Water Air Soil Pollut (2021) 232: 448
https://doi.org/10.1007/s11270-021-05375-x

Alterations in the Development and Gonadal Structure of Nile Tilapia (*Oreochromis niloticus*) Exposed to Natural and Synthetic Estrogens

Oscar Pacheco Passos Neto · André Bezerra dos Santos · José Roberto Feitosa Silva · Suetônio Mota

Received: 19 May 2021 / Accepted: 30 September 2021 / Published online: 24 October 2021
© The Author(s), under exclusive licence to Springer Nature Switzerland AG 2021

Abstract This study aimed to investigate the effects of endocrine disruptors 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) on Nile tilapia (*Oreochromis niloticus*) development, focusing on gonadal histological factors. Concentrations of estrogens E2 and EE2 ranging from 250 to 1000 ng·L^{-1} can produce intersex individuals, significantly decreasing the condition factor as the concentrations increased. These concentrations could also induce the development of morphological anomalies. Increasing the concentration of estrogens by one order of magnitude (μg·L^{-1}) was lethal for Nile tilapia larvae, with no effect on the incubation time and percentage of larvae hatching. Additionally, morphological anomalies and developmental problems were observed. Estrogens applied at a concentration of 160 μg·L^{-1} for 28 days caused the birth of a small number of intersex individuals in Nile tilapia, but generated almost entirely female populations in hormonal treatments. Furthermore, the gonads of fish exposed to the estrogens were smaller and less developed, without any significant difference between E2 and EE2. Variation in the exposure time (one to 4 weeks) of Nile tilapia to estrogens (160 μg·L^{-1}) resulted in the appearance of intersex individuals and the development of morphological anomalies, regardless of the exposure time.

Keywords Endocrine disruptors · Nile tilapia (*Oreochromis niloticus*) · Zootechnical aspects · Histological aspects

1 Introduction

Emerging micropollutants (EM), such as pharmaceuticals, hormones, and personal care products, among others, are being constantly released into the environment, affecting ecosystems and public health (Harb et al. 2019; Proctor et al. 2021). Among the main EM, antibiotics have received particular attention due to the possibility of the development of resistant pathogenic bacteria in the environment, as well as endocrine disrupters, such as the xenoestrogen bisphenol A, synthetic hormones (e.g., 17α-ethinylestradiol), and natural hormones (estrone and 17β-estradiol), as they can cause disorders in the reproductive systems of animals and humans (Adeel et al. 2017; Khanzada et al. 2019; Américo-Pinheiro et al. 2020).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11270-021-05375-x.

O. Pacheco Passos Neto · A. Bezerra dos Santos · S. Mota (✉)
Department of Hydraulic and Environmental Engineering, Federal University of Ceará, Campus do Pici, Bloco 713. Pici. CEP, Fortaleza, Ceará 60455-900, Brazil
e-mail: suetonio@ufc.br

J. R. Feitosa Silva
Department of Biology, Federal University of Ceará, Fortaleza, Ceará, Brazil

The European Union (EU), through the Directive 2013/39/EU, recently listed 45 priority substances, including polycyclic aromatic hydrocarbons (PAHs), phthalates, pesticides, metals, and endocrine-disrupting chemicals (EDCs) (Ribeiro et al., 2015). Seventeen compounds of emerging concern (CEC) were later included in EU−Decision 2015/495/EU. However, pharmaceuticals, personal care products, and steroid hormones have not yet been included (Goswami et al. 2018).

The main EM sources are sewage, pharmaceutical and industrial treatment plants, livestock, and agricultural wastewater. However, the occurrence, persistence, and adverse effects of EM are highly associated with wastewater treatment plants (WWTPs), which are the primary sources of EM in water bodies (Solaun et al. 2021; Proctor et al. 2021). EM are commonly found in concentrations ranging from a few nanograms to several micrograms per liter (Zhang et al. 2021; Ofrydopoulou et al. 2022), which depends on the water matrix being evaluated (Proctor et al. 2021). Studies conducted in some states in Brazil demonstrated that such concentrations can reach the order of $\mu g \cdot L^{-1}$ in surface waters close to large urban centers (Vidal et al. 2020; Coelho et al. 2020), especially due to the low sanitation coverage and WWTPs that are not designed to remove EDCs and other micropollutants.

In addition to the development of female characteristics in male individuals, which causes abnormal vitellogenin production, low sperm count, and the appearance of intersex fish, EDCs can also negatively affect the nervous and immune systems, cause behavioral disorders, and generally affect the homeostasis of organisms (Czarny et al. 2017). Therefore, studies in this area must be conducted to support discussions that may result in future public policies on water quality concerning these emerging pollutants.

The feminizing action of EDCs with estrogenic action has been observed in fish that live in rivers close to large urban centers worldwide (Meijide et al. 2016). Additionally, laboratory tests have confirmed that considerably low doses of hormones, in the order of $\mu g \cdot L^{-1}$ and $ng \cdot L^{-1}$, can negatively affect the development of several fish species (Vishnu Priyan et al. 2021; Adeel et al. 2017).

Nile tilapia (*Oreochromis niloticus*) is currently produced in more than 80 countries (FAO (Roma) 2020). As it is a very robust and prolific species, it can be found easily in the environment, such as in Brazil. Studies of this species indicated that the biotransformation of the herbicide diuron into active metabolites alters signaling pathways in the central nervous system, which may impact androgen and the stress response, as well as the behavior necessary for social dominance, growth, and reproduction (Boscolo et al. 2017).

Luzio et al. (2016) evaluated the endocrine effects of an estrogen (EE2-17-ethinylestradiol, 4 ng·L), an inhibitor of estrogen synthesis (Fad-fadrozole, 50 μg·L), or their binary mixture (Mix-EE2 + Fad, 4 ng·L + 50 μg·L) in zebrafish (*Danio rerio*) development. They found that fadrozole, alone or in combination with EE2, permanently disrupts sexual development, induces masculinization, and causes severe alterations in the testis, such as the formation of intersex characteristics associated with sperm duct enlargement, interstitial changes, asynchronous development, and basal membrane detachment. After the exposure, gonad histopathology revealed interstitial proteinaceous fluid deposits and, in the ovaries, atretic oocytes and presumably degenerative mineralization were observed. However, the gonadal changes induced by EE2 alone were partially reversible when the compounds were removed.

Tirado et al. (2017) evaluated the performance of 17β-estradiol (E2) and vitellogenin (Vtg) levels in plasma and prostaglandins in oocytes (PGE 2) during the reproductive period of Nile tilapia (*Oreochromis niloticus*) in a commercial farm in Ecuador. Adult female fish were treated with E2 and tilapia pituitary extract (TP) for 25 days, and E2 supplementation affected the levels of Vtg per treatment ($p < 0.05$), impacting gonadal growth. Moreover, the ovaries showed asynchronous development, and the proportions of mature oocytes were higher with E2 and TP than in the control. The PGE 2 concentrations differed between the treatments ($p < 0.05$).

