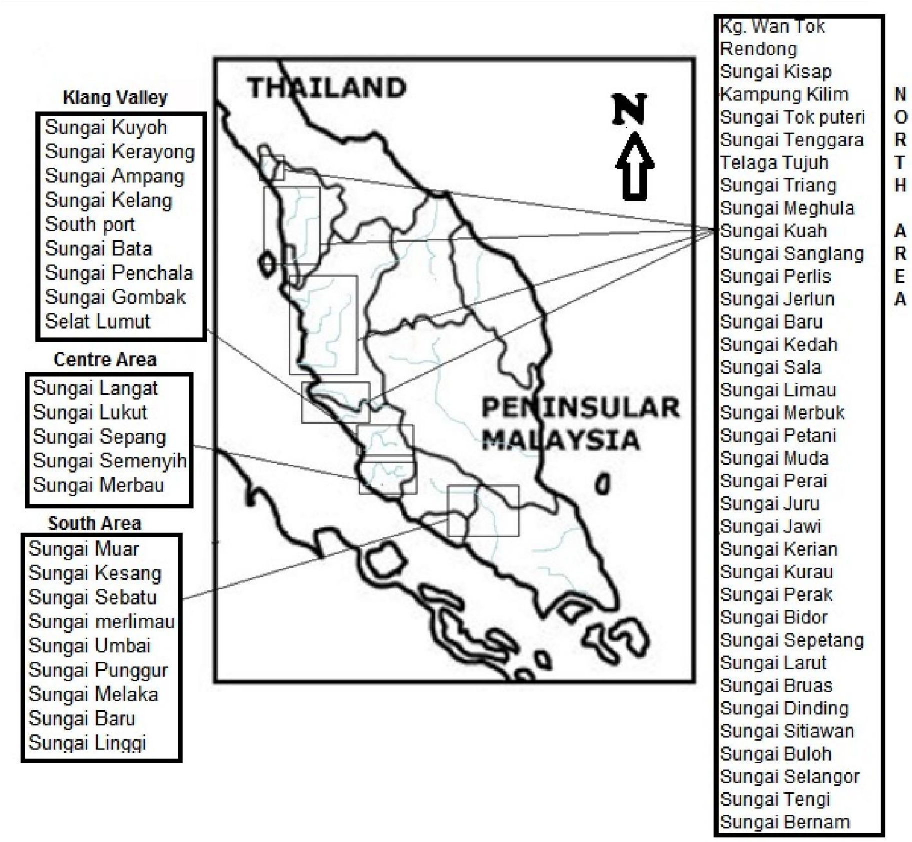

Figure1. Location of the water sampling sites from north to south of Peninsular Malaysia

3. Results

Results from this study reveal that concentration of E2 in aquatic environment of Peninsular Malaysia varies among localities. The data range between not detectable to almost 3700 ng/L (Table 1). Table 2 shows E2 mean concentrations based on zonation. E2 average concentration on the southern area is 15.66 ng/L. The central region with almost 150 ng/L of E2 concentration is the highest average among all other region. The northern region showed the highest average concentration of E2 in Malaysia which is 95.04 ng/L with max concentration of 3677.4 ng/L. The lakes have an average concentration of 11.83 ng/L. Even the lakes area is not the lowest average concentration but it have the lowest maximum concentration among the other maximum concentration which is 27.7 ng/L. The minimum value E2 concentration was around 2.1 ng/L to not detectable.

