Vitellogenin Induction in Male *Oreochromis niloticus*: Indication of Estrogenicity in Taal Lake, Philippines

Ailene H. Calamlam 1 and Blesshe L. Querijero 2

1 Department of Forestry and Environmental Science, College of Agriculture, Food, Environment and Natural Resources, Cavite State University,

Indang, 4122, Cavite, Philippines. Email ailene.calamlam@cvsu.edu.ph

2 Animal Biology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines – De La Salle University - Dasmariñas (DLSU-D)

Philippines. Email blquerijero@up.edu.ph

ABSTRACT

Vitellogenin (VTG) is an egg-yolk protein precursor normally produced by sexually mature female fish but can also be synthesized by males under the influence of endocrine-disrupting chemicals (EDCs). 17β-estradiol (E2), an EDC, has been reported to contaminate aquatic systems and cause the feminization of male fishes. The prevalence of EDCs in Taal Lake threatens its biodiversity. Taal Lake is the habitat of the endemic freshwater sardine, *Sardinella tawilis*. The continuous loading of EDCs might affect the population dynamics of such critical wild species, leading to a decline in the wild fish catch and extinction as a long-term effect. This study determined the contamination of Taal Lake with E2 and the VTG induction in male *Oreochromis niloticus*. Water samples were collected from Taal Lake for E2 analysis through ELISA. Also, sexually mature male *O. niloticus* were obtained, and concentrations of their blood plasma VTG were determined using ELISA. Water samples from Taal Lake had E2 concentrations at 1.22 ± 0.64 µg/L. The collected male *O. niloticus* had VTG concentrations at 478.90 ± 129.98 ng/mL. The results from this study provide evidence of vitellogenin production in male *O. niloticus*. However, the measured VTG induction is not limited to exposure to 17β-estradiol.

Keywords: vitellogenin induction, 17β-estradiol, estrogenicity, Taal Lake, male tilapia

INTRODUCTION

A large group of compounds called endocrine-disrupting chemicals (EDCs) released into the environment may lead to detrimental disorders in living organisms, due to the possibility of the interaction of these compounds with cellular components (Pamplona-Silva et al. 2018). These compounds can interact with estrogen receptors and interfere with the synthesis, secretion, transport, binding, action, and elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes, hence called endocrine disruptors (EPA 2016).

The prevalence of EDCs in water resources threatens aquatic biodiversity, affecting the development and reproduction of wildlife species (Bhandari et al. 2015). Some reported effects include disturbances in the development and expression of sexual characteristics. For example,

vitellogenin is an egg-yolk protein precursor normally produced by adult female fish; however, it can also be synthesized by males under the influence of EDCs (Caspillo et al. 2014; Depiereux et al. 2014; Davis et al. 2009; Rodas-Ortiz et al. 2008). Experimental studies revealed that concentrations as low as one ng/L of 17β-estradiol can induce vitellogenin production in exposed male fish (Hansen et al. 1998; Purdom et al. 1994). In other experimental studies, high incidences of feminization and intersex were observed in male fish after chronic exposure to estrogen (Green et al. 2015; Caspillo et al. 2014; Depiereux et al. 2014; Adebiyi et al. 2013; Kosai et al. 2011). Taal Lake has high biological significance because of its unique collection of fauna. The freshwater sardine, *Sardinella tawilis,* is endemic to this lake. The continuous loading of these contaminants might significantly disrupt the reproduction and population dynamics of such critical wild species leading to a decline in the wild fish catch and extinction as a long-term effect.

Many different chemicals have been recognized as EDCs. Classical examples that are frequently found in the environment, include natural and synthetic hormones, BPA, phthalates, alkylphenols (APs), polycyclic aromatic hydrocarbons (PAHs), and agricultural chemicals (Pamplona-Silva et al. 2018). Among the different EDCs, estrogenic compounds have merited the most concern. Paraso and Capitan 2012, have studied the estrogenic contamination of Laguna de Bay and vitellogenin induction has also been observed in male common carp caged in Laguna de Bay, Philippines. The detected VTG in the male common carp established the estrogenic contamination in Laguna de Bay. Williams et al. (2003) identified natural estrogens, such as estrone and 17β-estradiol, and synthetic ethinylestradiol contribute the most significant endocrine disruption. These estrogenic substances are released into the freshwater systems through municipal sewage effluents, livestock waste, agricultural discharge, and industrial effluents (Ying et al. 2002). Human and animal excretion is considered the primary source of estrogen in aquatic ecosystems (Matozzo et al., 2008). These substances are difficult to detect and impossible to remove once they reach the aquatic environment.

In this study, the vitellogenin induction in male *O. niloticus* is attributed as the observed effect to the presence of E2 and other estrogenic compounds since the induction of vitellogenin in male fish is considered one of the most common biomarkers for the detection of estrogenic

compounds in aquatic environments (Synder et al. 2003). Estrogenic compounds are those that have similar chemical properties to the hormone 17β-estradiol.