Alcántar-Vázquez (2018) conducted a study to evaluate the effects of different combinations of E2, EE2, and diethylstilbestrol (DES) on sex proportion, growth, and gonadal development of Nile tilapia. No significant differences in growth were observed between the groups. However, the proportion of deformed gonads was higher in the groups fed E2-DES and E2-DES-EE2.

Therefore, studies that evaluate the effects of EDCs in Nile tilapia (*Oreochromis niloticus*), a

known fish species resistant to adverse water environments, under controlled conditions are necessary, particularly for concentrations exceeding those found under normal conditions, but reported in the effluents of some treatment plants. Additionally, owing to the low dilution capacity of many surface waters, this problem may be potentialized. This study aimed to investigate the effects of endocrine disruptors E2 and EE2 on the development of Nile tilapia (*O. niloticus*), focusing on gonadal histological factors.

2 Materials and Methods

2.1 Research Location

This research was developed through a joint study between the Aquatic Resources Laboratory of the Department of Fisheries Engineering (LARAq/DEP), Sanitation Laboratory of the Department of Hydraulic and Environmental Engineering, and Department of Biology, located on the campus of Pici, of the Federal University of Ceará (UFC), Fortaleza, Ceará, Brazil. Nile tilapia eggs (*O. niloticus*) were obtained from the Aquaculture Station of the Department of Fisheries Engineering, UFC.

2.2 Experimental Design

Four experiments were conducted, each aiming to evaluate the effects of E2 and EE2 on Nile tilapia cultivation. The hormones were purchased from Sigma-Aldrich and used without any purification. Experiments 1 and 2 aimed to evaluate the effects of the estrogens on the sexual differentiation of Nile tilapia at concentrations ranging from $ng{\cdot}L^{-1}$ to $\mu g{\cdot}L^{-1}$ (Table 1). In experiment 3, the effect of hormones on the sexual differentiation of Nile tilapia was assessed after 28 days. Finally, in experiment 4, we aimed to further our understanding of the effects of hormones in weekly evaluations over a 28-day cycle (Table 1). All treatments were tested in quadruplicate.

The nomenclature used to designate the different treatments evaluated in this study was composed of three elements: (1) Arabic numerals indicating which experiment the treatment belongs to (1, 2, 3, or 4); (2) identification of the hormone used, i.e., E2 (17β-estradiol), EE2 (17α-ethinylestradiol), C + (positive control), and C- (negative control); and (3) the hormonal concentration evaluated, i.e., 250, 500, and 1000 $ng{\cdot}L^{-1}$ (experiment 1), 250, 500 and 1000 $\mu g{\cdot}L^{-1}$ (experiment 2), and 160 $\mu g{\cdot}L^{-1}$ (experiments 3 and 4). In experiment 4, a fourth element was

Table 1 Chronological order of the experiments developed in this research

Experiments	Estrogen concentration	Unit	Period (days)	Treatments	
Experiment 1				1.C +	
Hormonal exposure of fish	250, 500, and 1000	$ng{\cdot}L^{-1}$	28	1.E2-250	1.EE2-250
Growth period			60	1.E2-500	1.EE2-500
				1.E2-1,000	1.EE2-1,000
Experiment 2				2.C +	
Concentration test	250, 500, and 1000	$\mu g{\cdot}L^{-1}$	7	2.EE2-250	2.EE2-250
Egg incubation period			7	2.EE2-500	2.EE2-500
Hormonal exposure of fish			28	2.EE2-1,000	2.EE2-1,000
Experiment 3				3.C-	
Concentration test	160	$\mu g{\cdot}L^{-1}$	7	3.C +	
Hormonal exposure of fish			28	3.E2-160	
Growth period			122	3.EE2-160	
Experiment 4				4.E2-160–1	4.EE2-160–1
Hormonal exposure of fish	160	$\mu g{\cdot}L^{-1}$	7, 14, 21, and 28	4.E2-160–2	4.EE2-160–2
Growth period			217	4.E2-160–3	4.EE2-160–3
				4.E2-160–4	4.EE2-160–4

Estrogens: 17β-estradiol (E2) and 17α-ethinylestradiol (EE2)

added related to the period of hormonal exposure in weeks (1, 2, 3, or 4).

Table 2 presents a schematic representation of the experimental design and hormonal dose administration.

2.3 Procedures Common to All Experiments

2.3.1 Preparation of the Stock Solution and Administration of the Hormonal Dose

To prepare the stock solution, the hormones E2 and EE2 were diluted in ethyl alcohol PA in a 250-mL conical flask and processed in a 42-kHz ultrasonic processor for 120 s. Purified water (Milli-Q) was then added to each flask. The mixture was processed in an ultrasonic processor for 240 s, and a volume of this stock solution was carefully added to each experimental unit. The amounts of E2 and EE2, and volumes of ethyl alcohol PA, purified water (Milli-Q), and aliquots of the stock solution added to each experimental unit varied according to the experiment and desired final concentrations, as shown in Table 3.

To ensure that the positive control (C+) was subjected to the same conditions as the other treatments, without the presence of the hormone, ethyl alcohol PA was processed in an ultrasonic processor for 120 s in an Erlenmeyer flask; purified water was then added and processed for 240 s. A variable volume of this solution was added to each replicate. The experimental units for the negative control (C-) were prepared using water without adding any chemical substances.

2.3.2 Feeding and Maintaining Water Quality

During the period of exposure to the estrogens, which varied from one to 4 weeks, the individuals were kept in aquariums with a volume of 40 L (experimental unit). The photoperiod was artificially controlled (10 h of light and 14 h of darkness), and constant aeration was provided using an air compressor (nominal flow of 120 $L \cdot min^{-1}$) connected to several air stones. The aquariums were supplied with dechlorinated tap water.

The fish were fed four times a day (9 h, 12 h, 14 h, and 16 h 30 min) with powdered food for tilapia according to the manufacturer's recommendations. This amount was adjusted during the individuals' development. The dissolved oxygen (DO) content and temperature were monitored daily, and the pH, total ammoniacal nitrogen (NH_3/NH_4^+; TAN), and nitrite (NO_2^-) were measured every 5 days using colorimetric tests. Beginning from the TAN values and crossing the pH and temperature data, the concentration of

Table 2 Experimental design and hormonal dose administration in the four developed experiments

Egg/Aq, number of eggs per aquarium; *Larvae/Aq*, number of larvae per aquarium

Variable	Experiment			
	1	2	3	4
Egg/Aq	-	20	-	-
Larvae/Aq	10	10	12	12
Estrogen concentration	250, 500, and 1000 $ng \cdot L^{-1}$	250, 500, and 1000 $\mu g \cdot L^{-1}$	160 $\mu g \cdot L^{-1}$	160 $\mu g \cdot L^{-1}$