Table 1: Mean concentration of 17β-estradiol (ng/L) in all sampling locations around Peninsular Malaysia

Region	Site	Description	Mean (ng/L)	Region	Site	Description	Mean (ng/L)
Northern	Kg, Wan Tok Rendong	River	2.2	Central	Sungai Buloh	River	N.D
	Sungai Kisap	River	ND		Sungai Selangor	River	7.6
	Kampung Kilim	River	3.5		Sungai Tengi 1	River	3677.4
	Sungai Tok puteri	River	ND		Sungai Tengi 2	Estuary	13.6
	Sungai Tenggara1	River	ND		Sungai Bernam 1	Estuary	11
	Sungai Tenggara2	River	1.5		Sungai Bernam 2	River	6.7
	Telaga Tujuh	River	2.8		Sungai Bernam 3	River (upstream)	N.D
	Sungai Triang 1	River	7.3		Sungai Kuyoh	River	45.8
	Sungai Triang 2	Estuary	6.2		Sungai Kerayong	River	51.8
	Sungai Meghula	River	115.9		Sungai Ampang	River	40.8
	Sungai Kuah 1	River	17.8		Sungai Kelang 1	River (upstream)	13.7
	Sungai Kuah 2	Estuary	6.7		Sungai Kelang 2	River (central)	10.4
	Sungai Sanglang 1	River	37.1		Sungai Kelang 3	River(downstream)	8.2
	Sungai Sanglang 2	Estuary	18.5		Sungai Kelang 4	Estuary	14.6
	Sungai Perlis	Estuary	10		Sungai Bata	River	8.3
	Sungai Jerlun 1	River	31.3		Sungai Penchala	River	ND
	Sungai Jerlun 2	Estuary	15.5		Sungai Gombak	River	14.9
	Sungai Baru 1	River	63.1		Selat Lumut	River	11.7
	Sungai Baru 2	Estuary	9.1		Sungai Lukut 1	River	9.1
	Sungai Baru 3	River	7.4		Sungai Lukut 2	Estuary	10.8
	Sungai Kedah 1	River	34.2		Sungai Sepang Besar 1	River	12.3
	Sungai Kedah 2	Estuary	14.6		Sungai Sepang Besar 2	Estuary	9.6
	Sungai Sala 1	River	22.7		Sungai Sepang Kecil 1	River	9.7
	Sungai Sala 2	Estuary	12.8		Sungai Sepang Kecil 2	Estuary	10.8
	Sungai Limau 1	River	61.1		Sungai Langat 1	River	ND
	Sungai Limau 2	Estuary	10.2		Sungai Langat 2	Estuary	8.3
	Sungai Merbuk	River	8.5		Sungai Semenyih	River	21.1
	Sungai Petani	River	51.7	Southern	Sungai Merbau	River	9.8
	Sungai Muda	River	29.7		Sungai Muar 1	Town area	20.6
	Sungai Perai	River	14.6		SungaiMuar 2	River	11.9
	Sungai Juru 1	River	12		Sungai Muar 3	Estuary	13.6
	Sungai Juru 2	Estuary	9.5		Sungai Kesang	River	2.3
	Sungai Jawi 1	River	10.4		Sungai Sebatu 1	River	6.6
	Sungai Jawi 2	Estuary	16		Sungai Sebatu 2	Estuary	28.8
	Sungai Kerian 1	River	13.6		Sungai Merlimau	River	58.3
	Sungai Kerian 2	Estuary	4		Sungai Umbai 1	River	12.9
	Sungai Kurau 1	River	113.3		Sungai Umbai 2	Estuary	20.1
	Sungai Kurau 2	Estuary	7.7		Sungai Punggur	River	8.5
	Sungai Perak 1	River	4.4		Sungai Melaka	River	3.3
	Sungai Perak 2	River	7.7		Sungai Baru	Estuary	2.1
	Sungai Perak 3	River	N.D		Sungai Linggi	Estuary	14.5
	Sungai Bidor 1	River	31	Lakes	Tasik Titiwangsa	Lake	ND
	Sungai Bidor 2	River	7.2		Tasik Perdana	Lake	ND
	Sungai Sepetang 1	River	115.5		Tasik MARDI	Lake	2.5
	Sungai Sepetang 2	Estuary	16.5		Tasik Sri Serdang	Lake	27.7
	Sungai Larut	River	13		Tasik Mines	Lake	ND
	Sungai Bruas 1	River	26.9		Tasik IPS	Lake	7.4
	Sungai Bruas 2	Estuary	ND		Tasik Permaisuri	Lake	ND
	Sungai Dinding	Estuary	6.8		Tasik Kelana Jaya	Lake	9.7
	Sungai Sitiawan	Estuary	4.2				