MATERIALS AND METHODS

The study determined the concentration of 17β-estradiol (E2) in the surface water of Taal Lake, in a region where tilapias are produced commercially in floating net cages. It also determined the vitellogenin induction in male tilapias *(O. niloticus)* by measuring the vitellogenin level in blood plasma. In this study, 17β-estradiol served as a model compound for environmental estrogens, while the determination of other environmental estrogens was not included. Tilapia is considered the relevant fish species for this study because they are not known to be hermaphrodites; they are resilient to environmental pollutants and an important food fish that are grown extensively in aquaculture enclosures in Taal Lake and other Philippine freshwater lakes. This study has not included controlled exposure where E2 was used exclusively to induce VTG production in male Tilapias since exposure to estrogens in the environment is never single-chemical.

Site Description

Water and fish samples were collected from Taal Lake. Surface water samples were collected from four sampling sites, and each sampling site has a distance of at least 50 meters from each other (coordinates: 14°02'29.1"N 121°00'08.4"E; 14°02'32.5"N 121°00'24.0"E; 14°02'30.3"N 121°00'27.1"E; 14°02'24.4"N 121°00'24.4"E). The sampling sites were located near the volcano island with high stocking density fish cages and populated land areas along the shoreline. Fish samples were collected in one of these cages.

Figure 1. Sampling points in Taal Lake (Google Maps 2016).

Fish Sampling and Blood Plasma Extraction

Sexually mature male tilapias, grown in floating fish cages for about eight months, were randomly collected on-site during the summer of April 20, 2016, using cast nets. The fish were kept in large, aerated tanks with water collected from the sampling sites and were immediately transported to the laboratory. Eight fish were anesthetized with 100 mg/L tricaine methanesulfonate solution (Himedia® MS222 - ethyl 3-aminobenzoate methanesulfonate salt). Fish were patted dry, and the fork length, total length, and weight of the fish were recorded. Fish fork length ranged from 170 – 190 mm, total length ranged from 245 – 270 mm, and weight went from 300 – 400 g.

One milliliter of the blood sample from each fish was collected from the caudal vasculature using 3-mL heparinized syringes. The blood samples were centrifuged at 3000 x g with a maintained temperature of -10˚C for 10 minutes to separate the blood components. The blood plasma was collected and transferred into sterile 1-mL Eppendorf tubes using sterile pipette tips. The fresh blood plasma samples were immediately stored in the freezer at -40 ºC until the assay was performed (Asem-Hiablie et al. 2013; Paraso et al. 2019).

Quantification of Vitellogenin (VTG) in Blood Samples of Male Fish

An immunochemical method, Enzyme-Linked Immunosorbent Assay (ELISA), was used to quantify the concentration of VTG in the blood plasma of male *O. niloticus*. ELISA has been the preferred method in determining VTG and E2, due to its good sensitivity, which is generally in the range of ng/mL. ELISA is a highly sensitive method that uses antibodies specific to VTG and is usually not subject to interference from other proteins (Jones et al. 2000). The technique involves coating the wells with VTG from the samples.

A commercially available VTG ELISA kit – TECO® REACH Perch (Perciformes) Vitellogenin ELISA (TECO® medical Group, Gewerbestrasse, Switzerland) was used to quantify the concentration of VTG present in the previously obtained blood plasma samples. All the reagents needed for the assay, including a set of standardized reagents and specific antibody-coated microplate wells, are in the test kit. Other materials and equipment required for the test were micropipettes, multichannel pipettes, a vortex mixer, an orbital shaker, and a microplate reader capable of measuring at 450 nm.

Assay Procedure

The frozen blood plasma samples were thawed in normal tap cold water (15-20˚C) within 10-15 minutes. The prepared blood plasma samples were subjected to Enzyme-Linked Immunosorbent Assay (ELISA). All samples were analyzed in duplicates. The test protocol was done under the protocol set by the manufacturer of the ELISA kit. All blood plasma samples were prepared before the start of the assay by diluting it to a dilution of 1:1000 with a Dilution Buffer provided in the kit. A 1000 µL dilution buffer was combined with a 1 µL blood plasma sample. The microplate wells were first allocated for NSB/blank (Non-Specific Binding), standards, controls, and blood plasma samples (with unknown VTG concentration).