Table 3 Amounts of estrogen (E2 or EE2), ethyl alcohol PA (Al), and purified water (Milli-Q) used to prepare the stock solutions in experiments 1, 2, 3, and 4. V/Aq is the volume of stock solution added in each 40-L experimental unit and (estrogen) is the final target estrogen concentration in each experimental unit

Experiment	Estrogen (mg)	Al (mL)	Milli-Q (mL)	V/Aq (mL)	(Estrogen)
1	2	10	90	0.5	250 $ng \cdot L^{-1}$
	4				500 $ng \cdot L^{-1}$
	8				1,000 $ng \cdot L^{-1}$
2	30	10	200	70	250 $\mu g \cdot L^{-1}$
	60				500 $\mu g \cdot L^{-1}$
	120				1,000 $\mu g \cdot L^{-1}$
3 and 4	25.6	10	230	60	160 $\mu g \cdot L^{-1}$

non-ionized ammonia (NH_3) was obtained. The water quality was maintained daily by siphoning the feces and food remains before the first feeding. This management was divided into two stages: (1) water quality maintenance during hormonal exposure, and (2) water quality maintenance after hormonal exposure.

During hormonal exposure, the siphoned water, containing feces and uneaten feed, was filtered with an acrylic blanket and returned to its respective aquarium. The siphoning time was sufficient to remove debris with no control over the drained volume. Once per week, all of the water in the aquarium was renewed, and the hormonal dosage was readministered.

After hormone exposure, fish were transferred to 250-L circular tanks to assess their development (external morphological characteristics) and histological changes in the gonads. The fish remained under this condition until they reached an adequate size to allow the removal of the gonads for histological analysis, according to the needs of each experiment developed. The siphoned volume was replaced daily with fresh tap water to maintain the water quality, equivalent to 10% of the total volume.

2.3.3 Histological Studies

After reaching the appropriate size, the fish were euthanized by desensitization on ice. The gonads were removed (Fig. 1), stored in histological microcassettes, labeled, submerged in 10% formalin solution for 24 h, transferred to 70% alcohol for preservation, and later analyzed.

The gonads were dehydrated, diaphanized, embedded in paraffin, subjected to microtomy, and stained. Hematoxylin and Gomori's trichrome were used to differentiate the gonadal components better, thereby facilitating the identification of the sexual cells' maturation stages. The routine histological protocol was adapted from Tolosa et al. (2003) and is shown in Supplementary Material.

2.4 Estrogen Measurement Methodology

The samples (500 mL) were initially filtered through a glass fiber filter with a diameter and porosity of 47 mm and 0.45 µm, respectively, using a vacuum pump (MFS, VP-24). The filtrate was transferred to a flat-bottomed volumetric flask for the next stage.

Strata-X cartridges were used for solid-phase extraction (SPE), which were coupled to a vacuum manifold manual processor (Applied Separations, Speed Mate 12) connected to a vacuum pump. Initially, the cartridges were conditioned with methanol (10 mL). The hormones were then extracted from the filtrate through the cartridges with a flow rate of 1.5–2.0 $mL \cdot min^{-1}$. The compounds present in the eluate were identified and quantified

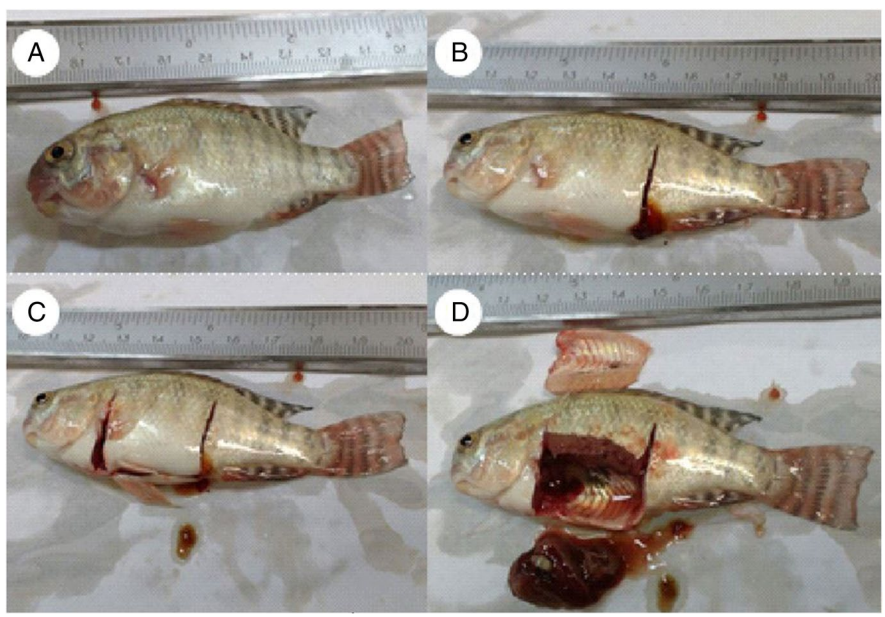

Fig. 1 Sequence of incisions and removal of the viscera to access the gonads of Nile tilapia (*Oreochromis niloticus*) exposed to the hormones 17β-estradiol (E2) and 17α-ethinylestradiol (EE2). **A** Intact fish. **B** Incision in the posterior region. **C** Incisions in the ventral and anterior regions. **D** Removal of the musculature and viscera

by high-performance liquid chromatography (20A Prominence, Shimadzu) equipped with a UV–vis detector (SPD-20A; 215 nm) and a C18 column (15 cm × 4.6 mm DI, 0.4 µm, Shimadzu). The gradient elution cycle applied was as follows (acetonitrile/HCl 0.1%): increase from 15 to 80% acetonitrile in 10 min and return to 15% in 4 min. The initial flow rate was 1.0 mL·min^{-1}, and after 5 min, the flow was increased to 2.0 mL·min^{-1}. The furnace temperature was maintained at 35 °C, and the injection volume was 20 µL.

The limit of detection (LOD) and limit of quantification (LOQ) for E2 were 0.3 and 1.2 µg·L^{-1}, respectively, while those for EE2 were 1.8 and 6.0 µg·L^{-1}, respectively (Vidal et al., 2020).

Table 4 Dissolved oxygen (DO), temperature (Temp.), pH, nitrite (NO_2^-), total ammoniacal nitrogen (TAN), and non-ionized ammonia (NH_3) content of the water used in juvenile Nile tilapia culturing (*Oreochromis niloticus*)

Parameter	Range
OD (mg·L^{-1})	6.4–7.8
Temp. (°C)	26–28
pH	7–8
NO_2^- (mg·L^{-1})	0.2–0.5
NAT (mg·L^{-1})	0.2–1.2
NH_3 (mg·L^{-1})	< 0.03

3 Results and Discussion

3.1 Water Quality Parameters

The water quality parameters did not vary greatly between the treatments (Table 4). Dissolved oxygen (OD) is considered the main water quality parameter that should be observed to maintain aquatic organisms in captivity. The cultivation values for DO exceeded 6.0 mg·L^{-1}, indicating a good aerobic environment for fish culture. In all the experiments conducted, the pH remained at an average value close to 7.5, which is also adequate for Nile tilapia culture.