Remark: ND refers to non-detectable

Table 2: Mean concentration of 17β-estradiol around Peninsular Malaysia based on regions

Zonation	No of site	17β-estradiol (ng/L)		
		Min	Max	Average
South	13	2.1	58.3	15.66
Center	10	ND	3677.4	149.19
North	57	ND	115.9	95.04
Lakes	8	ND	27.7	11.83

Remark: ND refers to non-detectable

4. Discussions

There is no definite value range of E2 concentrations in the aquatic environment because its concentration depends on the activities exist around the water bodies and its sources. In some studies reported that the concentrations of E2 in receiving waters of European countries and the United States have been measured at 1 ng/L to 160 ng/L (Baronti et al., 2000; Kolpin et al., 2002; Lintelmann et al., 2003; Cargouët et al., 2004). In Malaysia, Koyama et al. (2006) reported river and estuarine waters around Kuala Lumpur contained E2 concentrations ranging from 6.1 to 284 ng/L, while Ismail et al. (2007c) found the value ranged from non-detectable to 27700 ng/L for river around the Klang Valley. If all E2 data of present and previous studies are compiled together, it can be said that Malaysia has a very high E2 in the aquatic environment.

E2 in aquatic environment are reported derived from wastewater. This is because E2 or generally estrogen itself can be found in many of personal care and pharmaceutical products (PCPP). Even animal manure and urine contribute to the release of E2 into environment (Ismail et al., 2007a). According to Nagae et al. (2010), large influxes of natural and synthetic chemicals, derived from domestic, industrial and agricultural discharge, are released into the aquatic environment. So, the high amount of E2 in environment probably due to improper management and technology of sewage system. Developed countries are known for its good management on its water sewer and treatment technologies. The amount of E2 release in environment is low cause it does not leached out to the river or other water bodies. E2 just contained within the water sewer system. So this statement also can be applied to the E2 concentration in this study. The highest E2 concentration in this study is on the North Part. There are still many villages around the north area which showed that there is no systematic water management in most north area. So, all the waste are not well manage & most of the time end up in the river. E2 concentration on center area and Klang Valley area, which most of the area are urban area with a planned sewage system are not as high as north area. As for lakes, most lakes show non-detectable value of E2, this is maybe because most of the lakes chosen are actually a control area like Tasik Titiwangsa and Tasik Perdana. Usual lakes are well manage, so when a lake is actually under control, no wastewater will be channel to the lake to preserved the quality of the water.

E2 show a lot of adverse effects and post a threat towards living organisms. So to see the effect of E2 a biomarker can be used since biomarkers offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed between tissues and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990). Biomarkers are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response (CBM-NRC, 1987). Sequential order of responses to pollutant stress within a biological system is molecular, cellular, tissue, systemic, organisms, population & community (Bayne et al., 1985).

On molecular level, E2 can affect the gene expression of male organisms. It Is able to express vitellogenin and choriogenin. Through gene-array we found vitollegenin is highly regulated in the male Largemouth Bass (Larkin et al., 2003), Japanese medaka (Lei at el., 2013), Java medaka (Koyama et al., 2006) and common goby (Nagae at el., 2010). This compund is regulated in the male test animal body by E2 since it is known that these genes are involved in the oogenis and are regulated by estrogenic stimulus (Arukwe et al., 2001). Female organism that is exposed to E2 reacted differently than male. Lei at el. (2013) shows that vitollegenin were significantly down regulated in female java medaka compared to the male. These results indicate that E2 stimulation at high concentration interferes with reproductive phenomena through delayed response by inhibit the synthesis of vitellogenin in this species.