The 50 µL matrix solution was added to all wells using a multichannel pipette. Then, 50 µL dilution buffer was added to the assigned NSB wells. 50 µL of each prepared standard VTG solution (80 ng/mL, 27 ng/mL, 9 ng/mL, 3 ng/mL, 1ng/mL, and 0 ng/mL), prepared controls (C1 and C2), and pre-diluted male blood plasma samples were added into the corresponding wells. The wells were then covered and incubated for 120 ± 5 min at room temperature (18-30˚C) on a shaker (500 rpm). After incubation, the contents of the wells were aspirated and washed three times using 350 µL diluted wash buffer. After washing, 100µL Biotinylated AB was added to

each well. The wells were again incubated for 60 ± 5 min at room temperature (18-30˚C) on a shaker (500rpm). After the incubation, the wells were washed three times using a wash buffer. Following the washing step, 100 µL of SA-HRP Conjugate was added to each well. The wells were again covered and incubated for 30 ± 5 min at room temperature (18-30˚C) on a shaker (500 rpm). After the incubation, the wells were washed five times with wash buffer. A 100 µL of the TMB Substrate Solution was added to each well, and the plate was incubated for 15 – 30 minutes, in the dark, at room temperature (18-30˚C) on a shaker (500 rpm). The microplate was covered with aluminum foil to prevent light exposure. To stop the reaction, 100 µL stop solution was added to the wells. Color development was measured 10 minutes after adding the stop solution using a microplate absorbance reader (iMark™, Bio-Rad Laboratories, Inc.) at 415 nm with the reference filter at 655 nm. The values of absorbance were analyzed by using MyAssays Online Software ("Four-Parametric Logistic Curve" online data tools, MyAssays Ltd., 2016).

Water Collection

Water samples were collected from Taal Lake for 17β-estradiol (E2) analysis. 100 mL grab samples of lake water were collected five centimeters below the water surface in amber glass bottles with polytetrafluoroethylene caps (Asem-Hiablie, 2013; Jurgens, et. al, 1999). The water samples were transferred to the bottles using a glass funnel. The funnel and grab collector were rinsed thoroughly using lake water from each site before the sample was collected. All water samples were contained in an insulated container and were immediately transported to the laboratory. Water samples were then filtered using a 1 µm pore diameter glass fiber filter (Pall®; Pall Corporation, Michigan, Mexico). A minimal volume of methanol (AR, RCI Labscan) was poured on the residue on the filter to maximize the extraction of the analyte. One percent (1%) of formaldehyde was added to the filtered water samples and was stored at 4^{o}C before use.

Pre-treatment of Water Samples

Solid-phase extraction (SPE) of the water samples was performed. The SPE procedure used C18 SPE cartridges (Supelco®, Bellefonte, PA, USA) following the water pre-treatment procedures specified in the cartridge user manual. Each cartridge was preconditioned with 5 mL methanol and 10 mL deionized water. Water samples (100 mL) were loaded into SPE cartridges under gentle vacuum pressure at a flow rate of 20 mL/min. The cartridges were washed with deionized water at a flow rate of 20 mL/min and were then vacuum-dried for one minute. Drying

was followed by washing 5 mL of 95% n-hexane (AR, RCI Labscan) at a flow rate of 20 mL/min. The 17β-estradiol was eluted from the C18 cartridge using 5 mL dichloromethane (Baker Analyzed®, USA) at a flow rate of 3-5 mL/min. The solvent was evaporated to dryness under the fume hood. The resulting residue, which contains the 17β-estradiol, was dissolved using 100 µL methanol, and the mixture was stirred using a vortex mixer to dissolve the residue completely. Deionized water (900 µL) was added to achieve a concentration of 10 % (v/v) methanol.

Determination of 17β-estradiol Concentration

Detection and quantification of 17β-estradiol were accomplished using an immunochemical method, Enzyme-Linked Immunosorbent Assay (ELISA). This was done using a commercially available ELISA kit (EcologienaÒ, Tokiwa Chemical Industries Co., Ltd, Japan). The 17β-estradiol assay standards were prepared. All water samples were analyzed in duplicates following the protocol set by the manufacturer of the 17β-estradiol ELISA kit.

Assay Procedure

The microplate wells were first allocated for blank, standard, and water samples (unknown concentration). The conjugate solution, 17β-estradiol assay standards (0 µg/L, 0.05 µg/L, 0.15 µg/L, 0.40 µg/L, 1.00 µg/L), and wash solution were prepared before the start of the assay. Conjugate solution (100 µL) was mixed with 100 µL of each 17β-estradiol standard and water samples to form a 200 µL conjugate-standard solution or conjugate-sample solution. 100 µL aliquots of the mixture were dispensed into each allocated microplate well. The microplate was covered with a film to prevent contamination and evaporation and was incubated for 60 minutes at room temperature (18-25 ˚C). After the incubation, the microplate was rinsed three times with 300 µL of the wash solution. Color solution (100 µL) was dispensed into each microplate well and was then incubated for 30 minutes at room temperature (18-25 ˚C). Then, 100 µL stop solution was added into each microplate well to terminate the reaction. Water samples and standards absorbance were read at 450 nm wavelength using a microplate absorbance reader (iMarkTM, Bio-Rad Laboratories, Inc.). The values of absorbance were then analyzed using MyAssays Online Software (“Four-Parametric Logistic Curve” online data tools, MyAssays Ltd., 2016).