For all the experiments, the water quality parameters were always within the comfort range of Nile tilapia. Thus, it can be inferred that any losses caused to the individuals' development were due to the hormone exposure.

3.2 Gonadal Histology

The gonads of fish obtained in experiment 1 are presented in Table 5. Intersex individuals subjected to EE2 treatment were observed, even at the lowest concentrations, 250 ng·L^{-1}. In treatment 1.E2-500, one intersex individual was found, while none were observed in the other treatments. However, the gonads of a considerable number of individuals in treatments 1.E2-250 and 1.E2-1.000 could not be removed due to their small size and fragility (seven and five individuals, respectively).

In experiment 2, increasing the concentration of estrogens by one order of magnitude (µg·L^{-1}) was

Table 5 Classification of Nile tilapia (*Oreochromis niloticus*) from experiment 1 regarding their gonadal characteristics (male, female, and intersex). The individuals were exposed to concentrations of 250, 500, and 1,000 ng·L^{-1} of the hormones 17β-estradiol and 17α-ethinylestradiol during the hatching phase

Treatment	Gonadal characteristics				Total (*n*)
	Male (*n*)	Female (*n*)	Intersex (*n*)	Lost (*n*)	
1.C	8	5	0	3	16
1.E2-250	1	2	0	7	10
1.E2-500	2	4	1	2	9
1.E2-1.000	1	7	0	5	13
1.EE2-250	2	2	2	3	9
1.EE2-500	0	6	4	4	14
1.EE2-1.000	1	6	2	1	10

Legend: Experiment compound concentration in ng·L^{-1}; *C*, control

lethal for Nile tilapia larvae, with no effect on the incubation time and percentage of larvae hatching. Additionally, morphological anomalies and developmental problems were observed.

In experiment 3, with 122 days of growth after the end of hormonal exposure, the number of gonads lost during the extraction process was considerably reduced: one gonad was lost in treatment 3.E2-160 and two were lost in treatment 3.EE2-160. No gonads were lost in treatments 3.C- and 3.C+, and no intersex individuals were observed. Only one intersex fish was observed among all survivors, which was observed in treatment 3.E2-160 (Table 6).

In treatments 3.C- and 3.C+, approximately equal numbers of males and females were observed. However, in the hormonal treatments, females were almost absolutely predominant. In treatment 3.E2-160, only two males and 27 females were observed of 31 surviving fish. In treatment 3.EE2-160, of the 15 survivors, there were 13 females and no males.

Owing to the advanced stage of development of the individuals in experiment 4 (217 days of growth after the end of hormonal exposure for the last group), in most cases, the external sex of each specimen could be evaluated based on the general characteristics and anatomy of the urogenital papilla. The stages of gametogenesis in male and female germ cells could also be visualized in greater detail in the histological sections. External sex was classified as undefined in one, one, and two individuals from treatments 4.E2-160–2, 4.EE2-160–1, and 4.EE2-160–2, respectively. An individual with a male urogenital papilla was only observed in treatment 4.E2-160–2. In this case, the histology of the gonad confirmed that it was a male individual with mature testicles.

In treatments 4.E2-160–1, 4.E2-160–3, 4.E2-160–4, 4.EE2-160–3, and 4.EE2-160–4, all individuals were classified as females, according to their external characteristics. However, for all five treatments mentioned above, the histology of the gonads showed that, among the possible females, there were intersex and male individuals (Table 7).

Intersex fish were observed in treatments 4.E2-160–1 (one individual), 4.E2-160–3 (one individual), 4.EE2-160–2 (one individual), 4.EE2-160–2 (three individuals), and 4.EE2-160–4 (two individuals), while no intersex individuals were observed in treatments 4.E2-160–2, 4.E2-160–4, and 4.EE2-160–1.

Although the gonads shown in Figs. 2 to 4 were from fish from experiment 4, they illustrate the classic characteristics of the gonads of male, female, and intersex individuals, regardless of which experiment the fish underwent. Figure 2 shows histological sections of the normal mature testicle of Nile tilapia in the fit phase to release sperm. The lumen of dilated seminiferous tubules in the presence of sperm, discontinuous germinal epithelium, and blood cells (erythrocytes) could be observed. Figure 3 shows the histological sections of the normal mature gonad of a female Nile tilapia in the phase suitable for spawning. Primary and fully developed oocytes could be observed in the presence of cortical alveoli, calf granules, and nuclei peripheral to the nucleus.

The gonads of intersex individuals, which have both male and female characteristics, are shown in Fig. 4. For male characteristics, this gonad can be classified as in the development phase, initial development subphase, with a predominant presence of spermatocytes, and imperceptible lumens of seminiferous tubules. For female characteristics, the gonad can be classified as immature, with the presence of previtellogenic oocytes in primary growth.

In all of the experiments conducted during this study, a delay in the gonadal development of the Nile tilapia was observed, which resulted in difficulty in removing the gonads in very young individuals, such as those in experiments 1 and 2 and, to a lesser extent, experiment 3. For this reason, the development times after hormonal exposure were gradually increased until they culminated in 217 days in experiment 4. In this way, it was possible to not only remove the

Table 6 Classification of Nile tilapia (*Oreochromis niloticus*) from experiment 3 regarding their gonadal characteristics (male, female, and intersex). The individuals were exposed 160 $\mu g \cdot L^{-1}$ of the hormones 17β-estradiol and 17α-ethinylestradiol during the hatching phase

Treatment	Gonadal characteristics				Total (*n*)
	Male (*n*)	Female (*n*)	Intersex (*n*)	Lost (*n*)	
3.C-	11	10	0	0	21
3.C+	7	9	0	0	16
3.E2-160	2	27	1	1	31
3.EE2-160	0	13	0	2	15

C-, negative control; *C*+, positive control; experimental compound concentration in $\mu g \cdot L^{-1}$

Table 7 Classification of Nile tilapia (*Oreochromis niloticus*) in experiment 4 regarding their external (male, female, and undefined) and gonadal (male, female, and intersex) characteristics. The individuals were exposed to 160 μg·L^{-1} of the hormones 17β-estradiol and 17α-ethinylestradiol for periods of one, two, three, and 4 weeks

Treatment	External sex			Gonadal			Total (*n*)
	Male (*n*)	Female (*n*)	Undefined (*n*)	Male (*n*)	Female (*n*)	Intersex (*n*)	
4.E2-160–1	0	9	0	0	8	1	9
4.E2-160–2	1	9	1	1	10	0	11
4.E2-160–3	0	8	0	0	7	1	8
4.E2-160–4	0	10	0	1	9	0	10
4.EE2-160–1	0	3	1	1	3	0	4
4.EE2-160–2	0	7	2	0	8	1	9
4.EE2-160–3	0	9	0	0	6	3	9
4.EE2-160–4	0	10	0	1	7	2	10