On systemic level, E2 can cause interruption on the reproduction systems where obvious adverse effects on the fecundity, hatchability and time to hatching of an embryo have been reported. Hatchability of fertilized eggs exposed to E2 was significantly decreased and time to hatching was significantly delayed (Imai et al., 2005; Lei at el., 2013; Jukosky et al., 2008). This is maybe due to disturbances of metabolic processes and damage to the cell membrane of the eggs. A study indicates that 27.9 ng/L E2 can reduce the fecundity and fertility of Japanese medaka when the fish were exposed continuously from hatching to 100 days post-hatch (Seki et al., 2005). The lowest concentration of E2 to affect fecundity is 463 ng/L when the adults of Japanese medaka are exposed to E2 (Kang et al., 2002). The abnormal features observed in larvae were scoliosis and abdominal swelling (Lei at el., 2013). The eggs production and the successful number of spawn rate are also decreased (Jukosky et al., 2008).

On organism level, E2 cause development of ova-testis and by that cause the sex transformation or feminization of the male testing organism. Allen et al. (1999) and Hashimoto et al. (2000) observed relatively high vitellogenin as well as the presence of testis-ova in male flounders from England and Japan, respectively. All males fish were found to have sex transformation at 1 ng/L which is a very low concentration of E2 (Lei at al., 2013). Following oral administration of 20 mg/kg 17β-estradiol-valerate to a rainbow trout, the sex composition of fish was found to be 97% of female and 3% intersex (Senol et al., 2008). Most genetic males of Japanese medaka were transformed to phenotypic females by E2 following both short-term and long-term exposure (Hirai et al., 2006a). In a different study, Hirai et al. (2006b) found the formation of ova-testis started during the larval period (approximately 0–20 days post-hatch) and sex transformation from male to female occurred at the transforming phase from larva to juvenile (approximately 14–20 days post-hatch). This could be because the early life stage of organism is very sensitive to the effects of chemicals, as reported by Iwamatsu (1999).

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References

1. Gutendorf B, Westendorf J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 2001;166:79-89.
2. Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicology Science 2000;54:154-167.
3. Lee HB, Peart TE. Determination of 17 beta-estradiol and its metabolites in sewage effluent by solid-phase extraction and Gas chromatography mass spectrometry. Journal AOAC International 1998;81: 1209-1215.
4. Aris AZ, Shamsuddin AS, Praveena SM. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. Environment International 2014;69:104-119.
5. Imai S, Koyama J, Fujii K. Effects of 17beta-estradiol on the reproduction of Java-medaka (*Oryzias javanicus*) a new test fish species. Marine Pollution Bulletin 2005;51(8-12):708-714.
6. Ismail A, Zulkifli SZ, Yusuff FM, Yasuteru U, Sachiko K, Koji A. Assessment of the 17β-estradiol in the aquatic environments around Klang Valley, Malaysia. Wetland Science 2007a;5(4):322-325.
7. Zulkifli SZ, Mohamat-Yusuff F, Ismail A, Miyazaki N. Food preference of the giant mudskipper *Periophthalmodon schlosseri* (Teleostei: Gobiidae). Knowledge and Management of Aquatic Ecosystems 2012;405:07p1-10.
8. Zulkifli SZ, Mohamat-Yusuff F, Mukhtar A, Ismail A, Miyazaki N. Determination of food web in intertidal mudflat of tropical mangrove ecosystem using stable isotope markers: A preliminary study. Life Science Journal 2014;11(3):427-431.
9. Ismail A, Badri MA, Ramlan MN. Heavy metal contamination in fiddler crabs (*Uca annulipes*) and hermit crabs (*Clibanarius* sp.) in a coastal area of northern Peninsular Malaysia. Environmental Technology 1991;12(10):923-926.