RESULTS AND DISCUSSION

Plasma Vitellogenin (VTG) Concentrations

The male *O. niloticus* samples had detectable concentrations of plasma VTG. The fish collected from Taal Lake had an average VTG concentration of 478.90 ± 129.98 ng/mL.

Table 1. Vitellogenin concentration of Male tilapia (*O. niloticus*) from Taal Lake

Fish Number	VTG Concentration in Fish Sample (ng/mL)	Average ± SD VTG (ng/mL)
1	386.9	478.90 ± 129.98
2	449.9	
3	448.5	
4	318.9	
5	17.8	
6	643.6	
7	625.6	
8	1980.0	

**Data were analyzed using software capable of generating a 4-Parameter Logistic curve – "MyAssays".*

The detected vitellogenin in male *O. niloticus* establishes the contamination with estrogen in Taal Lake. Under normal circumstances, male fish may have little or undetectable VTG levels in their blood plasma (Ankley and Johnson 2004) and VTG induction by 17β-estradiol has been reported to be dose-dependent Berg et al. 2004). The detected VTG concentration in males indicates an unnatural response to estrogens present in Taal Lake. The measured VTG concentration in male tilapia in this study, which is 478.90 ± 129.98 ng/mL shown in Table 1, is similar to those detected in the adult male tilapia cultured in the lakes of San Pablo City, Laguna with extensive aquaculture activities, which ranges from 40.68 ± 40.97 µg/mL to 57.22 ± 52.71 µg/mL (Mabansag, 2019). The observed vitellogenin induction can be attributed to the presence of E2 as well as to other estrogenic compounds.

17β-estradiol (E2) Concentrations in Water Samples

Environmental pollution by estrogenic compounds can have a potential risk of endocrine disruption to exposed aquatic organisms. The concentration of 17β-estradiol (E2) was measured in the surface water samples from selected sites of the lake to verify the presence of estrogens.

Results showed that water samples from Taal Lake had an E2 concentration of 1.22 ± 0.64 µg/L, as shown in Table 2. This measured E2 concentration in Taal Lake was higher than those detected in Laguna de Bay. Water samples collected from the East Bay and West Bay of Laguna de Bay had E2 concentrations of 0.39 ± 0.15 µg/L and 0.40 ± 0.16 µg/L, respectively (Paraso and Capitan 2012). The difference between the surface area of the two lakes could explain the higher E2 concentrations observed in Taal Lake. Laguna de Bay is the largest lake in the Philippines covering a surface area of 911.7 km^2 while Taal Lake has a surface area of 234.2 km^2. Taal Lake is four times smaller than Laguna de Bay, which could concentrate the estrogens on the surface water. It is important to note that estrogenic compounds are mostly nonpolar and have lower densities, which tend to float on the surface of the water.

Table 2. Concentrations of 17β-estradiol in water samples from Taal Lake

Sampling Station Number	17β-estradiol in Water Samples (µg/L)	Average ± SD 17β-estradiol (µg/L)
1	0.840692	1.216441 ± 0.64
2	2.09528	
3	0.6551	
4	1.27469	

**Data were analyzed using software capable of generating a 4-Parameter Logistic curve – "MyAssays".*

There are many pathways for estrogenic contaminants to enter the lake by natural and/or anthropogenic origins. The presence of E2 in aquatic environments is highly associated with wastewater effluents, direct discharges, and run-offs from domestic, agricultural, and industrial sources (Praveena et al. 2016; Rocha et al. 2015; Studer 2011). In this study, the incidence of E2 detection can be related to the distribution characteristics of residential and agricultural areas established around the lake as indicated by the color-coded land use in the map (Figure 2).

The selected fish cage in Taal Lake was located near the volcano island, in an area close to households. According to ADB (2014), only three to five percent of households in the Philippines have access to a sewage system network. On an island with no access to a municipal sewage system, individual or communal septic tanks are utilized, these are usually below the standards set by the Department of Environment and Natural Resources. Consequently, sewage leaching into the water systems could emit considerable amounts of E2 into the lake. It has been

studied that the primary source of E2 in municipal wastewater is urine, which contains 67 – 80% of estrogens excreted daily (Maurer et al. 2006). The other primary source of hormone steroids is livestock waste (Ying et al. 2002). Non-point sources, like run-offs loaded with animal waste from agricultural lands and facilities surrounding the lake, could have reached the surface water.