Legend: Experiment compound-concentration in μg L^{-1}-weeks of exposure

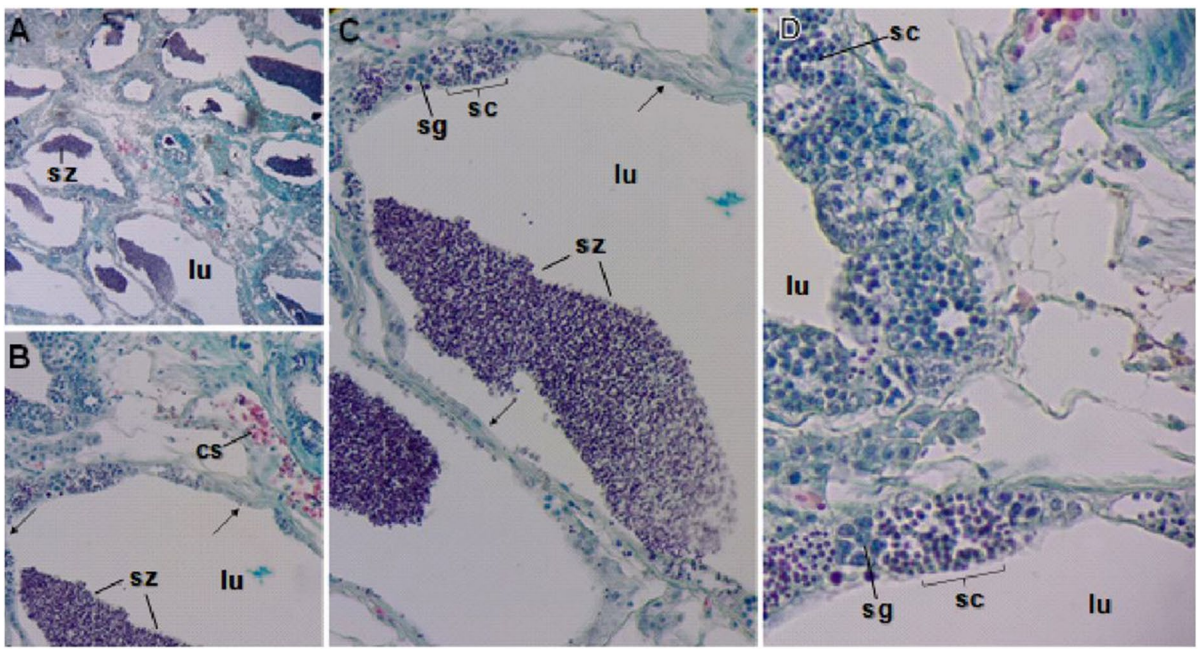

Fig. 2 Histological sections of the normal testis of sexually mature male Nile tilapia (*Oreochromis niloticus*) from wxperiment 4. **A**–**D** Different enlargements and details of a testicle at the fit to release sperm stage. lu, lumen of the seminiferous tubule; sc, spermatocyte; sg, spermatogonia; sz, sperm; cs, blood cells; ↑, germline epithelium discontinuity;}, spermatocyte; staining, hematoxylin and Gomori trichrome

gonads more successfully but also determine the external sex and compare the results with the results obtained from the histological sections of the gonads.

In fish, exposures to EDCs during critical developmental life stages can alter sex phenotypes, impair gonad development, and disturb the progress of the reproductive process (Luzio et al. 2016). Hamid et al. (2021) reported that EDC mixtures caused severe malformations, including epididymal or gubernacular lesions, and deciduous spermatids in the testis,

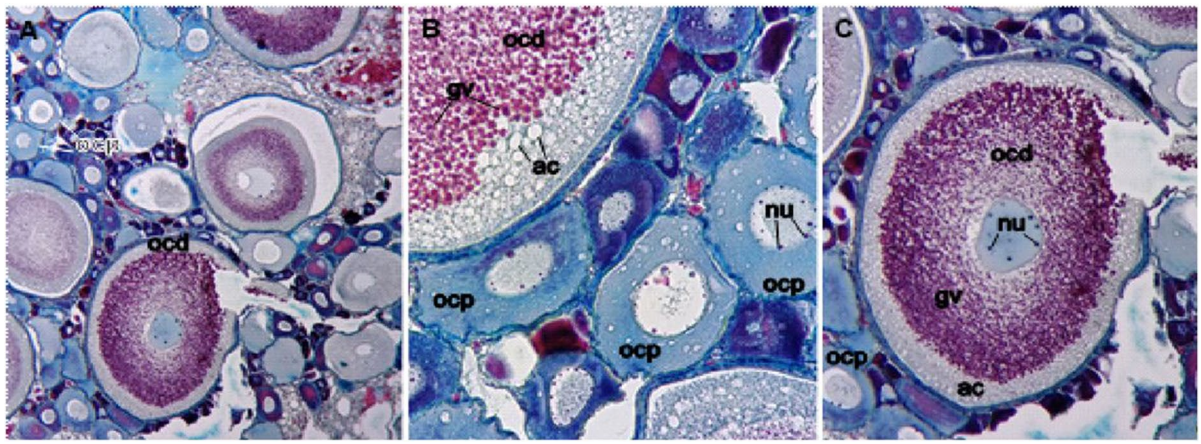

Fig. 3 Histological sections of a normal female Nile tilapia ovary (*Oreochromis niloticus*) from experiment 4. **A–C** Different enlargements and details of an ovary at a stage able to spawn. nu, nucleolus; ocp, primary oocyte; ocd, fully developed oocyte; ac, cortical socket; gv, calf granules; staining, hematoxylin and Gomori trichrome