10. Ismail A, Jusoh NR, Ghani IA. Trace metal concentrations in marine prawns off the Malaysian coast. Marine Pollution Bulletin 1995;31(1):108-110.
11. Ismail A, Mohamat-Yusuff F, Zulkifli SZ. Imposex in *Thais* spp. along the Straits of Malacca. In: C. Nitithamyong (Ed.), Proceedings of the First Joint Seminar on Coastal Oceanography. 2004. Chiang Mai: Chulalongkorn Univeristy and Japan Society for the Promotion of Science (JSPS).
12. Ismail A, Ramli R. Trace metals in sediments and molluscs from an estuary receiving pig farms effluent. Environmental Technology 1997;18(5):509-515.
13. Mohamat-Yusuff F, Zulkifli SZ, Ismail A, Harino H, Yusoff MK, Arai T. Imposex in *Thais gradata* as a biomarker for TBT contamination on the southern coast of Peninsular Malaysia. Water, Air & Soil Pollution 2010;11(1):443-457.
14. Mohamat-Yusuff F, Zulkifli SZ, Ismail A. Imposex study in *Thais tuberosa* from port and non-port areas along the west coast of Peninsular Malaysia. Journal of Tropical Marine Ecosystem 2011;2:1-9.
15. Mohamat-Yusuff F, Zulkifli SZ, Otake T, Harino H, Ismail A. Study on a new mechanism of sterilization in imposex affected females of tropical marine neogastropod, *Thais* sp. Journal of Environmental Biology 2014;35.
16. Sow AY, Ismail A, Zulkifli SZ. Heavy metals uptake by Asian swamp eel, *Monopterus albus* from paddy fields of Kelantan, Peninsular Malaysia: Preliminary study. Tropical Life Sciences Research 2012;23(2):27-38.
17. Sow AY, Ismail A, Zulkifli SZ. An Assessment of Heavy metal bioaccumulation in Asian swamp eel, *Monopterus albus*, during plowing stages of a paddy cycle. Bulletin of Environmental Contamination and Toxicology 2013;91(1):6-12.
18. Khodadoust D, Ismail A, Zulkifli SZ. Application of Java medaka (*Oryzias javanicus*) fish and surface sediments to assess Cd and Cu contamination in Linggi Estuary, Malaysia. Acta Biologica Malaysiana 2012;1(2):46-54.
19. Khodadoust D, Ismail A, Zulkifli SZ. Short time effect of cadmium on juveniles and adults of java medaka (*Oryzias javanicus*) fish as a bioindicator ecotoxicological studies. Life Science Journal 2013;10(1):1857-1861.
20. Rainbow PS. Bio-monitoring of heavy metal availability in the marine environment. Marine Pollution Bulletin 1995;31(4):183-192
21. Otsuka Lifescience Initiative. 17b-estradiol EIA kit: Instruction for use. Otsuka Pharmaceutical Co., Ltd., Tokushima, 2003.
22. Ismail A, Zulkifli SZ, Yasuteru U, Sachiko K, Koji A. The level of 17β-Estradiol in aquatic environment around Klang Valley. Malaysian Applied Biology 2007b;36(1):85-87.
23. Baronti C, Curini R, D'Ascenzo G, DiCorcia A, Gentili A, Samperi R. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in receiving river water. Environmental Science & Technology 2000;24:5059-5066.
24. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB. Pharmaceuticals hormones and other organic wastewater contaminants in U.S. streams, 1999−2000: a national reconnaissance. Environmental Science & Technology 2002;36:1202-11.
25. Lintelmann J, Katayama A, Kurihara N, Shore L, Wenzel A. Endocrine disruptor in the environment. Pure Applied Chemistry 2003;75(5):631–681.
26. Cargouët M, Perdiz D, Mouatassim-Souali A, Tamisier-Karolak S, Levi Y. Assessment of river contamination by estrogenic compounds in Paris area (France). Science of the Total Environment 2004;324:55-66.
27. Koyama J, Imai S., Fujii K, Kawai S, Yap CK, Ismail A. Pollution by estrogen in river and estuarine waters around Kuala Lumpur, Malaysia, and their effects on the estuarine Java medaka, *Oryzias javanicus*. Japan Journal of Environmental Toxicology 2006;9(2):141-147.
28. Ismail A, Zulkifli SZ, Yasuteru U, Sachiko K, Koji A. The levels of 17β-estradiol in surface water of main rivers in Selangor, Malaysia. Science Putra Research Bulletin 2007c;15(1):38-42.
29. Nagae M, Yuji Takao Y, Ohkubo N, Mastsubara T, Soyano K. Estrogenic activity in estuaries by measuring serum vitellogenin concentration of japanese male common goby in northwestern part of Kyushu. Coastal Environmental and Ecosystem Issues of the East China Sea. 2010;205-213.
30. McCarthy JF, Shugart LR. Biological markers of environmental contamination. Lewis Publisher, Chelsea, Michigan, 1990;457.
31. Committee on Biological Markers of the National Research Council (CBM-NRC). Biological markers of the biomarkers. Environmental Health Perspectives 1987;74:3-9.
32. Bayne BL, Brown DA, Burns K, Dixon DR, Ivanovici A, Livingstone DA, Lowe DM, Moore MN, Stebbing ARD, Widdings J. The effects of stress and pollution on marine animals. Praeger, New York, USA. 1985; 141-157.