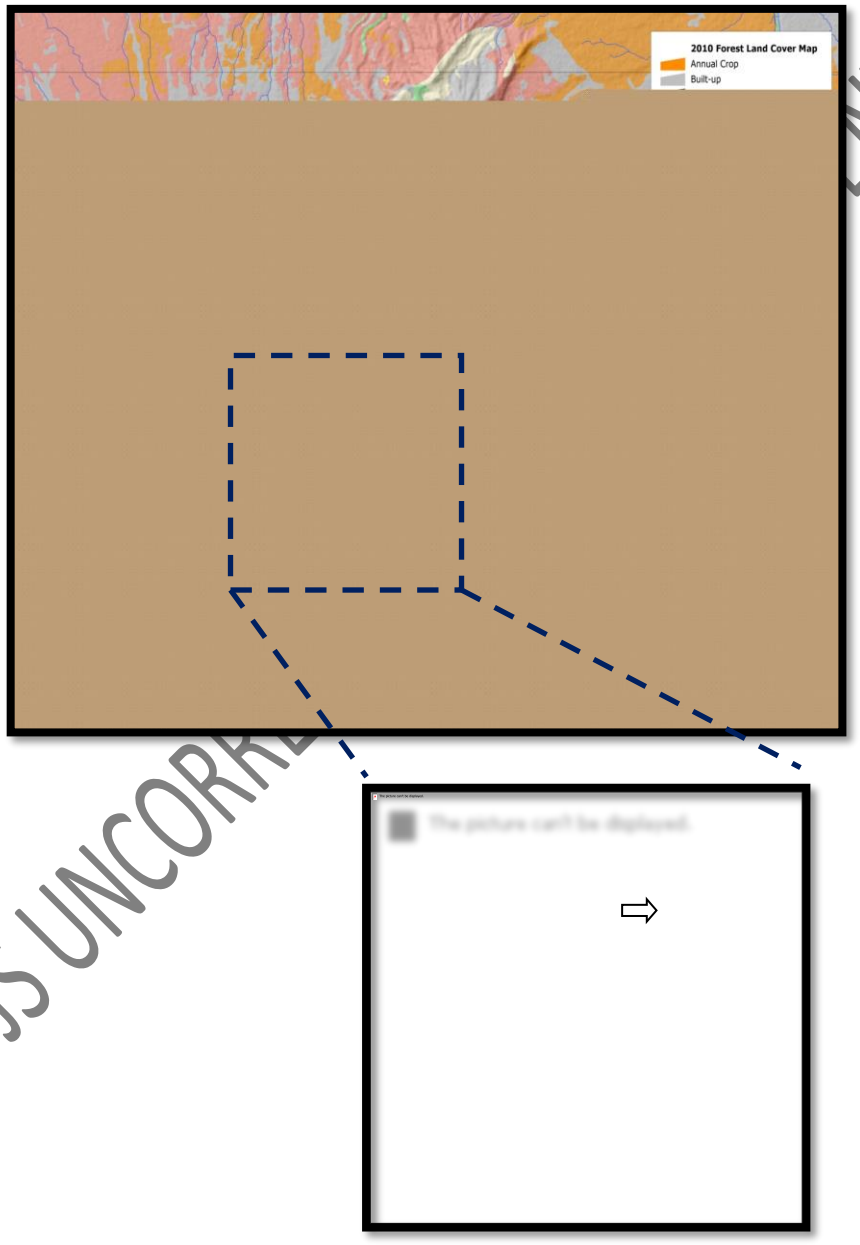

Figure 2. Land-use map of Taal Lake. (Ecosystem Research Development Bureau, ERDB-UPLB, generated, 2010). Indicated is the limnological sampling site (white arrow).

Fish in cages or fences set up in lakes are exposed to a wide range of contaminants under field conditions, some of which may have the same effect as EDCs. Unknown contaminants that mimic estrogen may have feminizing effects. EDCs encompass a variety of substances, aside from hormones synthesized by vertebrates; it includes phytoestrogens, mycotoxins, synthetic hormones, pesticides, detergent components, and persistent environmental pollutants such as PCBs, PCDDs, PCDFs (Cargouet et al. 2007).

The selected sampling site in Taal Lake is surrounded by mainly agricultural lands characterized as an annual crop in the land-use map. The use of pesticides in cultivated lands could have contributed to the presence of other EDCs. In a study by Rodas-Ortiz et al., (2008), they correlated the VTG induction and sexual steroid alterations in male tilapias with the organochlorine pesticide concentrations in the liver and polycyclic aromatic hydrocarbon (PAH) metabolites in bile. Their study revealed significant correlations between plasma VTG and E2 levels with the pesticide hexachlorobenzene (HCB) in the liver and BaP metabolites in bile.

CONCLUSIONS AND RECOMMENDATIONS

The collected male *O. niloticus* from Taal Lake had detectable concentrations of plasma vitellogenin. The male tilapia samples had an average vitellogenin concentration of 478.90 ± 129.98 ng/mL. Under normal circumstances, male fish may have minor or undetectable vitellogenin. Thus, the detectable concentrations of plasma vitellogenin in the male tilapia samples indicate induction from estrogenic compounds.