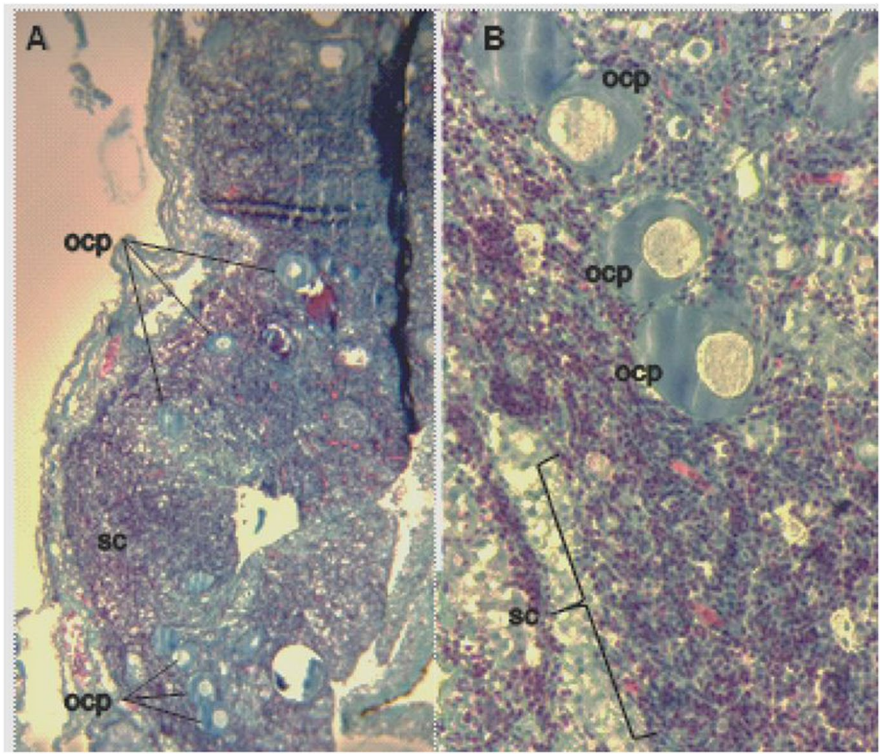

Fig. 4 Histological section of the gonad of an intersex Nile tilapia (*Oreochromis niloticus*) from experiment 4. ocp, primary oocyte; sc, spermatocyte. Staining, hematoxylin and Gomori trichrome

and perturbed the androgen axis in a cumulative, dose additive manner when compared to single chemical exposure. As far we are concerned, there are very few studies on the estrogenic action of EDCs in sexual differentiation in any of the tilapia species under laboratory conditions; the studies that have explored this subject involved the capture of wild specimens, together with water and soil analysis.

Studying two tilapia species (*Sarotherodon melanotheron* and *Tilapia guineensis*) captured in the

Awba Dam, Nigeria, Adeogun et al. (2016) found an average frequency of 34.8% of intersex individuals between the two species evaluated. Researchers have reported the presence of heavy metals (As, Cd, Pb, Hg, and Ni) in the substrate of the dam, along with organochlorines (polychlorinated bisphenyl) and phenolic compounds (4-iso-nonylphenol).

Woodling et al. (2006) also reported atrophy in gonadal development in fish exposed to EDCs collected downstream from a sewage treatment plant effluent discharge. They also reported difficulty in removing the gonads and visually identifying the individual's sex. Dammann et al. (2011) reported a decrease in the gonadosomatic index in *Pimephales promelas* as the concentrations of estrone and E2 increased from 10 to 100 ng·L^{-1} and 5 to 50 ng·L^{-1}, respectively. Lange et al. (2001) also observed gonadal atrophy in *P. promelas* exposed to concentrations of EE2 ranging from 0.2 to 64 ng·L^{-1}.

Fish are significantly more sensitive to the action of EDCs during the early stages of development, particularly during the period of sexual differentiation, which occurs between fertilization and 25 days post-hatching (Martinez-Bengochea et al. 2020; Song et al. 2020). Pawlowski et al. (2004) found that a period of exposure of only 10 to 15 days to a low concentration of EE2 (10 ng·L^{-1}) was sufficient to cause the feminization of *P. promelas*. In contrast, they did not observe a feminizing effect in *P. promelas* exposed to the same concentration of EE2 for 3 weeks after the natural period of sexual differentiation. Niemuth et al. (2014) exposed sexually differentiated *P. promelas* males to 40 µg·L^{-1} of the endocrine disruptor metformin and observed the production of plasma vitellogenin, but no occurrence of feminized fish. In another study involving the same species, chemical compound, and concentration, Niemuth and Klaper (2015) observed feminized individuals when exposed from the early stages of development.

Several other authors have observed that the presence of E2 in water causes the feminization of males, including *Oryzias latipes* Koger et al. 2000), South American cichlid *Cichlasoma dimerus* (Meijide et al. 2016), *Poecillia reticulata* (Toft and Baatrup 2003), and *Fundulus heteroclitus* (Urushitani et al. 2002).

Although the estrogenic potential of EE2 is usually reported to be lower than that of E2, in this study, there was no difference in the number of intersex individuals when comparing the same concentrations of both hormones. This may indicate that, for E2 and EE2, the minimum concentrations capable of generating a response were lower than the lowest tested concentration.

A serious ecological consequence of these substances in the environment is the development of phenotypic females (XY), also known as neo-females. Mating a normal male (XY) with a neo-female produces 25% normal females (XX), 50% normal males (XY), and 25% males with the YY genotype, which are called supermales. Mating a supermale with a normal female produces a 100% male population. In this context, Hassell et al. (2016) and Huang et al. (2016) stated that long-term exposure to estrogenic substances can destroy entire populations and cause subsequent species extinction.

Xie et al. (2021) investigated the potentially toxic effects of E2 exposure on little yellow croaker (*Larimichthys polyactis*, L. poliactis), which have a unique gonadal development pattern in which males undergo a hermaphroditic stage. Fish were maintained in tanks and exposed to E2 concentrations of 10 µg·L or not (ethanol and control groups) from 30 to 90 days post-hatching (dph). After exposure, the E2 was withdrawn, and the fish continued to culture to 150 and 365 dph. Morphological and histological analyses were conducted to compare changes in fish. The results showed that E2 exposure caused three major phenotypes at 30 and 60 days after treatment (dat), including ovary, ovotestis, and gonadal development retardation. The average ratios of these three phenotypes were 60.6%, 11.97%, and 27.43%, respectively. The body length and weight of the E2 exposure groups were repressed during the E2 exposure period, while they recovered after E2 withdrawal. However, gonadal development (gonadosomatic index) of testis in the E2 exposure groups was retarded at 60 days and did not recover until 365 dph. The sex determination/differentiation-related genes erα, erβI, erβII, fshβ, and cyp11b2 were significantly decreased in male fish exposed to E2. Therefore, E2 exposure led to feminization and disrupted testis maturation and spermatogenesis, which persisted into the sexual maturity stage.

In addition to hormones, other chemical compounds, such as drugs, have shown to cause endocrine disorders in the sexual differentiation of fish. Niemuth and Klaper (2015) exposed *Pimephales promelas* to 40 µg·L^{-1} of metformin, an oral antidiabetic medication, and

observed the development of intersex individuals with different degrees of feminization. The authors observed males with the occasional and dispersed presence of perinucleolar follicles to individuals with male external characteristics with 100% female gonads, with oocytes at different developmental stages.

4 Conclusions

Concentrations of the hormones 17β-estradiol and 17α-ethinylestradiol from 250 to 1,000 ng·L^{-1} can produce intersex individuals in Nile tilapia (*O. niloticus*), with a significant decrease in the condition factor as the concentrations increased. In addition, these concentrations could also induce the development of morphological anomalies without any significant difference between the concentrations evaluated. Increasing the hormone concentration by one order of magnitude (μg·L^{-1}) was lethal for Nile tilapia fry, with no effect on the incubation time and the percentage of larvae hatching. Morphological anomalies and developmental problems were also observed in histological studies.