33. Larkin P, Sabo-Attwood T, Kelso J, & Denslow D. Analysis of Gene Expression Profiles in LargeMouth Bass Exposed to 17-B estradiol & to anthrophigenic contaminant that behave as estrogen. Ecotoxicology. 2003; 12: 463-468.
34. Lei B, Jia Kanga J, Yua Y, Zhab J, Li W and Wang Z. Estradiol 17-valerate affects embryonic development and sexual differentiation in Japanese medaka (Oryzias latipes). Aquatic Toxicology. 2013; 134-135:128-134.
35. Arukwe A, Kullman SW and Hinton DE (2001). Differential Biomarker gene & protein expression in nonyl-phenol and estradiol-17 beta treated juvenile rainbow trout. Comp Biochem. Physiol. C. Toxicol. Pharmacol. 2001; 129:1-10.
36. Jukosky JA, Watzin MC and Leiter JC. The effects of environmentally relevant mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction. Aquatic Toxicology. 2008; 86: 323–331.
37. Seki M, Yokota H, Maeda M and Kobayashi K. Fish full life-cycle testing for 17 -estradiol on medaka (*Oryzias latipes*). Environmental Toxicology & Chemistry. 2005; 24: 1259–1266.
38. Kang IK, Yokota H, Oshima Y, Ysuruda Y, Yamaguchi T, Maeda M, Imada N, Tadokoro H and Honjo T. Effect of 17b-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). Chemosphere. 2002; 47: 71–80.
39. Allen Y, Matthiesen P, Scott AP, Haworth S, Feist S , and Thain JE.The extent of estrogenic contamination in the UK estuarine and marine environments further survey of flounder. Science of the Total Environment. 1999; 233: 5-10.
40. Hashimoto S, Bessho H, Nakamura M, Iguchi T, and Fujita K. Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. Marine Environmental Research. 2000; 49: 37-53.
41. Senol G, Mecit Y, Kenan G and Turan K. Effects of oral administration of estradiol valerate on gonadal sex differentiation in the rainbow trout, *Oncorhynchus mykiss*. Journal of Animal and Veterinary Advances. 2008; 7(11): 1400–1404.
42. Hirai N, Nanba A, Koshio M, Kondo T, Morita M and Tatarazako N. Feminization of Japanese medaka (*Oryzias latipes*) exposed to 17-estradiol: Effect of exposure period on spawning performance in sex-transformed females. Aquatic Toxicology. 2006a; 79: 288–295.
43. Hirai N, Nanba A, Koshio M, Kondo T, Morita M and Tatarazako N. Feminization of Japanese medaka (*Oryzias latipes*) exposed to 17B-estradiol: formation of testis–ova and sex-transformation during early-ontogeny. Aquatic Toxicology. 2006b; 77: 78–86.
44. Iwamatsu T. Convenient method for sex reversal in a freshwater teleost, the medaka. Experimental Zoology. 1999; 283: 210–214.

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