17β-estradiol concentration of 1.22 ± 0.64 µg/L was detected in Taal Lake. The results suggest the detected vitellogenin in male *O. niloticus* is a biological response to estrogen exposure. The results from this study provide evidence of vitellogenin production in male *O. niloticus*. However, the measured vitellogenin is not limited to exposure to 17β-estradiol. Fishes and other animals are exposed to various pollutants under field conditions. Other environmental pollutants may increase or inhibit vitellogenin production in fish.

Continuing research on the detection, quantification, and monitoring of EDCs in the environment is needed to provide a further understanding of environmental concerns. Further research should consider the change in seasons since it could be a factor in the varying concentrations of EDCs in water. Further research may include a whole-lake field study, investigating other endocrine disruptors released into the environment and assessing whether VTG induction is responsive to estrogenic EDCs alone.

REFERENCES

Adebiyi FA, Siraj SS, Harmin SA, Christianus A. 2013. Plasma sex steroid hormonal profile and gonad histology during the annual reproductive cycle of river catfish *Hermibagrus nemurus* in captivity. Fish Physiol Biochem. 39:547-557.

Ankley GT, Johnson RD. 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR J 45:469-483.

Asem-Hiablie S, Church CD, Elliott HA, Shappell NW. Schoenfuss HL, Drechsel P, Williams CF, Knopf AL, Dabie MY. 2013. Serum estrogenicity and biological responses in African catfish raised in wastewater ponds in Ghana. Science of the Total Environment 463-464: 1182-1191.

Beresford N, Brian JV, Runnalls TJ, Sumpter JP, Jobling S. 2011. Oestrogenic Activity of Tropical Fish Food Can Alter Baseline Vitellogenin Levels in Male Fathead Minnow (*Pimephales promelas*). Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, United Kingdom.

Berg H, Modig C, Olsson P-E. 2004. 17beta-estradiol induced vitellogenesis is inhibited by cortisol at the post-transcriptional level in Arctic char (Salvelinus alpinus). Reprod Biol Endocrinol. 2:62. https ://doi.org/10.1186/1477-7827-2-62

Bhandari RK, Deem SL, Holliday DK, Jandegian CM, Kassotis CD, Nagel SC, and Rosenfeld CS. 2015. Effects of the environmental estrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. General and Comparative Endocrinology, 214195-219.

Borgert CJ, Baker SP, Matthews JC. 2013. Potency matters: Threshold governs endocrine activity. Regulatory Toxicology and Pharmacology 67: 83-88.

Cargouet M, Perdiz M, Levi Y. 2007. Evaluation of the estrogenic potential of river and treated waters in the Paris area (France) using in vivo and in vitro assays. Ecotoxicology and Environmental Safety 67: 149–156.

Caspillo NR, Volkova K, Hallgren S, Olsson PE, Porsch-Hallstrom I. 2014. Short-term treatment of adult male zebra (*Danio Rerio*) with 17α – ethinyl estradiol affects the transcription of genes involved in development and male sex differentiation. Comparative Biochemistry and Physiological, Part C 164:35-42.

Castillo B, Castillo A, and Gonzales C. 1974. Tawilis fishery resources investigation of Taal Lake. Terminal report. Bureau of Fisheries and Aquatic Resources. 19pp.

Castillo B and Gonzales C. 1976. Hydrology of Taal Lake. Fish. Res. J. Phil. 1: 62-75.

Chighizola C and Meroni PL. 2012. Review: The role of environmental estrogens and autoimmunity. *Autoimmunity Reviews*, *11* (Special Issue on Gender, Sex Hormones, Pregnancy and Autoimmunity), A493-A501.

Cruz G, Foster W, Paredes A, Yi KD, & Uzumcu M. 2014. Long-Term Effects of Early-Life Exposure to Environmental Oestrogens on Ovarian Function: Role of Epigenetics. *Journal Of Neuroendocrinology*, *26*(9), 613-624.

Davis LK, Fox BK, Lim C, Hiramatsu N, Sullivan CV, Hirano T, Grau EG. 2009. Induction of vitellogenin production in male tilapia *(Oreochromis mossambicus)* by commercial fish diets. Comparative Biochemistry and Physiology, Part A 154: 249-254.

Deksissa, Tolessa. 2008. Fate and Transport of Steroid Hormones in the Environment. Paper 17. Available from: http://opensiuc.lib.siu.edu/ucowrconfs_2008/17

Depiereux S, Liagre M, Danis L, De Meulder B, Depiereux E, Segner H, Kestemont P. 2014. Intersex Occurrence in Rainbow Trout (*Oncorhynchus mykiss*) Male Fry Chronically Exposed to Ethynylestradiol. PLoS ONE 9(7): e98531.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30, 293–342.

Ecosystem Research Development Bureau (ERDB-UPLB). 2010. Generated Land-Use Maps.