Hormones applied at a concentration of 160 μg·L^{-1} for 28 days caused a small number of intersex Nile tilapia individuals to develop but generated almost entirely female populations in hormonal treatments. Additionally, morphological anomalies were observed, in which the gonads of fish exposed to the estrogens were smaller and less developed, without any significant difference between E2 and EE2.

The variation in the exposure time (one to 4 weeks) of Nile tilapia to the hormones (160 μg·L^{-1}) resulted in the appearance of intersex individuals and the development of morphological anomalies, regardless of exposure time.

Funding The authors would like to thank the National Council for Scientific and Technological Development (CNPq), Process nº 481985/2012–3, for the financial support.

References

Adeel, M., Song, X., Wang, Y., Francis, D., & Yang, Y. (2017). Environmental impact of estrogens on human, animal and plant life: A critical review. *Environment International, 99*, 107–119. https://doi.org/10.1016/j.envint.2016.12.010

Adeogun, A. O., Onibonoje, K., Ibor, O. R., Omiwole, R. A., Chukwuka, A. V., Ugwumba, A. O., & Arukwe, A. (2016). Endocrine-disruptor molecular responses, occurrence of intersex and gonado-histopathological changes in tilapia species from a tropical freshwater dam (Awba Dam) in Ibadan, Nigeria. *Aquatic Toxicology, 174*, 10–21. https://doi.org/10.1016/j.aquatox.2016.02.002

Alcántar-Vázquez, J. P. (2018). Sex proportion in Nile tilapia *Oreochromis niloticus* fed estrogen mixtures: A case of paradoxical masculinization. *Latin American Journal of Aquatic Research, 46*(2), 337–345. https://doi.org/10.3856/vol46-issue2-fulltext-9

Américo-Pinheiro, J. H. P., da Cruz, C., Aguiar, M. M., Torres, N. H., Ferreira, L. F. R., & Machado-Neto, J. G. (2020). Histological changes in targeted organs of Nile tilapia (*Oreochromis niloticus*) exposed to sublethal concentrations of the pesticide carbofuran. *Water, Air, and Soil Pollution, 231*, 228. https://doi.org/10.1007/s11270-020-04628-5

Boscolo, C. N. P., Pereira, T. S. B., Batalhão, I. G., Dourado, P. L. R., Schlenk, D., & de Almeida, E. A. (2017). Diuron metabolites act as endocrine disruptors and alter aggressive behavior in Nile tilapia (*Oreochromis niloticus*). *Chemosphere, 191*, 832–838. https://doi.org/10.1016/j.chemosphere.2017.10.009

Coelho, L. H. G., de Jesus, T. A., Kohatsu, M. Y., Poccia, G. T., Chicarolli, V., Helwig, K., Roberts, C. H. J., Teedon, P., & Pahl, O. (2020). Estrogenic hormones in São Paulo waters (Brazil) and their relationship with environmental variables and Sinapis alba phytotoxicity. Water Air Soil Pollut 231, 150 (2020). https://doi.org/10.1007/s11270-020-04477-2

Czarny, K., Szczukocki, D., Krawczyk, B., Zieliński, M., Miękoś, E., & Gadzała-Kopciuch, R. (2017). The impact of estrogens on aquatic organisms and methods for their determination. *Critical Reviews in Environmental Science and Technology, 47*(11), 1–55. https://doi.org/10.1080/10643389.2017.1334458

Dammann, A. A., Shappell, N. W., Bartell, S. E., & Schoenfuss, H. L. (2011). Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, *Pimephales promelas*. *Aquatic Toxicology, 105*, 559–568. https://doi.org/10.1016/j.aquatox.2011.08.011

FAO (Roma), 2020. The State of World Fisheries and Aquacultura 2020: Meeting the sustainable development goals. Access: Aug 30th 2021.

Goswami, L., Vinoth Kumar, R., Borah, S. N., Arul Manikandan, N., Pakshirajan, K., & Pugazhenthi, G. (2018). Membrane bioreactor and integrated membrane bioreactor systems for micropollutant removal from wastewater: A review. *Journal of Water Process Engineering, 26*, 314–328. https://doi.org/10.1016/j.jwpe.2018.10.024

Hamid, N., Junaid, M., & Pei, D. S. (2021). Combined toxicity of endocrine-disrupting chemicals: A review. *Ecotoxicology and Environmental Safety, 215*, 112136. https://doi.org/10.1016/j.ecoenv.2021.112136

Harb, M., Lou, E., Smith, A. L., & Stadler, L. B. (2019). Perspectives on the fate of micropollutants in mainstream anaerobic wastewater treatment. *Current Opinion in Biotechnology, 57*, 94–100. https://doi.org/10.1016/j.copbio.2019.02.022

Hassell, K., Pettigrove, V., Beresford, N., Jobling, S., & Kumar, A. (2016). No evidence of exposure to environmental estrogens in two feral fish species sampled from the Yarra River, Australia: A comparison with Northern Hemisphere studies. *Ecotoxicology and Environmental Safety, 131*, 104–117. https://doi.org/10.1016/j.ecoenv.2016.05.004

Huang, G. Y., Liu, Y. S., Chen, X. W., Liang, Y. Q., Liu, S. S., Yang, Y. Y., Hu, L. X., Shi, W. J., Tian, F., Zhao, J. L., Chen, J., & Ying, G. G. (2016). Feminization and masculinization of western mosquitofish (*Gambusia affinis*) observed in rivers impacted by municipal wastewaters. *Scientific Reports, 06*(01), 1–11. https://doi.org/10.1038/srep20884

Khanzada, N. K., Farid, M. U., Kharraz, J. A., Choi, J., Tang, C. Y., Nghiem, L. D., Jang, A., & An, A. K. (2019). Removal of organic micropollutants using advanced membrane-based water and wastewater treatment: A review. *Journal of Membrane Science, 598*, 117672. https://doi.org/10.1016/j.memsci.2019.117672

Koger, C. S., The, S. J., & Hinton, D. E. (2000). Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17β-estradiol or testosterone. *Marine Environmental Research, 50*(1–5), 201–206. https://doi.org/10.1016/S0141-1136(00)00068-4

Lange, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H., & Sumpter, J. P. (2001). Effects of the synthetic estrogen 17 α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology Chemistry, 20*, 1216–1227. https://doi.org/10.1002/etc.5620200610

Luzio, A., Monteiro, S. M., Rocha, E., Fontaínhas-Fernandes, A. A., & Coimbra, A. M. (2016). Development and recovery of histopathological alterations in the gonads of zebrafish (*Danio rerio*) after single and combined exposure to endocrine disruptors (17α-ethinylestradiol and fadrozole). *Aquatic Toxicology, 175*, 90–105. https://doi.org/10.1016/j.aquatox.2016.03.014