El-Sayed TA, Abdel-Aziz SH, El-Sayed AFM, Zeid S. 2014. Structural and functional effects of early exposure to 4-nonylphenol on gonadal development of Nile tilapia (*Oreochromis niloticus*): b-histological alterations in testes. Fish Physiol Biochem 40:1495–1507.

Environmental Protection Agency [EPA]. 2016. "What Are Endocrine Disruptors?" http://www.epa.gov/endo/pubs/edspoverview/whatare.htm [accessed: January 2016].

Fisch H, Hyun G, Golden R. 2000. The Possible Effects of Environmental Estrogen Disrupters on Reproductive Health. Current Science Inc. ISSN 1527-2737. 1:253-261.

"Four-Parametric Logistic Curve" online data tools, MyAssays Ltd., 2016. http://www.myassays.com/four-parameter-logistic-curve.assay

Ghoshdastidar AJ, Fox S, Tong AZ. 2014. The presence of the top prescribed pharmaceuticals in treated sewage effluents and receiving waters in Southwest Nova Scotia, Canada. Environ. Sci. Pollut. Res. Int. 22, 689–700.

Green C, Briana J, Kanda R, Scholzea M, Williams R, Jobling S. 2015. Environmental concentrations of anti-androgenic pharmaceuticals donot impact sexual disruption in fish alone or in combination withsteroid oestrogens. Aquatic Toxicology 160: 117–127.

Gross-Sorokin MY, Roast SD, Brighty GC. 2006. Assessment of feminization of male fish in English rivers by the environment agency of England and Wales. Environ Health Perspect 114 (Suppl 1):147-151.

Guevarra1 RD, Paraso MGV, Lola MSEG. 2020. Biomarker Evaluation in Nile Tilapia (*Oreochromis niloticus*) to Assess the Health Status of Aquaculture Areas in the Seven Lakes of San Pablo. Philippine Journal of Science. Vol. 149 No. 3-a, October 2020

Hansen PD, Dizer H, Hock B, Marx A, Sherry J, McMaster M. 1998. Vitellogenin—a biomarker for endocrine disruptors. Trends Anal Chem; 17:448–51.

Hinck JE, Blazer VS, Schmitt CJ, Papoulias DM, Tillitt DE. 2009. Widespread occurrence of intersex in black basses (Micropterus spp.) from U.S. rivers, 1995–2004. Aquatic Toxicology 95:60–70.

Hung DQ, Thiemann W. 2002. Contamination by selected chlorinated pesticides in surface waters in Hanoi, Vietnam. Chemosphere 47, 357–367.

Johnson AC, Belfroid A, Di Corcia A. 2000. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sci Total Environ; 256:163–73.

Jones PD, Tremblay LA, De Coen WM, Giesy JP. 2000. Vitellogenin as a biomarker for Environmental Estrogens. Australasian Journal of Ecotoxicoloy. Vol. 6, pp. 45-58.

Kabir ER, Rahman MS, and Rahman I. 2015. Review: A review on endocrine disruptors and their possible impacts on human health. Environmental Toxicology And Pharmacology, 40241-258.

Keller VDJ, Lloyd P, Terry JA, Williams, RJ. 2015. Impact of climate change and population growth on a risk assessment for endocrine distruption in fish due to steroid estrogens in England and Wales. Environmental Pollution 197: 262-268.

Khalil FF, Farrag FH, Mehrim AI, Refaey MA. 2014. Pawpaw (Carica papaya) seeds powder in Nile tilapia (Oreochromis niloticus) diets: 2 Liver status, sexual hormones and histological structure of the gonads. Egypt. J. Aquat. Biol. & Fish., Vol. 18, No. 1: 97- 113 (2014) ISSN 1110 – 6131.

Kosai P, Jiraungkoorskul W, Sachamahithinant C, Jiraungkoorskul K. 2011. Induction of testis-ova in nile tilapia (*Oreochromis niloticus*) exposed to 17β-estradiol. Natural Science. Vol.3, No.3, 227-233.

Lee O, Takesono A, Tada M, Tyler CR, and Kudoh T. 2012. Biosensor Zebrafish Provide New Insights into Potential Health Effects of Environmental Estrogens. *Environmental Health Perspectives*, *120*(7), 990-996.

Limpiyakorn T, Homklin S, Ong SY. 2011. Fate of Estrogens and Estrogenic Potentials in Sewerage Systems. Critical Reviews in Environmental Science and Technology, 41:1231–1270.

Mabansag CJA, Paraso MGV, Marcelino RT, Clavecillas AA, Lola, MSEG. 2019. A Preliminary Survey of Estrogenic Effects in Cultured Adult Male Nile Tilapia (Oreochromis niloticus) in the Seven Lakes of San Pablo City, Philippines. Bulletin of Environmental Contamination and Toxicology. https://doi.org/10.1007/s00128-019-02685-z

Mannelli C, Ietta F, Avanzati AM, Skarzynski D, and Paulesu L. 2015. Biological Tools to Study the Effects of Environmental Contaminants at the Feto-Maternal Interface. *Dose-Response*, *13*(4), 1-11.