Martinez-Bengochea, A., Doretto, L., Rosa, I. F., Oliveira, M. A., Silva, C., Silva, D. M. Z. A., Santos, G. R., Santos, J. F. S., Avelar, M. M., Silva, L. V., Lucianelli-Junior, D., Souza, E. R. B., Silva, R. C., Stewart, A. B., Nakaghi, L. S. O., Valentin, F. N., & $ Nóbrega, R. H. . (2020). Effects of 17β-estradiol on early gonadal development and expression of genes implicated in sexual differentiation of a South American teleost *Astyanax Altiparanae*. *Comparative Biochemistry and Physiology Part b: Biochemistry and Molecular Biology, 248–249*, 110467. https://doi.org/10.1016/j.cbpb.2020.110467

Meijide, F. J., Rey Vázquez, G., Piazza, Y. G., Babay, P. A., Itria, R. F., & Lo Nostro, F. L. (2016). Effects of waterborne exposure to 17β-estradiol and 4-tert-octylphenol on early life stages of the South American cichlid fish *Cichlasoma dimerus*. *Ecotoxicology and Environmental Safety, 124*, 82–90. https://doi.org/10.1016/j.ecoenv.2015.10.004

Niemuth, N. J., & Klaper, R. D. (2015). Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. *Chemosphere, 135*, 38–45. https://doi.org/10.1016/j.chemosphere.2015.03.060

Niemuth, N. J., Jordan, R., Crago, J., Blanksma, C., Johnson, R., & Klaper, R. D. (2014). Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish. *Environmental Toxicology and Chemistry [s.l.], 34*(2), 291–296. https://doi.org/10.1002/etc.2793

Ofrydopoulou, A., Nannou, C., Evgenidou, E., Christodoulou, A., & Lambropoulou, D. (2022). Assessment of a wide array of organic micropollutants of emerging concern in wastewater treatment plants in Greece: Occurrence, removals, mass loading and potential risks. *Science of the Total Environment, 802*, 149860. https://doi.org/10.1016/j.scitotenv.2021.149860

Pawlowski, S., van Aerle, R., Tyler, C., & Braunbeck, T. (2004). Effects of 17α-ethinylestradiol in a fathead minnow (Pimephales promelas) gonadal recrudescence assay. *Ecotoxicology and Environmental Safety [s.l.], 57*(3), 330–345. https://doi.org/10.1016/j.ecoenv.2003.07.019

Proctor, K., Petrie, B., Lopardo, L., Camacho Munoz, D., Rice, J., Barden, R., Arnot, T., & Kasprzyk-Hordern, B. (2021). Micropollutant fluxes in urban environment – A catchment perspective. *Journal of Hazardous Materials, 401*, 123745. https://doi.org/10.1016/j.jhazmat.2020.123745

Ribeiro, A. R., Nunes, O. C., Pereira, M. F., & Silva, A. M. (2015). An overview on the advanced oxidation processes applied for the treatment of water pollutants defined in the recently launched Directive 2013/39/EU. *Environment International, 75*, 33–51. https://doi.org/10.1016/j.envint.2014.10.027

Solaun, O., Rodríguez, J. G., Menchaca, I., Lopez-Garcia, E., Martinez, E., Zonja, B., Postigo, C., de Alda, M. L., Barcelo, D., Borja, A., Manzanos, A., & Larreta, J. (2021). Contaminants of emerging concern in the Basque coast (N Spain): Occurrence and risk assessment for a better monitoring and management decisions. *Science of the Total Environment, 765*, 142765. https://doi.org/10.1016/j.scitotenv.2020.142765

Song, W., Lu, H., Wu, K., Zhang, Z., Shuk-Wa Lau, E., & Ge, W. (2020). Genetic evidence for estrogenicity of bisphenol A in zebrafish gonadal differentiation and its signalling mechanism. *Journal of Hazardous Materials, 386*, 121886. https://doi.org/10.1016/j.jhazmat.2019.121886

Tirado, J. O., Valladares, L., Muñoz, D., Caza, J., Manjunatha, B., & Kundapur, R. R. (2017). Levels of 17β-estradiol, vitellogenin, and prostaglandins during the reproductive cycle of *Oreochromis niloticus*. *Latin American Journal of Aquatic Research, 45*(5), 930–936. https://doi.org/10.3856/vol45-issue5-fulltext-8

Toft, G., & Baatrup, E. (2003). Altered sexual characteristics in guppies (*Poecilia reticulata*) exposed to 17β-estradiol and 4-tert-octylphenol during sexual development. *Ecotoxicology and Environmental Safety, 56*(2), 228–237. https://doi.org/10.1016/S0147-6513(02)00138-0

Tolosa, E. M. C., Behmer, O. A., & Rodrigues, C. J. (2003). Manual de técnicas para histologia normal e patológica. *São Paulo: Manole, 2003*, 341.

Urushitani, H., Shimizu, A., Katsu, Y., & Iguchi, T. (2002). Early estrogen exposure induces abnormal development of *Fundulus heteroclitus*. *Journal of Experimental Zoology, 293*(7), 693–702. https://doi.org/10.1002/jez.10161

Priyan, V. V., Shahnaz, T., Suganya, E., Sivaprakasam, S., & Narayanasamy, S. (2021). Ecotoxicological assessment of micropollutant Diclofenac biosorption on magnetic sawdust: Phyto, Microbial and Fish toxicity studies. *Journal of Hazardous Materials, 403*, 123532. https://doi.org/10.1016/j.jhazmat.2020.123532

Vidal, C. B., Barbosa, P. G. A., Pessoa, G. P., Buarque, P. C., Nascimento, J. G. S., Farias-Filho, A. L., Paz, M. S., dos Santos, A. B., Cavalcante, R. M., & Nascimento, R. F. (2020). Multiresidue determination of endocrine disrupting compounds in sewage treatment plants (SPE-HPLC-DAD). *J. Braz. Chem. Soc., 31*(12), 2518–2530. https://doi.org/10.21577/0103-5053.20200127

Woodling, J. D., Lopez, E. M., Maldonado, T. A., Norris, D. O., & Vajda, A. M. (2006). Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. *Comparative Biochemistry and Physiology, Part C, 144*, 10–15. https://doi.org/10.1016/j.cbpc.2006.04.019

Xie, Q. P., Li, B. B., Wei, F. L., Yu, M., Zhan, W., Liu, F., & Lou, B. (2021). Growth and gonadal development retardations after long-term exposure to estradiol in little yellow croaker Larimichthys Polyactis. *Ecotoxicology and Environmental Safety, 222*, 112462. https://doi.org/10.1016/j.ecoenv.2021.112462

Zhang, D., Liu, W., Wang, S., Zhao, J., Xu, S., Yao, H., Wang, H., Bai, L., Wang, Y., Gu, H., Tao, J., & Shi, P. (2021). Risk assessments of emerging contaminants in various waters and changes of microbial diversity in sediments from Yangtze River chemical contiguous zone, Eastern China. *Science of the Total Environment, 803*, 149982. https://doi.org/10.1016/j.scitotenv.2021.149982

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.