Matozzo V, Gagné F, Marin MG, Ricciardi F, Blaise C. 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. Environment International 34: 531–545.

Matsumoto T, Kobayashi M, Moriwaki T, Kawai S, Watabe S. 2004. Survey of estrogenic activity in fish feed by yeast estrogen-screen assay. Comp. Biochem. Physiol. C 139, 147–152.

Maurer M, Pronk W, Larsen TA. 2006. Treatment processes for source-separated urine. Water Res. 40 (17), 3151–3166.

Niemuth NJ, Klaper RD. 2015. Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. Chemosphere 135: 38-45.

Oosterhuis M, Sacher F, Ter Laak TL. 2013. Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data. Sci. Total Environ. 442, 380–388.

Pamplona-Silva MT, Mazzeo DEC, Bianchi J, Marin-Morales MA. 2018. Estrogenic Compounds: Chemical Characteristics, Detection Methods, Biological and Environmental Effects. Water Air Soil Pollut (2018) 229:144. https://doi.org/10.1007/s11270-018-3796-z

Paraso MGV. 2018. Estrogenic pollutants: impacts on reproductive health of fish in Laguna de Bay [Philippines]. AGRIS - International System for Agricultural Science and Technology.

Paraso MGV, Capitan SS. 2012. Vitellogenin Induction and Gonad Abnormalities in Male Common Carp (*Cyprinus carpio* L.) Introduced to Laguna de Bay, Philippines. Philipp J Vet Anim Sci 38 (1): 34-44

Pontelli RN, Nunes AA, de Oliveira SB. 2016. Impact on human health of endocrine disruptors present in environmental water bodies: is there an association with obesity? *Ciencia & Saude Coletiva*, (3), 753.

Praveena SM, Hamin N, Razak SQNA, Aris AZ. 2016. Analysis of steroid estrogens in river sediment by high performance liquid chromatography-electrospray ionization-mass spectrometry. Iranian Journal of Science and Technology, Transactions A: Science. https://doi.org/10.1007/s40995-016-0109-5.

Purdom CE, Hardiman PA, Byre VJ, Eno NC, Tyler CR, Sumpter JP. 1994. Estrogenic effects of effluents from sewage treatment works. Chem Ecol; 8:275 – 85.

Rocha MJ, Cruzeiro C, Reis M, Pardal MÂ, Rocha E. 2015. Toxicological relevance of endocrine disruptors in the Tagus River estuary (Lisbon, Portugal). Environmental Monitoring and Assessment, 187(8), 483. https://doi.org/10.1007/s10661-015-4679-z.

Rodas-Ortı´z JP, Ceja-Moreno V, Chan-Cocom ME, Gold-Bouchot G. 2008. Vitellogenin Induction and Increased Plasma 17β-Estradiol Concentrations in Male Nile Tilapia, Oreochromis niloticus, Exposed to Organochlorine Pollutants and Polycyclic Aromatics Hydrocarbons. Bull Environ Contam Toxicol 81:543–547.

Scheurer M, Michel A, Brauch HJ, Ruck W, Sacher F. 2012. Occurrence and fate of the antidiabetic drug metformin and its metabolite guanylurea in the environment and during drinking water treatment. Water Res. 46, 4790–4802.

Snyder, S. A., Westerhoff, P., Yoon, Y., & Sedlak, D. L. 2003. Pharmaceuticals, personal care products, and endocrine disruptors in water: implications for the water industry. Environmental Engineering Science, 20(5), 449–469. https://doi.org/10.1089/109287503768335931.

Studer KE. 2011. Investigating Estrogenic Endocrine Disrupting Compounds and Their Disinfection Byproducts Within Drinking Water Treatment. Environmental & Water Resources

Engineering Masters Projects. Paper 58. Available from:
http://scholarworks.umass.edu/cee_ewre/58

Turgut C. 2003. The contamination with organochlorine pesticides and heavy metals in surface water in Kucuk Menderes River in Turkey, 2000-2002. Environ. Int. 29, 29–32.

Williams RJ, Johnson AC, Smith JJL, Kanda R. 2003. Steroid Estrogens Profiles along River Stretches Arising from Sewage Treatment Works Discharges. Environ. Sci. Technol. 37, 1744-1750.

Ying GG, Kookana RS, Ru YJ. 2002. Occurrence and fate of hormone steroids in the environment. Environmental International 28: 545-551.

Yuan X, Li T, Zhou L, Zhao X. 2014. Characteristics and Risk Assessment of Estrogenic Compounds in Rivers of Southern Jiangsu Province, China. IERI Procedia 9: 176-184.